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Restart of DNA replication in Gram-positive bacteria: functional characterisation of the *Bacillus subtilis* PriA initiator

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ABSTRACT

The PriA protein was identified in *Escherichia coli* as a factor involved in the replication of extrachromosomal elements such as bacteriophage φX174 and plasmid pBR322. Recent data show that PriA plays an important role in chromosomal replication, by promoting reassembly of the replication machinery during reinitiation of inactivated forks. A gene encoding a product 32% identical to the *E.coli* PriA protein has been identified in *Bacillus subtilis*. To characterise this protein, designated $PriA_{Bs}$, we constructed $priA_{Bs}$ mutants. These mutants are poorly viable, filamentous and sensitive to rich medium and UV irradiation. Replication of pAMβ1-type plasmids, which is initiated through the formation of a D-loop structure, and the activity of the primosome assembly site *ssiA* of plasmid pAMβ1 are strongly affected in the mutants. The purified $PriA_{Bs}$ protein binds preferentially to the active strand of *ssiA*, even in the presence of *B.subtilis* SSB protein (SSB_{Bs}). $PriA_{Bs}$ also binds stably and specifically to an artificial D-loop structure *in vitro*. These data show that $PriA_{Bs}$ recognises two specific substrates, *ssiA* and D-loops, and suggest that it triggers primosome assembly on them. $PriA_{Bs}$ also displays a single-stranded DNA-dependent ATPase activity, which is reduced in the presence of SSB_{Bs}, unless the *ssiA* sequence is present on the ssDNA substrate. Finally, $PriA_{Bs}$ is shown to be an active helicase. Altogether, these results demonstrate a clear functional identity between $PriA_{Ec}$ and $PriA_{Bs}$. However, $PriA_{Bs}$ does not complement an *E.coli priA* null mutant strain. This host specificity may be due to the divergence between the proteins composing the *E.coli* and *B.subtilis* PriA-dependent primosomes.

INTRODUCTION

The *Escherichia coli* PriA protein was characterised as being required for replication of bacteriophage φX174 and plasmid ColE1 (1–3). In these extra-chromosomal elements PriA promotes the initiation of replication through its specific binding to DNA, followed by the ordered assembly of several other proteins, PriB, PriC, DnaT, DnaC, DnaB replicative helicase and DnaG primase. This particular nucleoprotein complex has been referred to as the φX174-type primosome (4,5 and references therein; for recent reviews see 3,6). This primosome can be sequentially assembled on two distinct DNA sites, specifically bound by PriA. One, designated *pas* (primosome assembly site), was characterised in φX174 and ColE1. PriA binds to the *pas* in the single-stranded DNA (ssDNA) form, when it folds into a particular structure not bound by the SSB protein (7). The second type of PriA binding site is a D-loop structure (8,9). This three-stranded molecule is an early intermediate of the replication of ColE1-type plasmids (10). The cellular function of PriA has emerged more recently, following the identification of its gene. PriA is not essential in *E.coli*, suggesting that initiation of DNA replication promoted by this protein is accessory (11–14). However, disruption of the *priA* gene decreases cell viability, causes sensitivity to rich medium, filamentation, UV sensitivity, deficiency in recombination and constitutive induction of the SOS response. These phenotypes led to the hypothesis that the cellular role of the φX174-type primosome is to restart stalled DNA replication, as well as to repair some types of DNA damage by linking DNA recombination to replication (6,15–17). The structural nature of the DNA specifically recognised by PriA supports this proposal: the three-stranded DNA molecules generated by recombinational repair of the DNA triggers the ordered cascade of primosomal proteins, inaugurated by PriA, to recruit the DNA replication machinery (18,19). To better indicate its cellular

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function, the ϕX174-type primosome has been renamed the replication restart primosome (17).

In addition to specific binding of DNA, PriA is also a 3′→5′ helicase, translocating along ssDNA in the direction opposite to the replication fork helicase. This DNA melting activity was shown to be dispensable for the central role of PriA in *E.coli* (20). Nevertheless, it has recently been proposed that PriA helicase activity would generate the ssDNA needed for the loading of DnaB when the forked substrate specifically targeted by PriA is double stranded (21–24).

Current knowledge about primosomal proteins in *Bacillus subtilis* is less detailed. Counterparts of the *E.coli* replicative helicase and the primase are known in *B.subtilis* (25,26), but there are no obvious homologues of the PriB, PriC, DnaT and DnaC primosomal proteins (27). Characterisation of the sole primosome assembly site isolated so far in Gram-positive bacteria, the *ssiA* sequence carried by the plasmid pAMβ1, has pointed to the existence of a ϕX174-type primosome in *B.subtilis* (28). Three *B.subtilis* essential proteins, DnaB, DnaD and DnaI, which are not encoded in the *E.coli* genome, are required for chromosomal replication (29) and for *ssiA* activity (28). A potential PriA analogue was tentatively identified more recently in *B.subtilis* on the basis of sequence homology (27,30,31). Masai *et al.* purified this protein from an insoluble fraction and, following renaturation, showed it to be a DNA-dependent ATPase displaying helicase activity and able to bind to an artificial D-loop structure (31). Nevertheless, this initial characterisation did not show that the protein was involved in replication restart in *B.subtilis*. In this report we address precisely this question in order to establish the existence of a PriA-dependent primosome in *B.subtilis*. We present *in vivo* evidence demonstrating functional analogy between this *B.subtilis* protein and *E.coli* PriA. We also report purification of this protein, which we designate $PriA_{Bs}$, in a soluble form, with which we confirm and extend a previous *in vitro* study (31). More particularly, we report that $PriA_{Bs}$ displays a much stronger affinity for ssDNA than its *E.coli* counterpart. Altogether, this study confirms the existence of a PriA-dependent primosome in *B.subtilis*, built from a conserved initiator. Finally, we show that $PriA_{Bs}$ does not substitute for $PriA_{Ec}$ *in vivo*, suggesting a host specificity for this protein which may be due to the divergence between the primosomal partners acting after PriA in the two bacteria.

MATERIALS AND METHODS

Bacterial strains and growth media

The strains used in this study are listed in Table 1. *Bacillus subtilis* strains are all derivatives of strain 168. They were cultivated either in LB medium or in minimal medium (Spizizen's minimal salts) (32) supplemented with 0.1% D-glucose, 0.01% L-tryptophan, 0.1% casamino acids, 18 mg l^{-1} ammonium iron(III) citrate (~17% iron; Merck), as indicated in the text, and, when required, with 0.6 μg ml^{-1} erythromycin (Em), 4 μg ml^{-1} chloramphenicol (Cm) and 0.5 or 1 mM IPTG. Competent cells were prepared as described in Bron (32). The restriction map of the $priA_{Bs}$ chromosomal region in strains PPBJ65, PPBJ69, PPBJ117 and PPBJ120 was verified by Southern analysis. Strain CBB294 is a derivative of PPBJ120 disrupted for $priA_{Bs}$ but carrying a mutation suppressing the lack of $PriA_{Bs}$ (*dnaB75*) (33).

Plasmid constructions and preparations were done in *E.coli* strain MiT898. *Escherichia coli* strains were grown on Luria broth supplemented with 25 mg ml^{-1} thymine or minimal medium M63 (34) supplemented with 0.2% D-glucose. Spectinomycin (Spc) (60 μg ml^{-1}), ampicillin (100 μg ml^{-1}), kanamycin (Km) (50 μg ml^{-1}) and IPTG (for concentrations see Table 3) were added when required.

Plasmids and M13 derivatives

The plasmids used in this study are listed in Table 1. Plasmids of the pAPJ series were constructed by inserting various PCR fragments digested with *Eco*RI and *Bam*HI between the *Eco*RI and *Bam*HI sites of pMUTIN2. These PCR fragments were generated using chromosomal DNA of strain 168 as template and the following oligonucleotides as primers: pAPJ11, O_{PP8} and O_{PP9}; pAPJ12, O_{PP10} and O_{PP11}; pAPJ13, O_{PP12} and O_{PP14}; pAPJ14, O_{PP13} and O_{PP14}. O_{PP8}, 5′-CCGGAATTC*GCTCTG-TAACCATCAAAACCC*-3′ (+689 to +710); O_{PP9}, 5′-CGCGGATCC*GAAGCGGCCCTTGAAGCGG*-3′ (+1023 to +1002); O_{PP10}, 5′-CCGGAATTC*GCATGAAATGGCGCACCAGG*-3′ (+2079 to +2098); O_{PP11}, 5′-CGCGGATCC*TTACATCATCAT-ATAAGG*-3′ (+2419 to +2401); O_{PP12}, 5′-CCGGAATTC*T-CAAAACAAACCGGAGAGCGC*-3′ (–25 to –3); O_{PP13}, 5′-CCGGAATTC*AATTTTGCAGAAGTCATCGTTG*-3′ (+4 to +26); O_{PP14}, 5′-CGCGGATCC*GGAAGATCGGCTCCGT-GTGC*-3′ (+376 to +355). The $priA_{Bs}$ sequence is italicised and the *Eco*RI and *Bam*HI sites are underlined. Arbitrary coordinates for the $priA_{Bs}$ sequence retained in the oligonucleotides are indicated in parentheses, giving the value +1 to the A of the proposed translational start of the ORF (27). The sequence of the insert in pAPJ13 has been checked to ascertain the absence of mutation.

For construction of pSMG3, the $PriA_{Bs}$ coding sequence was PCR amplified from chromosomal DNA of strain 168 with O_{PP17} and O_{PP18} as primers and inserted into the *Nde*I and *Sap*I sites of pCYB1 (both blunted by Klenow filling in): O_{PP17}, 5′-TGAATTTTGCAGAAGTCATCG-3′; O_{PP18}, 5′-CATCAT-CATATAAGGATTCATATC-3′. This generated a triple fusion protein, $PriA_{Bs}$–intein–chitin binding domain (CBD), expressed under the control of *E.coli* transcriptional (the P_{tac} promoter, inducible by IPTG) and translational signals. The sequence of $priA_{Bs}$ carried by pSMG3 has been verified.

For construction of pSMG19, the SSB_{Bs} coding sequence was PCR amplified from chromosomal DNA of strain 168 with O_{SMG18} and O_{SMG19} as primers, digested with *Nde*I and *Sap*I and inserted in the sames sites of pTYB1: O_{SMG18}, 5′-GAATTCCATATGCTTAACCGAGTTGTATTAG-3′; O_{SMG19}, 5′-GAATTCGCTCTTCCGCAGAATGGAAGATC-ATCATCCGAGATG-3′ (sequences underlined in O_{SMG18} and O_{SMG19} represent a *Nde*I and a *Sap*I site, respectively). This generated a triple fusion protein SSB_{Bs}–intein–CBD, expressed under the control of transcriptional and translational signals of bacteriophage T7. The sequence of ssb_{Bs} carried by pSMG19 has been verified.

pAPJ41 is a derivative of the pGB2 vector (35) which allows inducible expression of $PriA_{Bs}$ in *E.coli* and which does not need $PriA_{Ec}$ for replication. This plasmid was constructed in several steps. First, the chromosome of strain PPBJ69 was digested with *Swa*I, ligated and transformed into *E.coli* to

Table 1. Plasmids, bacteriophages and strains used in this study

Strains, plasmids and phages	Description	Reference
***E.coli* strains**		
MiT898	Δ*endA araD139* Δ[*ara-leu*] *galU galK hsdM hsdS rpsL* Δ[*lacIOPZYA*] X74	(56)
B834(DE3)	*hsdS gall cIts857 ind1 metSam7 nin5 lacUV5-T7 gene 1*	(57)
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	(38)
DM4000	(*lac-pro*)XIII *hisG4 argE3 ara14 xyl5 mtl1 rpsL31 sulA*::*Mu-d* (*Ap*, *lac*, *B*::Tn*9*)	(49)
JC18983	DM4000 *priA2*::*Km*	(49)
***B.subtilis* strains**		
168	*trpC2*	C. Anagnostopoulos
PPBJ65	168 $priA_{Bs}$::pAPJ12	This work
PPBJ69	168 *priAind*::pAPJ13	This work
PPBJ117	168 $priA2_{Bs}$::pAPJ14	This work
PPBJ120	168 $priA1_{Bs}$::pAPJ11	This work
CBB294	168 $priA1_{Bs}$::pAPJ11 *dnaB75*	(55)
Plasmids and phages		
pCYB1	pBR322-based vector (IMPACT system)	New England Biolabs
pTYB1	pBR322-based vector (IMPACT system)	New England Biolabs
pVA798ΔRCR	pIP501 derivative	(48)
pMUTIN2	pBR322 *lacZ lacI* Em^R P_{spac}	(58)
pGB2	pSC101-based vector	(35)
pADG6406-1	pADG6406 *ssiA*$^+$ carrying vector	(28)
pADG6406-2	pADG6406 *ssiA*$^-$ carrying vector	This work
pAPJ11	pMUTIN2 P_{spac}: (nt +689 to +1023 of $priA_{Bs}$)	This work
pAPJ12	pMUTIN2 P_{spac}: (nt +2079 to +2419 of $priA_{Bs}$)	This work
pAPJ13	pMUTIN2 P_{spac}: (nt –25 to +376 of $priA_{Bs}$)	This work
pAPJ14	pMUTIN2 P_{spac}: (nt +4 to +376 of $priA_{Bs}$)	This work
pSMG3	pCYB1::$priA_{Bs}$	This work
pSMG19	pTYB1::ssb_{Bs}	This work
pAPJ2	pC194 and pBSSK$^-$ joined by *Hin*dIII site	This work
pAPJ9	pAPJ2 carrying the *ssiA* active strand on ssDNA	This work
pAPJ10	pAPJ2 carrying the *ssiA* inactive strand on ssDNA	This work
pAPJ41	pGB2, P_{tac}-$priA_{Bs}$	This work
pAPJ43	pGB2, P_{tac}-$priA_{Ec}$	This work
M13-*ssiA*$^+$	M13mp19 carrying the *ssi*A active strand on ssDNA	This work
M13-*ssiA*$^-$	M13mp19 carrying the *ssiA* inactive strand on ssDNA	This work

isolate a plasmid, pAPJ19, which contains the whole $priA_{Bs}$ ORF and additional 3′-flanking sequences. Second, the 3′ end of the $priA_{Bs}$–*intein*–*CBD* ORF in pSMG3 (*Sac*I–*Bam*HI) was exchanged for the 3′ end of the $priA_{Bs}$ ORF of pAPJ19 (*Sac*I–*Bgl*II) to generate plasmid pSMG4. Finally, to obtain pAPJ41 the *Eco*47III–*Pst*I restriction fragment of pSMG4 carrying the *laci*^{q} gene and the $priA_{Bs}$ ORF placed under the control of the P_{tac} promoter was cloned in the pGB2 vector between the *Sma*I and *Pst*I sites located in the polylinker. pAPJ43 is almost identical to pAPJ41 except that it carries the $PriA_{Ec}$ coding sequence in place of $PriA_{Bs}$. It was constructed in two steps. First, the *Nde*I–*Pvu*I fragment of the $PriA_{Ec}$-expressing plasmid described in Nurse *et al*. (36) was exchanged with a similarly cleaved fragment of pSMG3 to give plasmid pAPJ42. Then the *Mlu*I–*Hin*dIII fragment of pAPJ42 carrying the whole P_{tac}–$priA_{Ec}$ artificial gene was exchanged for the corresponding *Mlu*I–*Hin*dIII fragment of pAPJ41 to give pAPJ43.

The two M13mp19 derivatives carrying the *ssiA* sequence in both orientations, M13-*ssiA*$^+$ and M13-*ssiA*$^-$, were constructed in two steps. The *ssiA* sequence (145 nt, coordinates 4712–4856 in pAMβ1) (37) was generated by PCR using pIL253 as template and O_{PP1} and O_{PP3} as primers, and inserted in both orientations in the *Sma*I site of the polylinker of plasmid pAPJ2, giving plasmids pAPJ9 and pAPJ10: O_{PP1}, 5′-TAATTATTAG-GGGGAGAAGGAGAGAG-3′; O_{PP3}, 5′-CCTATAAAAGAT-AGAAAATTAAAAAATC-3′. The sequence of *ssiA* has been

verified by DNA sequencing. The activity of *ssiA* has been verified in *B.subtilis* by showing that the ssDNA of plasmid pAPJ9 is efficiently converted into double-standed DNA (dsDNA) *in vivo*. Finally, the *Sal*I–*Eco*RI fragments carrying *ssiA* from pAPJ9 and pAPJ10 were cloned into M13mp19 similarly cut, to give M13-*ssiA*$^+$ and M13-*ssiA*$^-$, respectively.

Plating efficiency and UV survival tests

Bacillus subtilis strains were grown to mid-log phase in minimal medium containing Em with and without IPTG (1 mM) as indicated (see legend to Fig. 2 and Table 2). To measure the plating efficiency, cultures were diluted appropriately and plated on minimal medium and on LB similarly supplemented with Em and IPTG, and incubated for 16–40 h at 37°C. To measure UV survival, minimal medium plates were irradiated immediately after plating with a 2 J m^{-2} dose of UV for different time periods and incubated for 16–40 h at 37°C. The tests with *E.coli* were performed similarly. Strains were grown in minimal medium containing Spc, supplemented or not with IPTG, as indicated (see Table 3), and spread on plates not supplemented with IPTG.

DNA manipulation and analysis

Standard techniques were used for DNA manipulation and cloning in *E.coli* (38). Total DNA from exponentially growing *B.subtilis* cells was extracted as described (28). After agarose gel electrophoresis, plasmid DNA was revealed by Southern blotting with α-^{32}P-radiolabelled probe generated with a nick translation kit (Roche) with purified plasmid DNA in the presence of [α-^{32}P]dATP (ICN). The different plamid species were revealed and quantified with a Storm apparatus (Molecular Dynamics) and ImageQuant software.

DNA sequencing of PCR products or plasmid templates was done with the PRISM sequencing kit (Applied Biosystems) and resolved on an automated DNA sequencer (Applied Biosystem 377A).

M13 ssDNA was prepared from TG1 cells as described (38). pAPJ9 ssDNA was similarly prepared from TG1 cells containing the helper phage M13K07 (38).

DNA probes used for the electrophoretic mobility shift assays were prepared by several means. The 174 nt long *ssiA*$^{+/-}$ ssDNA probes were excised by restriction from M13-*ssiA*$^+$ and M13-*ssiA*$^-$ ssDNA with the use of the following oligonucleotides complementary to restriction sites flanking *ssiA*: O_{SMG42}, 5′-CGTCGACCTGCAGCATGCA-3′; O_{SMG43}, 5′-GCCGATGAATTCGATCCT-3′; O_{SMG44}, 5′-GCCGATGAATTCGATTAAT-3′. The underlined sequences represent a *Pst*I site in O_{SMG42} and an *Eco*RI site in O_{SMG43} and O_{SMG44}. The O_{SMG42}/O_{SMG43} pair was used to excise *ssiA*$^+$ from the M13-*ssiA*$^+$ ssDNA template and, similarly, the O_{SMG42}/O_{SMG44} pair to excise *ssiA*$^-$ from M13-*ssiA*$^-$. The ssDNA (50 nM) was heated at 65°C for 10 min in a 1 ml solution containing 100 mM NaCl, 20 mM Tris, pH 7.5, 1 mM DTT and 10 mM $MgCl_2$ and the complementary pair of oligonucleotides (500 nM) were allowed to anneal by cooling the mixture slowly to 37°C. The DNA was then digested to completion with *Pst*I and *Eco*RI. The 174 nt fragments released were purified from the larger bacteriophage DNA fragment and smaller oligonucleotides by gel filtration on Superose 6 (Pharmacia). They were then treated with shrimp phosphatase (Pharmacia) before 5′-end-labelling using [γ-^{32}P]ATP (ICN) and T4 polynucleotide kinase. Finally, both fragments were purified by electrophoresis on 5% (w/v) polyacrylamide gels and recovered by passive elution in buffer E (10 mM Tris, pH 8, 1 mM EDTA, 0.2% SDS, 0.3 M NaCl) overnight at 30°C. The size and uniformity of the fragments was verified on a denaturing polyacrylamide gel using a sequence size ladder.

dsDNA and branched DNA molecules were prepared by annealing the following purified oligonucleotides, which are identical to those used by McGlynn *et al.* (8) for the study of $PriA_{Ec}$ and RecG binding to D-loop and bubble structures: O_{SMG27}, 5′-GACGCTGCCGAATTCTACCAGTGCCTTGCTAGCATCTTTGCCCACCTGCAGGTTCACCC-3′; $O_{SMG27comp}$, 5′-GGTGAACCTGCAGGTGGGCAAAGATGTCCTAGCAAGGCACTGGTAGAATTCGGCAGCGTC-3′; O_{SMG28}, 5′-GGGTGAACCTGCAGGTGGGCGGTGCTCATCGTAGGTTAGTTGGTAGAATTCGGCAGCGTC-3′; O_{SMG29}, 5′-AAAGATGTCCTAGCAAGGCAC-3′. The D-loop was made by annealing O_{SMG27}, O_{SMG28} and O_{SMG29} mixed at a molar ratio of 1:2:3, respectively. The bubble was made by annealing O_{SMG27} and O_{SMG28} and the dsDNA with O_{SMG27} and $O_{SMG27comp}$ at a molar ratio of 1:2. Annealing was performed by heating the oligonucleotides in buffer A (10 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl) for 5 min at 95°C, then they were left for 10 min at 65°C, followed by slow cooling to room temperature. In each combination, the O_{SMG27} oligonucleotide was 5′-end-labelled with [γ-^{32}P]ATP (ICN) and T4 polynucleotide kinase prior to annealing. The expected synthetic DNA substrates were purified by elution in buffer E after separation from free oligonucleotides by native electrophoresis in a 5% polyacrylamide gel. The Ost4 oligonucleotide was used as a ssDNA probe, prepared as for the other DNA substrates: Ost4, 5′-GCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTG-3′.

For each probe, the concentrations of DNA substrates used in the gel mobility assay were estimated by monitoring the specific activity of the labelled oligonucleotide after end labelling and the final activity of the purified substrate.

Purification of $PriA_{Bs}$, $PriA_{Ec}$ and SSB_{Bs}

$PriA_{Bs}$ and SSB_{Bs} were expressed and purified using the IMPACT system (New England Biolabs). $PriA_{Bs}$ and SSB_{Bs} proteins fused to the intein–CBD tag were overproduced in strains MiT898 and B834 (DE3), respectively. To limit protein aggregation, cell growth was carried out at 25°C. Optimal conditions for $PriA_{Bs}$ production were IPTG induction for 3 h at the end of exponential growth, and for SSB_{Bs} production overnight growth without induction. Cells were harvested, resuspended in HEN_{500}-T buffer (20 mM HEPES pH 7.6, 0.1 mM EDTA, 500 mM NaCl, 0.1% Triton X-100) and broken by sonication (Bioblock Vibracell 72408 sonicator, used as recommended by the supplier). The lysate was centrifugated at 4°C for 1 h at 20 000 *g*, the supernatant loaded onto chitin beads and the protein separated from intein by addition of 30 mM DTT and incubation overnight at 4°C. The protein was eluted and further purified by conventional chromatography. In the case of $PriA_{Bs}$, the protein in HEN_{100}-D buffer (20 mM HEPES pH 7.6, 0.1 mM EDTA, 100 mM NaCl, 1 mM DTT), was loaded onto a Hi-Trap SP-Sepharose column (Pharmacia) and eluted with a linear NaCl gradient in HED

buffer. The fractions containing $PriA_{Bs}$ were loaded onto a Hi-Trap heparin column (Pharmacia) and the protein bound was eluted with HEN_{100}-D. It was diluted twice with 100% glycerol and stored at –20°C. The yield of $PriA_{Bs}$ was ~2 mg protein l^{-1} of culture and its purity was estimated to be 95%. In the case of SSB_{Bs}, the protein eluted from the chitin beads was further purified by successive chromatography on Hi-Trap Q-Sepharose and heparin columns (Pharmacia). The eluted proteins were finally treated by heat (at 85°C for 5 min) to eliminate by precipitation the contaminants which co-purify with SSB_{Bs}. We have shown that such a heat treatment does not modify SSB_{Bs} binding activity to ssDNA, as shown previously for SSB of *E.coli* (10). We have also observed by gel filtration on a Superose 12 column (Pharmacia) that purified SSB_{Bs} is a tetrameric protein, like its *E.coli* counterpart. The yield of SSB_{Bs} was ~0.5 mg protein l^{-1} and its purity was estimated to be 95%.

The same purification procedure was used for $PriA_{Ec}$ as for $PriA_{Bs}$, except that expression was in strain JC19008 carrying plasmid pSMG24 (39). The yield of purified soluble protein was similar in both cases.

Immunodetection of $PriA_{Bs}$

Immunisation against $PriA_{Bs}$ and serum preparation in the rabbit was entrusted to Eurogentec. Prior to injection, $PriA_{Bs}$ protein was further purified to homogeneity by electrophoresis on a SDS–polyacrylamide gel. The antibodies directed against $PriA_{Bs}$ were purified from serum by the method described in Pringle *et al.* (40) after coupling $PriA_{Bs}$ to Affi-Gel10 as recommended by the supplier (Bio-Rad). The relative levels of $PriA_{Bs}$ in the different strains used were determined by immunoblot analysis with the purified antibodies. Cells were grown exponentially in LB medium suplemented with IPTG. Cell lysates were prepared by lysozyme treatment of the harvested cells followed by brief sonication. The same amounts of total cellular proteins of each strain were then fractionated by SDS–PAGE on 8% gels and transferred to a Hybond PVDF membrane (Amersham) by electroblotting using a semi-dry transfer system. $PriA_{Bs}$ immunodetection was carried out as described in the ECL^+ kit (Amersham). Purified anti-$PriA_{Bs}$ antibodies were diluted 1/500 for the hybridisation step. Protein G–horseradish peroxidase (Bio-Rad) was used to reveal anti-$PriA_{Bs}$ with a Storm apparatus (Molecular Dynamics) and quantification was with ImageQuant software.

Gel mobility shift assays

For some experiments reported (see Figs 4 and 5) the reaction mixture (40 µl) contained 10 mM HEPES pH 7.5, 3 mM DTT, 200 mM NaCl, 0.2 mg/ml bovine serum albumin (BSA) and γ-^{32}P-labelled DNA, at the concentrations specified in the figure legends. The amounts of purified $PriA_{Bs}$ and SSB_{Bs} (expressed in nM) are indicated in the figures. Reaction mixtures were incubated at 30°C for 15 min and analysed by gel electrophoresis through a 5 or 4% (80:1) polyacrylamide gel as indicated in the figure legends, following addition of 10 µl of 50% glycerol (supplemented with 0.04% xylene cyanol and 1 mg ml^{-1} BSA). In the case of the binding experiments performed with SSB_{Bs} and $PriA_{Bs}$, SSB_{Bs} was pre-incubated for 15 min at 30°C with the ssDNA substrates prior to addition of $PriA_{Bs}$, which was then allowed to further interact for 15 min at 30°C. For other experiments (see Fig. 6), various amounts of $PriA_{Bs}$ and $PriA_{Ec}$ were incubated with labelled DNA substrates (0.1 nM) in 20 µl of R buffer (50 mM HEPES, 1 mM DTT, 1 mM EDTA, 0.1 mg ml^{-1} BSA, 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^{-1} BSA) was added and the samples were loaded on a 5% polyacrylamide gel (30:1) containing 5% glycerol and 0.25× TBE. Three different electrophoresis buffers were used: TAM (6 mM Tris, 5 mM Na acetate, 2 mM Mg acetate) (see Fig. 5), TEG (25 mM Tris, 0.19 M glycine, 1 mM EDTA) (see Figs 4 and 5) or 0.25× TBE (90 mM Tris–borate, 2 mM EDTA) (see Fig. 6). Electrophoresis was at 4°C at 12 V cm^{-1} for 2–4 h with circularisation of the buffer. Following electrophoresis, gels were dried under vacuum, revealed with a Storm apparatus (Molecular Dynamics), the radioactivity quantified with ImageQuant software and the apparent K_d determined according to Riggs *et al.* (41).

ATPase assay

ATPase activity was assayed by linking ATP hydrolysis to the oxidation of NADH as described previously (42). The dependence of the ATPase reaction on ssDNA cofactor was examined by the above method at 37°C in a buffer containing 50 mM HEPES pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 0.2 mg ml^{-1} BSA. The concentrations of DNA (expressed in nM nt), ATP and proteins $PriA_{Bs}$ and SSB_{Bs} (in nM) used in the assays are indicated in the figure legends, as well as the time course of the reaction. Kinetic experiments were performed in a UV/VIS spectrometer Lambda 20 (Perkin Elmer). Values for the Michaelis–Menten constants k_{cat} and K_m for ATP at saturating amounts of ssDNA were derived by fitting data directly to the Michaelis–Menten equation.

Helicase assay

The same standard forked DNA used for study of the *Thermus aquaticus* helicase (43) was used to assay the helicase activity of $PriA_{Bs}$. It was similarly prepared by annealing the following two purified oligonucleotides, after labelling of the 5′ end of oligonucleotide O_{PP210} with T4 polynucleotide kinase (NEB): O_{PP210}, 5′-T_{30}CGAGCACCGCTGCGGCTGCACC-3′; O_{PP211}, 5′-GGTGCAGCCGCAGCGGTGCTCG T_{30}-3′.

Helicase assays were performed with the indicated amount of $PriA_{Bs}$ added to 1 nM DNA substrate in 20 µl of reaction buffer composed of 20 mM Tris pH 7.5, 50 mM NaCl, 3 mM $MgCl_2$, 4 mM DTT, 20 µg ml^{-1} BSA, with or without addition of ATP (5 mM) as indicated. After 30 min incubation at 30°C each reaction was stopped with 5 µl of S solution (3% SDS, 100 mM EDTA, 40% glycerol, 0.1% xylene cyanol) and run through a 12% polyacrylamide gel in 1× TBE. Following electrophoresis, gels were dried under vacuum, revealed with a Storm apparatus (Molecular Dynamics) and the radioactivity present in the forked substrate and in the ssDNA product quantified with ImageQuant software to calculate the level of helicase activity expressed as a percentage of ssDNA generated in the assay.

RESULTS

Bacillus subtilis encodes a homologue of the *E.coli* primosomal PriA protein

The sequence of a *B.subtilis* ORF encoding a homologue of $PriA_{Ec}$ has been reported (27,30). This ORF, designated $priA_{Bs}$, is located at 140° on the *B.subtilis* map. On the basis of sequence analysis, $priA_{Bs}$ is the second ORF of an operon including 12 ORFs. The level of homology of $PriA_{Bs}$ with the *E.coli* protein is highly significant, with 32% identity and 65% similarity distributed along the two proteins. However, $PriA_{Bs}$ contains 70 additional amino acids, clustered in the first third of the protein. Two distinct functional regions present in $PriA_{Ec}$ are conserved in the *B.subtilis* protein. One corresponds to the seven canonical motifs typical of many helicases and the other to a cysteine-rich region, which may be organised in two consecutive small zinc finger-like motifs (Fig. 1A).

To show that $priA_{Bs}$ is transcribed and translated, its putative product has been overproduced in *E.coli* and purified (Fig. 1B) and specific antibodies directed against this protein have been prepared and purified (see Materials and Methods). Whole cellular protein extracts from *B.subtilis* were analysed by western blotting using the anti-$PriA_{Bs}$ antibodies (Fig. 1C). A protein identical in size with the purified $PriA_{Bs}$ was detected in the *B.subtilis* wild-type strain (Fig. 1C, lane 5). The specifity of the signal was demonstrated by its disappearance in strain CBB294, in which $priA_{Bs}$ has been disrupted (see below; Fig. 1C, lane 8). Therefore, $PriA_{Bs}$ is expressed in *B.subtilis*. The number of $PriA_{Bs}$ molecules per cell is between 50 and 100, as deduced from the western blot analysis.

Bacillus subtilis priA mutants are poorly viable, sensitive to rich medium and UV irradiation

To study the role of $PriA_{Bs}$ in cell physiology, we constructed $priA_{Bs}$ mutants. For this purpose we disrupted $priA_{Bs}$ by transforming *B.subtilis* 168 cells with non-replicative Em^R plasmids carrying internal fragments of $priA_{Bs}$ (pAPJ11 and pAPJ14). As a control, we used a plasmid carrying the 3′ end of $priA_{Bs}$, and thus expected to preserve the integrity of $priA_{Bs}$ upon insertion (pAPJ12). As *E.coli* null *priA* mutants are viable on minimal medium but not on rich medium (13), disruption was carried out on minimal medium. Em^R transformants were obtained with the disrupting plasmids, giving strains PPBJ117 and PPBJ120, which carried $priA_{Bs}$ alleles designated $priA2_{Bs}$ and $priA1_{Bs}$, respectively (Fig. 1D). However, colonies were much smaller in size than upon transformation with the control plasmid. As expected from the size of the colonies, these strains grow slowly in minimal medium (doubling time >160 min) and microscopic examination of the bacteria revealed that they were filamentous. Measurements of the plating efficiencies of these strains showed that they were 40–100-fold less viable than the control strain on minimal medium and were sensitive to rich medium (Table 2), as well as to UV irradiation (Fig. 2). A strain in which $priA_{Bs}$ is under the control of the P_{spac} promoter was also constructed (PPBJ69 carrying the *priAind* allele) (Fig. 1D). This strain displayed wild-type phenotypes in the presence of IPTG (see Table 2 and Fig. 2), although its level of $PriA_{Bs}$ was 3-fold lower than in the control strains, as shown by western blot analysis (Fig. 1C,

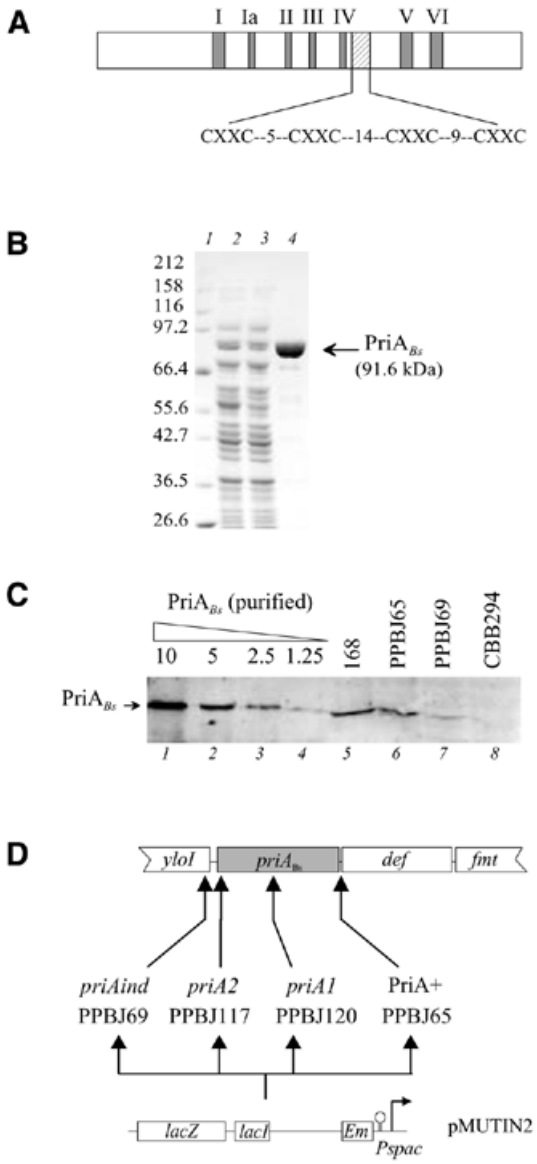

Figure 1. The *B.subtilis* PriA protein. (**A**) Schematic representation of the *B.subtilis* PriA protein. The double signature which characterises $PriA_{Ec}$ and which is conserved in $PriA_{Bs}$ is presented: (i) the seven conserved motifs (I–VI) of the 3′→5′ helicase subfamily into which $PriA_{Ec}$ is classified (filled boxes); (ii) the two putative zinc finger domains (hatched box); the spacing between the conserved cysteines is indicated. (**B**) Purification of $PriA_{Bs}$. The $PriA_{Bs}$ protein was purified in *E.coli* using the IMPACT system. Proteins were resolved by 8% SDS–PAGE and stained with Coomassie brilliant blue. Lane 1, molecular weight standards; lane 2, soluble proteins of *E.coli* cells induced to express the fusion $PriA_{Bs}$–intein–CBD; lane 3, flow-through of the chitin column; lane 4, elution from the chitin column after DTT cleavage. The purity obtained at this step has been estimated to be >95%. (**C**) Immunodetection of $PriA_{Bs}$ in *B.subtilis*. Protein preparations were resolved by 8% SDS–PAGE, transferred to PVDF membrane and probed with purified anti-$PriA_{Bs}$ antibodies. Lanes 1–4, the indicated amounts (in ng) of purified $PriA_{Bs}$; lanes 5–8, total proteins extracted from an equal amount of cells grown in the presence of IPTG from strain 168 and derivatives PPBJ65, PPBJ69 and CBB294 (a derivative of PPBJ120 carrying an extragenic mutation suppressing the lack of $PriA_{Bs}$, designated *dnaB75*) (33). The $priA_{Bs}$ allele present in each strain is indicated. (**D**) Schematic representation of the $priA_{Bs}$ chromosomal region in *B.subtilis* strains used in this study. $priA_{Bs}$ and flanking ORFs are represented by boxes. *yloI* is an ORF of unknown function; *def* and *fmt* are homologues of the *E.coli def* and *fmt* genes. In the lower part a schematic map of the pMUTIN2 integrative vector carrying the IPTG-inducible promoter P_{spac} is presented. Vertical arrows below the $priA_{Bs}$ ORF indicate the insertion sites of pMUTIN2 derivatives. The names of the resulting strains with their associated *priA* allele are indicated.

Table 2. Plating efficiency of *priA* mutants on minimal and rich medium

Strain	$priA_{Bs}$ genotype[a]	c.f.u. × 10^8 per OD_{650}[b]		
		IPTG	Minimal medium	Rich medium
PPBJ120	*1*	+	0.03	<0.0001
PPBJ117	*2*	+	0.07	<0.0001
PPBJ65	+	+	3.3	4.3
PPBJ65	+	–	2.6	1.9
PPBJ69	*ind*	+	1.9	1.5
PPBJ69	*ind*	–	0.19	0.06

[a]See Figure 1D.
[b]The strains were grown to mid-log phase in minimal medium in the presence (+) or absence (–) of IPTG (1 mM). Appropriate dilutions of the cultures were plated on either rich or minimal medium containing IPTG. c.f.u., colony forming units. Values are the average of between two and four independent determinations, except for PPBJ69 in rich medium + IPTG, which was tested only once.

compare lanes 5 and 6 with 7). In the absence of IPTG, however, this strain displayed phenotypes similar to those of disrupted strains: filamentation, small colonies and poor viability in minimal medium, as well as sensitivity to rich medium (Table 2) and sensitivity to UV irradiation (Fig. 2).

$PriA_{Bs}$ is required for primosome assembly on *ssiA* and D-loops *in vivo*

In *E.coli*, PriA was initially characterised as being required for two distinct modes of replication displayed by extrachromosomal elements. The first relies on a *pas* sequence required for replication of the ssDNA circular intermediate generated during rolling circle replication of bacteriophage ϕX174 (10). The second depends on a D-loop structure synthesised at an early step of the θ replication mode of ColEI-type plasmids (10). Interestingly, these two schemes of replication have been characterised in *B.subtilis* and have been shown to rely on identified primosomal proteins of this bacterium (28). Therefore, we tested $PriA_{Bs}$ dependence for these two modes of extrachromosomal replication. The *pas*-mediated conversion of ssDNA to dsDNA was measured in a plasmid rolling circle assay with the use of the *pas* sequence *ssiA* from plasmid pAMβ1 (28). D-loop-mediated replication was measured using an appropriate pAMβ1 derivative (28,44–46). The experiments were carried out in strains harbouring the conditional *priAind* allele, allowing modulation of $priA_{Bs}$ expression with the use of IPTG (Fig. 1).

To test the activity of *ssiA*, we used derivatives of the rolling circle plasmid pC194, which produce a ssDNA intermediate which is not efficiently converted to the dsDNA form in *B.subtilis* (47). This circular ssDNA molecule is detected by Southern blotting following electrophoresis of total DNA prepared from *B.subtilis* cells harbouring such plasmids (Fig. 3A). In the $PriA^+$ strain PPBJ65, conversion of ssDNA to the dsDNA form is promoted by *ssiA* in the active orientation ($ssiA^+$) but not in the inactive orientation ($ssiA^-$) (Fig. 3A, lanes 3 and 1). In contrast, in the *priAind* strain, conversion is inefficient, irrespective of the *ssiA* orientation. Conversion was inefficient both in the absence (Fig. 3A) and presence of IPTG (not

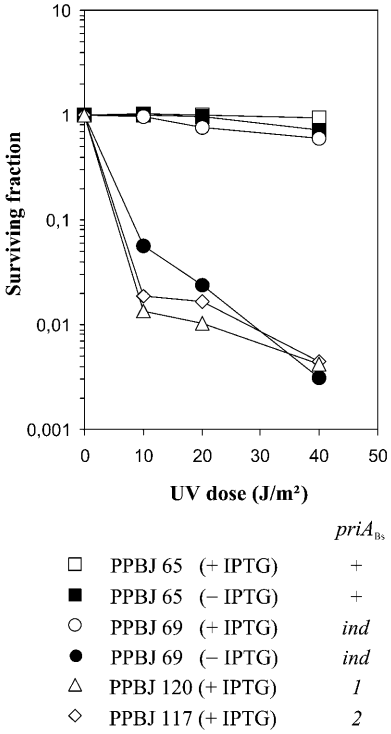

Figure 2. UV sensitivity of *B.subtilis priA* mutant strains. Strains were grown in minimal medium supplemented with Em in the presence or absence of IPTG (1 mM), as indicated. Appropriate dilutions were plated on the same medium and immediately irradiated with different doses of UV. After incubation for 2 days at 37°C, the fraction of surviving cells was determined and plotted against the UV dose. Each point is the mean value of two independent determinations.

shown), indicating that the diminished level of $PriA_{Bs}$ in the induced *priAind* strain is not sufficient to support *ssiA* activity on a multicopy extrachromosomal element, while it appears sufficient for chromosomal replication.

pAMβ1-type plasmids replicate by a θ mechanism that involves an early D-loop intermediate, in which the $ssiA^+$ sequence is present on the ssDNA portion of the molecule (44–46). In the *priAind* mutant grown without IPTG the copy number of plasmid pVA798ΔRCR (a pAMβ1-type plasmid; 48) was ~10-fold lower than in $PriA_{Bs}^+$ cells and the plasmid accumulated ssDNA (Fig. 3B). The ssDNA corresponded to the plasmid lagging strand template, as demonstrated by the use of strand-specific probes (not shown). A similar replication defect was observed when the cells were grown in the presence of IPTG (not shown), presumably reflecting the low $PriA_{Bs}$ level in the cells. The defect of pVA798ΔRCR replication in the absence of $PriA_{Bs}$ led to loss of the plasmid from *priAind* cells upon prolonged growth without IPTG and to its inability to become established in the $priA1_{Bs}$ strain (not shown). We conclude that $PriA_{Bs}$ is required for replication of pAMβ1-type plasmids and acts presumably by promoting primosome assembly on the D-loop intermediate. The *ssiA* sequence unmasked on the D-loop was previously shown not to be essential for pAMβ1 replication, suggesting a *ssiA*-independent mechanism(s) of primosome assembly (28). In *priAind* mutants pAMβ1 derivatives lacking *ssiA* exhibited a reduced copy number and accumulated ssDNA (not shown).

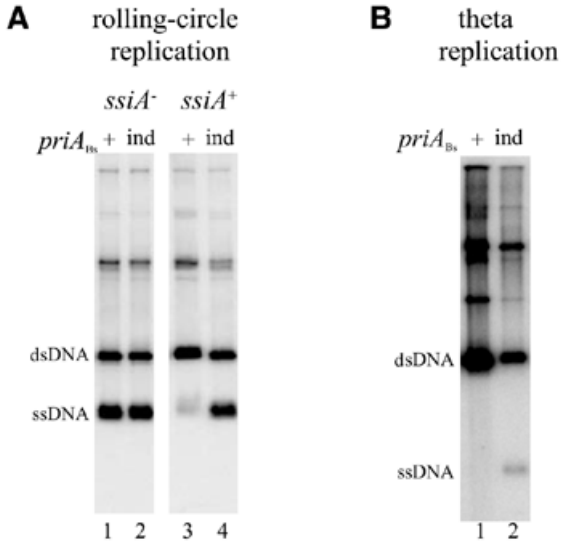

Figure 3. PriA$_{Bs}$ is required for *ssiA* activity and pAMβ1-type plasmid replication. (**A**) *Bacillus subtilis* PPBJ65 (PriA$_{Bs}^{+}$) and PPBJ69 (*priAind*) strains harbouring pC194-derived plasmid pADG6406-1 (*ssiA*$^{+}$) or pADG6406-2 (*ssiA*$^{-}$) were grown overnight in LB supplemented with IPTG, Em and Cm, then diluted 100-fold in fresh medium without IPTG and cultivated for ~5 h. Total DNA was extracted and analysed by Southern hybridisation using ^{32}P-labelled pC194 DNA as probe. (**B**) *Bacillus subtilis* PPBJ65 (PriA$_{Bs}^{+}$) and PPBJ69 (*priAind*) strains harbouring pAMβ1-derived plasmid pVA798ΔRCR were grown to mid-log phase without IPTG and their total DNA was extracted and analysed by Southern hybridisation, using ^{32}P-labelled pVA798ΔRCR as probe. ssDNA and dsDNA, single-stranded and double-stranded DNA, respectively.

We conclude that a *ssiA*-independent mechanism(s) of primosome loading during pAMβ1 replication is dependent on PriA$_{Bs}$ and assume that PriA$_{Bs}$, like PriA$_{Ec}$, triggers primosome assembly on D-loops.

PriA$_{Bs}$ binds preferentially to the primosome assembly site *ssiA*

Analysis of ssDNA conversion to dsDNA indicated that PriA$_{Bs}$ acts *in vivo* at *ssiA*. In order to test whether PriA$_{Bs}$ recognises the active strand of *ssiA*, we carried out gel shift analyses with the purified protein. For these experiments two 174 nt long ssDNA substrates, carrying either the active or inactive strand of *ssiA* (designated *ssiA*$^{+}$ and *ssiA*$^{-}$, respectively), were prepared and radiolabelled (see Materials and Methods). Somewhat surprisingly, the two substrates behaved differently in non-denaturing gels: the *ssiA*$^{+}$ strand migrated faster than the *ssiA*$^{-}$ strand (Fig. 4A, lanes 1 and 6) and was accompanied by a minor, slowly migrating product (Fig. 4A, lane 1). In contrast, the two strands appeared identical in size in a gel under denaturing conditions (data not shown). This suggests that the two strands might fold into different secondary or tertiary structures. Binding experiments conducted with *B.subtilis* SSB protein (SSB$_{Bs}$; see Materials and Methods for purification procedure) led to the same conclusion. Both strands were efficiently recognised by SSB$_{Bs}$, but the binding patterns were clearly different (Fig. 4A). The faster migrating form of *ssiA*$^{+}$ gave primarily two shifted bands while *ssiA*$^{-}$ gave an additional band at saturating amounts of SSB$_{Bs}$. The slower *ssiA*$^{+}$ form gave multiple shifted bands. This indicates

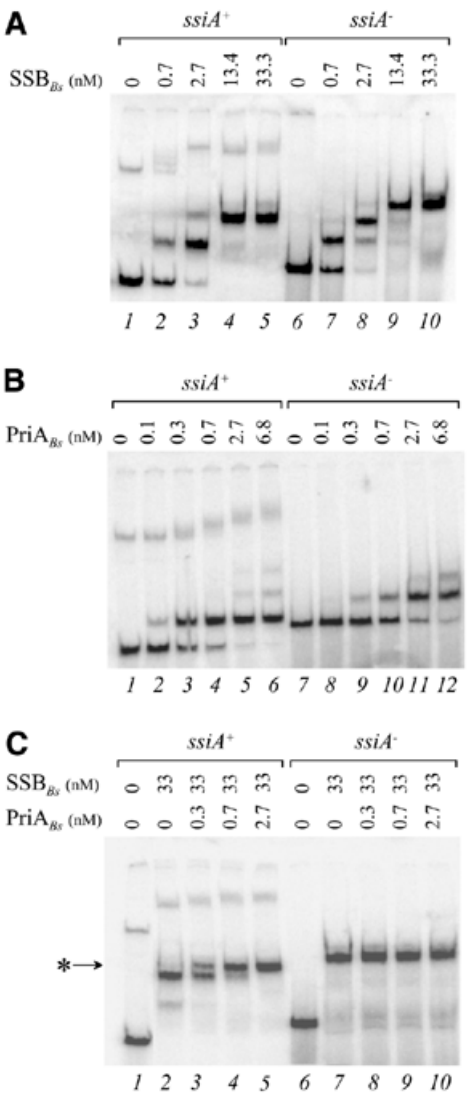

Figure 4. PriA$_{Bs}$ binds to the active strand of *ssiA* in the presence of SSB$_{Bs}$. Protein–DNA complexes were generated at 30°C for 30 min and electrophoresed on a 4% non-denaturing polyacrylamide gel in TGE buffer at 4°C. A 174 nt long ssDNA fragment containing *ssiA*$^{+}$ or *ssiA*$^{-}$ sequence was 5′ radiolabelled and used as a DNA binding substrate. Final protein concentrations of each binding experiment (expressed in nM) are indicated above each lane. SSB$_{Bs}$ and PriA$_{Bs}$ refer to *B.subtilis* SSB and PriA proteins, respectively. (**A**) Binding of SSB$_{Bs}$. (**B**) Binding of PriA$_{Bs}$. (**C**) Binding of PriA$_{Bs}$ to *ssiA* strands covered by SSB$_{Bs}$. The star indicates the PriA$_{Bs}$ supershift observed with *ssiA*$^{+}$ substrate covered by SSB$_{Bs}$.

that a part of the *ssiA*$^{+}$ sequence may be poorly accessible to a SSB$_{Bs}$ tetramer.

PriA$_{Bs}$ binding to the *ssiA* substrates indicated a 7-fold preference for *ssiA*$^{+}$ (K_d = 0.45 nM) over *ssiA*$^{-}$ (K_d = 3.1 nM) (Fig. 4B). PriA$_{Bs}$ generated one complex with the *ssiA*$^{+}$ substrate at low concentration and up to three complexes at higher concentrations. The slow migrating form of *ssiA*$^{+}$ was also shifted by PriA$_{Bs}$ to poorly resolved multiple bands. These experiments revealed that PriA$_{Bs}$ can bind stably to the inactive *ssiA* strand. This interaction does not depend on the presence of the *ssiA* sequence since it occurs with any ssDNA molecule longer than 41 nt, but not with dsDNA, to which PriA$_{Bs}$ binds poorly (data not shown).

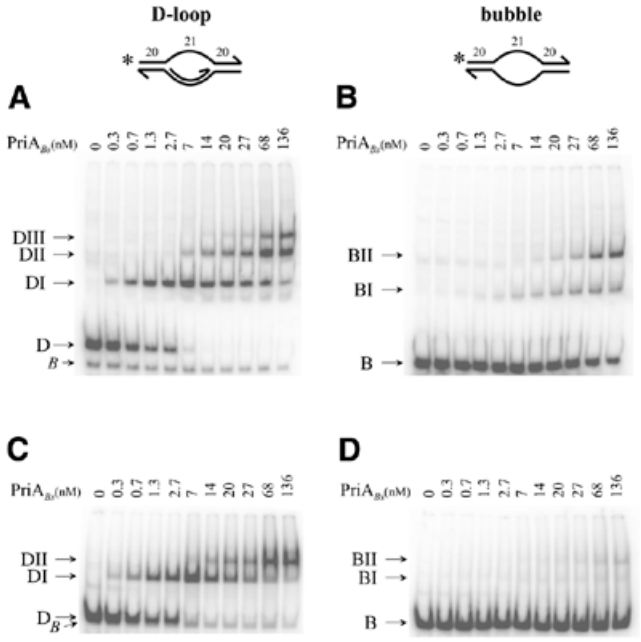

Figure 5. PriA$_{Bs}$ binds preferentially to a D-loop structure. The numbering on the schematic representation of the D-loop and bubble substrates indicates the size (in nt) of the dsDNA and ssDNA part, the star indicates the position of the ^{32}P radiolabelling present at the 5′ end. Purified PriA$_{Bs}$ protein at the indicated concentrations (expressed in nM) was added to the substrates, incubated at 30°C for 30 min and the protein–DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel run at 4°C in TGE buffer (A and B) or in TAM buffer (C and D). (**A** and **C**) PriA$_{Bs}$ binding to the D-loop. (**B** and **D**) PriA$_{Bs}$ binding to the bubble.

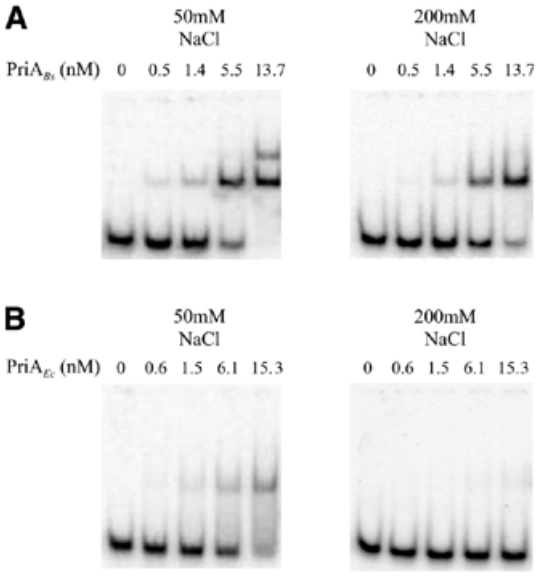

Figure 6. PriA$_{Bs}$ binds more stably to ssDNA than PriA$_{Ec}$. Protein–DNA complexes were electrophoresed on a 5% non-denaturing polyacrylamide gel containing 5% glycerol in 0.25× TBE buffer at 4°C. The 90 nt long Ost4 oligonucleotide was 5′ radiolabelled and used as a DNA binding substrate with the indicated concentrations of PriA$_{Bs}$ (**A**) or PriA$_{Ec}$ (**B**) (expressed in nM). Binding assays were performed in buffer containing either 50 (left) or 200 mM NaCl (right).

Finally, we carried out a binding experiment between PriA$_{Bs}$ and *ssiA* strands preincubated with an excess of SSB$_{Bs}$. Under these conditions only the *ssiA*$^+$ substrate was shifted by PriA$_{Bs}$ (Fig. 4C). Both fast and slow migrating forms of *ssiA*$^+$ were shifted, indicating that they have related structures (Fig. 4C).

PriA$_{Bs}$ binds specifically to a D-loop structure

Analysis of pAMβ1-related plasmids indicated that PriA$_{Bs}$ acts *in vivo* at D-loops (see above). We therefore investigated whether PriA$_{Bs}$ binds to the D-loop and bubble structures used previously to reveal PriA$_{Ec}$ binding (8). As shown in Figure 5, PriA$_{Bs}$ bound preferentially to the D-loop (K_d = 1.5 nM) in comparison to a bubble (K_d = 150 nM). Three complexes (DI, DII and DIII) (Fig. 5A) appeared consecutively with increasing amounts of PriA$_{Bs}$ added to the D-loop, whereas only two were observed with the bubble (BI and BII) (Fig. 5B). The appearance of DI at low protein concentrations, followed by that of DII, DIII, BI and BII at higher concentrations, clearly demonstrates the preferential binding of PriA$_{Bs}$ to the D-loop structure. A small amount of unbound contaminant bubble structure in the D-loop preparation (Fig. 5A, band B) provided an internal control which confirmed the preference for the D-loop. Furthermore, complexes of PriA$_{Bs}$ with the bubble substrate (BI and BII) were much less stable than those generated with the D-loop, since they almost completely disappeared when electrophoresed under destabilising conditions (i.e. in the presence of magnesium; Fig. 5D), whereas complexes with the D-loop remained stable (Fig. 5C). These results confirmed and detailed what was previously reported with PriA$_{Bs}$ purified differently from an insoluble form (31).

PriA$_{Bs}$ binds strongly to ssDNA

The above gel shift experiments indicated that PriA$_{Bs}$ displays a high affinity for ssDNA, which would clearly distinguish PriA$_{Bs}$ from its functional homologue PriA$_{Ec}$. To compare the two proteins with respect to their ssDNA binding activity, we performed gel shift experiments with the ssDNA substrate Ost4, a 90 nt long oligonucleotide. The PriA$_{Ec}$ protein was purified by a procedure similar to that used for PriA$_{Bs}$ (see Materials and Methods). As shown in Figure 6, PriA$_{Bs}$ binds with a better affinity than PriA$_{Ec}$ to the ssDNA probe (compare Fig. 6A and B, left; K_d = 4 and 20 nM, respectively). PriA$_{Bs}$ generated two discrete retarded bands, while a smear from the retarded band to the free DNA was observed with PriA$_{Ec}$. Such a gel shift pattern is strongly indicative that PriA$_{Ec}$ binding to ssDNA is unstable. Accordingly, increasing the ionic strength of the binding buffer nearly eliminated the band shift induced by PriA$_{Ec}$, while PriA$_{Bs}$ still bound to the substrate efficiently under those conditions (compare Fig. 6A and B, right).

PriA$_{Bs}$ is a ssDNA-dependent ATPase displaying helicase activity

PriA$_{Ec}$ is a ssDNA-dependent ATPase fueling its helicase activity (3). We have observed that PriA$_{Bs}$ induces ATP hydrolysis in the presence of naked ssDNA, but not dsDNA (Fig. 7A), indicating that it has a similar activity. Stable binding of PriA$_{Bs}$ to ssDNA is not required for this activity, because a 21 nt long oligonucleotide efficiently triggered PriA$_{Bs}$ ATPase activity (Fig. 7A) but did not form a stable complex with PriA$_{Bs}$ as judged by gel shift experiments (data

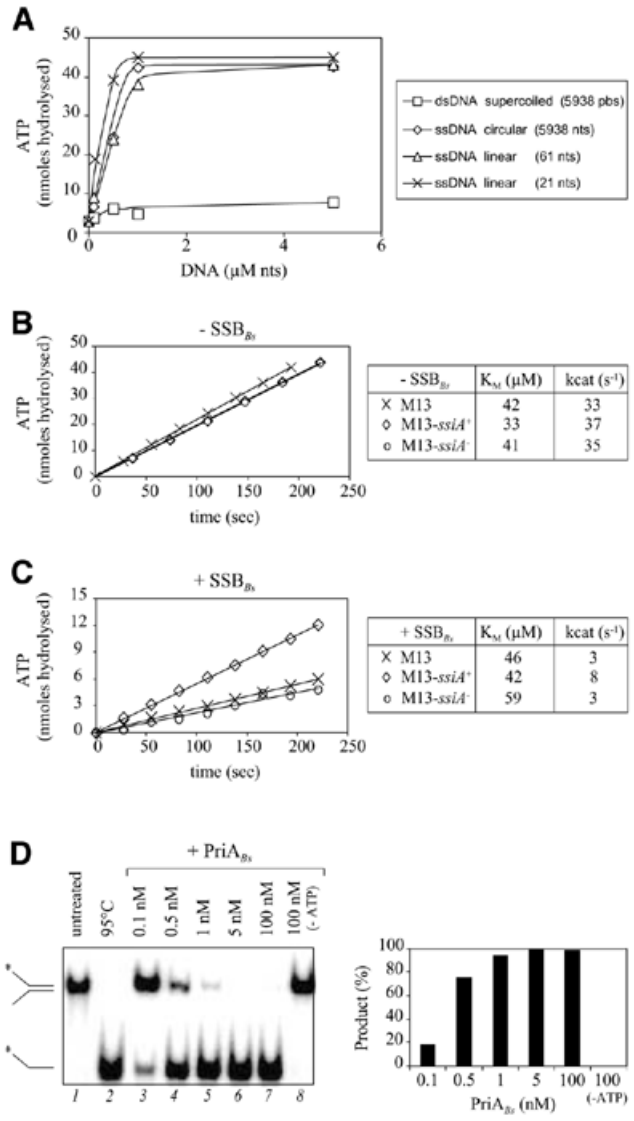

Figure 7. PriA$_{Bs}$ is a ssDNA-dependent ATPase displaying helicase activity. (**A**) PriA$_{Bs}$ (55 nM) was incubated at 37°C for 30 min in the presence of increasing amounts of DNA at a fixed concentration of ATP (45 μM). The calculated amount of ATP hydrolysed is plotted against DNA concentration. Supercoiled dsDNA and ssDNA were prepared from the pAPJ9 phagemid as described in Materials and Methods. Purified oligonucleotides used for construction of the D-loop substrate were used as linear ssDNA substrates (cf. Fig. 5). (**B** and **C**) ssDNA-dependent ATPase activity of PriA$_{Bs}$ was measured at 37°C in the presence of constant amounts (1 μM nt) of three ssDNA substrates, in the absence (B) or presence (C) of SSB$_{Bs}$ protein. In the experiment conducted with SSB$_{Bs}$, ssDNA substrates were incubated with this protein (0.5 monomer/nt) for 30 min at 37°C prior to addition of PriA$_{Bs}$. A representative kinetic experiment at 0.1 mM ATP is shown; similar experiments were carried out at different ATP concentrations in order to calculate the k_{cat} and K_m values of the enzyme for ATP. These values are reported in the adjacent table and are the average of two independent experiments. M13, M13-mp19 ssDNA; M13-*ssiA*$^+$, M13-mp19 ssDNA carrying the *ssiA*$^+$ sequence; M13-*ssiA*$^-$, M13-mp19 ssDNA carrying the *ssiA*$^-$ sequence. (**D**) PriA$_{Bs}$ displays helicase activity. (Left) Native gel analysis of PriA$_{Bs}$ unwinding activity. Lanes 1 and 2 contain, respectively, the synthetic forked DNA substrate and the labelled ssDNA liberated following heating at 95°C for 5 min, which are represented schematically on the left. Lanes 3–7 contain reactions performed with increasing amounts of PriA$_{Bs}$ (indicated in nM at the top of the gel). Lane 8 contains a reaction performed with the same amount of PriA$_{Bs}$ as in lane 7, but without ATP in the reaction buffer. (Right) Quantitation of PriA$_{Bs}$ unwinding activity. Helicase activity is expressed as the percentage of the liberated ssDNA strand quantified in each sample lane in the gel presented in (A) (see Materials and Methods).

not shown). The presence of *ssiA*$^+$ or *ssiA*$^-$ in naked M13 circular ssDNA did not affect the ATPase activity of PriA$_{Bs}$ (Fig. 7B). However, addition of SSB$_{Bs}$ protein prior to PriA$_{Bs}$, which reduced ATPase activity with all substrates, had less effect with the *ssiA*$^+$ than with the *ssiA*$^-$ substrate (Fig. 7C). We conclude that PriA$_{Bs}$ is a ssDNA-dependent ATPase and suggest that SSB$_{Bs}$ limits the accessibility of PriA$_{Bs}$ to ssDNA, unless a specialised DNA sequence, such as *ssiA*, is present in the ssDNA. The ATPase activity of PriA$_{Bs}$ is linked to the translocase/helicase activity already reported for PriA$_{Bs}$ (31). As illustrated in Figure 7D, we have also confirmed that our PriA$_{Bs}$ preparation displayed helicase activity: provided that ATP was included in the reaction, PriA$_{Bs}$ efficiently unwound a Y-shaped DNA molecule used in the helicase assay (43).

PriA$_{Bs}$ does not complement an *E.coli priA* null mutant

The results presented above show that PriA$_{Bs}$ is functionally equivalent to PriA$_{Ec}$ *in vivo* and *in vitro*, as was previously proposed by Masai *et al.* (31). A question raised by this identity is whether one PriA can substitute for the other *in vivo*. We tested this hypothesis with PriA$_{Bs}$ in *E.coli*. For this purpose we cloned *priA*$_{Bs}$ in pGB2, a plasmid which does not depend on PriA$_{Ec}$ for replication, and placed it under the control of *E.coli* translational and IPTG-inducible transcriptional signals to give plasmid pAPJ41 (see Materials and Methods). Another plasmid, pAPJ43, carrying the *priA*$_{Ec}$ coding sequence under the same expression signals, was constructed as a control. The *priA*$_{Ec}$ null mutant strain JC18983 and the wild-type isogenic strain DM4000 (49) were transformed by the two plasmids and tested for several phenotypes associated with the lack of PriA$_{Ec}$: viability, growth on rich medium, UV sensitivity and replication of the ColE1-type plasmid pBR322. As expected, the *priA*$_{Ec}$-carrying plasmid corrected the phenotypes of the *priA* mutant even without IPTG induction (Table 3) (only viability and sensitivity to rich medium were tested for this plasmid). In contrast, the *priA*$_{Bs}$-carrying plasmid did not correct any of the mutant phenotypes, either non-induced or induced with low IPTG concentrations (Table 3). At higher IPTG concentrations induction of PriA$_{Bs}$ was toxic (Table 3). The toxicity associated with PriA$_{Bs}$ expression was not observed in the wild-type strain (Table 3). These results show that PriA$_{Bs}$ cannot substitute for PriA$_{Ec}$ *in vivo*.

DISCUSSION

We report a detailed analysis of a *B.subtilis* protein proposed to be the counterpart of the *E.coli* PriA primosomal protein on the basis of sequence similarities (27,30,31), confirming and extending a previous biochemical analysis of this protein (31). Several lines of *in vivo* and *in vitro* evidence demonstrate that this protein is indeed PriA.

Typical phenotypes associated with the lack of PriA in *E.coli* have been observed with *B.subtilis priA* mutant cells. These include poor viability, slow growth, filamentation and sensitivity to rich medium and UV. In *E.coli* these defects of *priA* mutants are thought to be due to a deficiency in the repair of arrested replication forks (for reviews see 6,16,17). We therefore propose that PriA$_{Bs}$ plays a similar role in replication fork reactivation in *B.subtilis*.

Table 3. $PriA_{Bs}$ does not complement an *E.coli priA* null mutant

E.coli strain	$priA_{Ec}$ status	Plasmid		IPTG (μM)	Phenotype			
		Name	Induced protein		c.f.u. × 10^8 per OD_{600}[a]	Growth on rich medium[b]	UV resistance[c]	pBR322 replication[d]
DM4000	+	pGB2	none	0	2.4	+	0.6	+
JC18983	*2*	pGB2	none	0	0.7	–	0.0014	–
JC18983	*2*	pAPJ43	$PriA_{Ec}$	0	3.0	+	ND	ND
JC18983	*2*	pAPJ41	$PriA_{Bs}$	0	0.6	–	0.0006	–
				33	0.3	–	0.0004	–
				100	0.01	–	0.0010	–
DM4000	+	pAPJ41	$PriA_{Bs}$	0	3.1	+	0.7	+
				100	3.3	+	0.5	+
				300	2.6	+	0.5	ND

c.f.u., colony forming units; ND, not determined.
[a]Strains were grown exponentially at 37°C with the indicated dose of IPTG in minimal medium supplemented with spectinomycin. At OD_{600} = 0.4–0.6 cells were serially diluted, plated on minimal media without IPTG and with spectinomycin and c.f.u. were determined after 48 h incubation at 37°C.
[b]Cells grown in minimal medium without IPTG and with spectinomycin were streaked onto LB plates supplemented with spectinomycin and the indicated dose of IPTG, and incubated at 37°C for 48 h. + and – indicate the presence and absence of colonies, respectively.
[c]As in footnote a except that after plating, cells were irradiated at 20 J m^{-2}. Results are expressed as the fraction of surviving cells.
[d]Ability of pBR322 to replicate was measured by its ability to transform competent cells to tetracyclin resistance. +, ~10^6 transformants μg^{-1}; –, <100 transformants μg^{-1}.

$PriA_{Ec}$ is required for replication of several *E.coli* extrachromosomal elements (3). We report defects in two modes of extrachromosomal replication in *B.subtilis priA* mutants. One is the *ssiA*-dependent conversion of ssDNA to dsDNA. *ssiA* has been shown to act as a primosome assembly site in *B.subtilis* (28), similarly to the *pas* sequence of bacteriophage ϕX174 in *E.coli* (3). Moreover, we show that $PriA_{Bs}$ binds stably and specifically to the active strand of *ssiA in vitro*, as does the *E.coli* protein to the *pas* sequence of ϕX174 (5). $PriA_{Bs}$ still binds to *ssiA* in the presence of SSB_{Bs} protein. These results show that $PriA_{Bs}$ binds ssDNA carrying *ssiA* and suggest that it triggers primosome assembly at this site. Interestingly, *ssiA* appears to adopt a particular structure while its complementary strand does not. It has been shown that *pas* sites in *E.coli* are structurally distinct from their complementary strands and are resistant to melting by SSB and it is proposed that this feature determines their recognition by $PriA_{Ec}$ (3). Similarly, we propose that the structure adopted by *ssiA* is refractory to melting by SSB_{Bs} and that this contributes to its specific recognition by $PriA_{Bs}$ *in vivo*.

Another mode of extrachromosomal replication dependent on $PriA_{Bs}$ is that of the pAMβ1-type plasmids, which is similar to that described for the *E.coli* ColE1-type replicons (44). It involves the formation of a D-loop structure, to which $PriA_{Ec}$ binds specifically and promotes primosome assembly *in vitro* (8,9,18,19). $PriA_{Bs}$ protein efficiently binds an artificial D-loop structure *in vitro* (this study; 31). We observed that $PriA_{Bs}$ interaction with this three-stranded DNA molecule is more specific and much more stable than with a bubble structure. These combined *in vivo* and *in vitro* analyses suggest that $PriA_{Bs}$ triggers primosome assembly on such branched molecules, as reported for $PriA_{Ec}$. Such structures are thought to be targeted by PriA during DNA recombinational repair (3,6,16,17).

Another characteristic shared by the *B.subtilis* and *E.coli* PriA proteins is their low quantity in the cell, estimated to be 50–100 molecules per cell (50). We present evidence that this level can be reduced 3-fold in *B.subtilis* without the appearance of detectable cellular defects. However, the diminished quantity of $PriA_{Bs}$ is not high enough to sustain a normal level of $PriA_{Bs}$-dependent extrachromosomal replication.

During the course of this study the purification of $PriA_{Bs}$, a description of its binding to D-loop structures and its ssDNA-dependent ATPase and helicase activities have been reported (31). Our *in vitro* observations with a purified soluble form of $PriA_{Bs}$ confirm and extend this preliminary report. We have observed a more stable binding of $PriA_{Bs}$ than $PriA_{Ec}$ to ssDNA. Probably associated with this property is the capability of $PriA_{Bs}$ to bind to bubble structures, although less stably and with a lower affinity than to the D-loop structure. SSB_{Bs} prevents $PriA_{Bs}$ binding to ssDNA, but not to the *ssiA* sequence (this study) nor to a forked structure (data not shown). Therefore, we propose that SSB_{Bs} protein participates in the specific targeting of $PriA_{Bs}$-mediated primosome assembly to the DNA, as recently concluded for the *E.coli* primosomal restart machinery (24). PriA and SSB are two proteins highly conserved in bacteria and are two players involved in the early steps of replication fork re-activation. Therefore, an identical functional scaffold of replication restart appears conserved in these microorganisms in the initial stages.

Despite the strong similarities between the two bacterial PriA proteins, $PriA_{Bs}$ does not substitute for $PriA_{Ec}$ *in vivo*, which shows its host specificity. We have observed that $PriA_{Bs}$ is toxic in *E.coli priA* cells, but not in isogenic wild-type cells. It is possible that $PriA_{Bs}$ competes in the mutant with the primosomal pathways that operate in the absence of $PriA_{Ec}$ (51). Another possibility would be that production of $PriA_{Bs}$ adds yet another defect to the *priA* mutant cells, which are already affected in the metabolism of chromosomal DNA. The strong affinity of $PriA_{Bs}$ for ssDNA might be responsible for this toxicity. Nevertheless, the lack of toxicity of $PriA_{Bs}$ in

wild-type cells suggests that it neither competes efficiently with the endogenous $PriA_{Ec}$ for its regular chromosomal substrates nor titrates other protein partners.

The host specificity of the PriA protein raises the question of the protein content of the PriA-dependent primosome in *B.subtilis*. The *E.coli* PriA primosomal partners are PriB, PriC, DnaT, DnaC, DnaB (the replication fork helicase) and DnaG (the primase). Likely counterparts of the DnaB helicase and the DnaG primase are encoded respectively by *dnaC* and *dnaG* (formerly *dnaE*) in *B.subtilis* (25,26), but no obvious homologues of PriB, PriC and DnaT have been found in *B.subtilis* (27). We have suggested that three *B.subtilis* proteins, DnaB, DnaD and DnaI, initially identified as required for initiation of chromosome replication, are primosomal proteins. Indeed, they are required for the activity of the primosome assembly site *ssiA* and are involved in the replication of pAMβ1-type plasmids (28,52–54). Moreover, DnaI was shown to interact with DnaC in a two-hybrid assay and to co-localise with DnaB in the cell (55). Recently we isolated *dnaB* mutations that suppress the phenotypes of *B.subtilis priA* mutants. Interestingly, the *in vivo* defects of primosome assembly observed in a *priA* mutant were compensated for by a *dnaB* mutation, in a *dnaD*- and *dnaI*-dependent manner (33). Furthermore, we directly showed *in vitro* that purified PriA, DnaD and DnaB proteins specifically interact in this order on a forked DNA substrate, mimicking the product of recombinational repair of a stalled replication fork (39). Altogether, these genetic and biochemical observations suggest that $PriA_{Bs}$, DnaB, DnaD and DnaI could act together to load the replication fork helicase DnaC onto DNA during replication fork restart. Therefore, the *E.coli* and *B.subtilis* restart primosomes have apparently diverged at the proteins acting between the PriA initiator and the replicative helicase.

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