Anticipating chromosomal replication fork arrest: SSB targets repair DNA helicases to active forks

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In bacteria, several salvage responses to DNA replication arrest culminate in reassembly of the replisome on inactivated forks to resume replication. The PriA DNA helicase is a prominent trigger of this replication restart process, preceded in many cases by a repair and/or remodeling of the arrested fork, which can be performed by many specific proteins. The mechanisms that target these rescue effectors to damaged forks in the cell are unknown. We report that the single-stranded DNA binding (SSB) protein is the key factor that links PriA to active chromosomal replication forks *in vivo*. This targeting mechanism determines the efficiency by which PriA reaches its specific DNA-binding site *in vitro* and directs replication restart *in vivo*. The RecG and RecQ DNA helicases, which are involved in intricate replication reactivation pathways, also associate with the chromosomal replication forks by similarly interacting with SSB. These results identify SSB as a platform for linking a 'repair toolbox' with active replication forks, providing a first line of rescue responses to accidental arrest.

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Introduction

Accidental arrest of DNA replication is a major threat to genome stability and to the timely progression of the cell cycle in all organisms. Fork inactivation arises at random and by many different ways, and several mechanisms operate on damaged forks to restore DNA replication (Cox *et al*, 2000). A specific combination of proteins defines each of

these rescue pathways, providing an appropriate solution for the particular damage suffered by the multiprotein replication machinery (the replisome) or by the DNA of the fork (Michel *et al*, 2004). These repair proteins can be classified into two categories based on their mode of action. The first group acts to protect and/or eliminate the injury on the arrested fork. In this group are found many proteins of the DNA repair and recombination machineries of the cell, most of them being universally conserved (Kreuzer, 2005). The second group is composed of specific proteins that have the ability to promote the assembly of the replisome on forked DNA molecules. Their collective action at arrested forks allows DNA replication to resume from the site where it has been interrupted (Heller and Marians, 2006). Until now, these replication restart proteins have been identified and characterized only in bacteria (Kreuzer, 2005). These two groups of proteins are proposed to act either independently or coordinately to repair and reactivate the arrested fork (Michel *et al*, 2004; Heller and Marians, 2006). This results in a complex and intricate network of rescue pathways that might act on DNA replication forks in response to their inactivation. However, it remains unclear how the proteins of these rescue pathways reach the arrested forks in the cell. This is the central question of the present study conducted in the model bacterium *Bacillus subtilis*. We have focused on three proteins widely conserved in bacteria and known to intervene in the rescue of chromosomal DNA replication forks: the replication restart protein PriA and the recombination proteins RecG and RecQ. All three are members of the large SF2 family of DNA helicases (Gorbalenya and Koonin, 1993), and share the same property in binding specifically to forked DNA molecules, an activity through which they are proposed to come into play at arrested DNA forks (McGlynn and Lloyd, 2002; Hishida *et al*, 2004).

PriA_{Ec} (the suffixes *Bs* and *Ec* added to the proteins or genes names stand for *B. subtilis* and *Escherichia coli*, respectively.) executes two distinct functions on forked DNA molecules (Heller and Marians, 2006). The first is to trigger the replisome assembly process, in association with additional replication restart proteins. This consists of the recruitment and delivery of the DNA helicase of the replisome (DnaB_{Ec}) onto the single-stranded DNA (ssDNA) of the lagging strand at the head of the replication fork. Next, the other proteins of the replisome self-assemble around the loaded helicase, allowing DNA replication to proceed. The second action of PriA_{Ec} is to remodel forked DNA molecules that present a double-stranded (ds) lagging strand. PriA_{Ec} unwinds this dsDNA to generate the ssDNA template needed for the loading of DnaB_{Ec} (Jones and Nakai, 1999). The ssDNA binding (SSB) protein, which binds to ssDNA at the fork and with which PriA_{Ec} physically interacts, assists PriA_{Ec} in this fork remodeling activity (Jones and Nakai, 2001; Cadman and McGlynn, 2004). PriA_{Ec} is the sole *E. coli* replication restart protein widely conserved in bacteria. We have shown that PriA_{Bs} exhibits the same functional features as PriA_{Ec}

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in vitro and *in vivo* (Bruand *et al*, 2001; Polard *et al*, 2002). However, PriA_{BS} does not functionally substitute for PriA_{EC} in *E. coli*, probably due to evolutionary divergence of their replication restart partners (Marsin *et al*, 2001).

The role of RecG_{EC} and RecQ_{EC} at arrested DNA replication forks seems to involve their helicase activities, which act to remodel the fork. RecG_{EC} has been shown to couple its translocation along the parental dsDNA of the fork to the unwinding of the nascent strands of the leading and lagging strands (McGlynn and Lloyd, 2001). This could convert the three-way DNA junction that characterized the fork into a four-way DNA junction. RecG_{EC} can also drive the reverse reaction, which regenerates a forked-DNA substrate that can be converted into an active replication fork by the replication restart machinery. The RecQ_{EC}-directed remodeling of forked DNA molecules appears different, with a preferential unwinding of the parental and lagging strand dsDNA arms (Hishida *et al*, 2004). This is proposed to promote the loading of RecA recombinase onto the ssDNA stretches produced, which could then come into play at the blocked fork. Little is known about the requirement for RecG and RecQ homologues at arrested replication forks in other bacteria. RecG_{BS} has been shown to be a forked DNA-binding helicase able to complement some defects of a *recG_{EC}* mutant strain (Wen *et al*, 2005). *B. subtilis* encodes two RecQ-like proteins that both are needed for the full resistance of growing cells to DNA-damaging agents: YocI, named hereafter RecQ_{BS} (highly similar to RecQ_{EC}) and the more distantly related RecS (Sanchez *et al*, 2006).

The first action of PriA, RecG and RecQ at arrested chromosomal DNA replication forks appears to consist of their binding to a Y-shaped DNA structure originating from an active fork. The *B. subtilis* genome (like that of *E. coli*) is composed of a circular dsDNA molecule duplicated by a single pair of divergent forks. These two forks localize at discrete, often coincidental, positions in the cell where, together with the proteins of the replisome, they form a chromosomal DNA replication center called the replication factory (Lemon and Grossman, 1998, 2000). Here, we report that PriA_{BS} localizes continuously within the *B. subtilis* replication factory by interacting with the SSB protein. This specific targeting of PriA_{BS} in the cell appears to be required for the efficiency of its replication restart activity, which is crucial for optimal cell growth. RecG_{BS} and RecQ_{BS} are also shown to associate constitutively with the active chromosomal forks in the cell. As for PriA_{BS}, the targeting of RecG_{BS} and RecQ_{BS} to the replication factory relies on their interaction with SSB. Together, these results identify SSB as a common anchor for PriA, RecG and RecQ at active chromosomal forks in the cell. On the basis of this cytological, biochemical and genetic analysis, we propose that SSB equips replication forks with specific protein tools that can efficiently quickly bring about fork repair following accidental arrest.

Results

PriA_{BS} localizes continuously to the *B. subtilis* replication factory

To investigate PriA_{BS} localization, we inserted a construct encoding a GFP-PriA_{BS} fusion under the control of a promoter inducible by D-xylose at the chromosomal *amyE* locus. GFP-PriA_{BS} synthesis was monitored by Western blot analysis of

total protein extracts from exponentially growing cells, with the use of anti-PriA_{BS} (Figure 1A) or anti-GFP (Figure 1C) antibodies. When cells were grown without D-xylose, the amount of GFP-PriA_{BS} appeared to be as low as that of endogenous PriA_{BS} in wild-type cells (~50 molecules/cell; Polard *et al*, 2002; Figure 1A, compare lanes a and e). This concentration was notably increased (~30 fold) at the higher concentrations of inducer (i.e., 0.2% and above). This larger amount of the GFP-PriA_{BS} protein was not toxic for the cell, as judged by growth rate and viability. The *gfp-priA_{BS}* construct was fully functional because, when present in a *priA1* null mutant background (Figure 1A, lane c), several defects associated with the lack of PriA_{BS} were corrected by the basal level of expression of GFP-PriA_{BS}. These include the following: rich medium sensitivity; reduced viability in minimal medium; sensitivity to the DNA-damaging agent mitomycin C (MMC) (data not shown, but see Figure 4F). A protein similar in size and amount to endogenous PriA_{BS} was revealed with anti-PriA_{BS} antibodies in extracts prepared from *priA1* cells harboring the *gfp-priA_{BS}* construct and grown with D-xylose (Figure 1A, lane d). We suggest that it arises from proteolysis of the large amounts of the GFP-PriA_{BS} produced in those cells.

GFP-PriA_{BS} foci were detected in most of the *Pxyl:gfp-priA_{BS}* PriA⁺ cells grown in the absence of D-xylose (73.0% for 122 counted cells; Figure 1B). Cells grown in the presence of D-xylose exhibited a similar pattern of GFP-PriA_{BS} foci, except that they were much brighter and clearly visible in nearly all cells (>99.0% for 97 counted cells; Figure 1B). In this latter condition, background fluorescence was also increased and appeared to cover the nucleoid. The same localization pattern for GFP-PriA was observed in the *Pxyl:gfp-priA_{BS}* *priA1* strain (Supplementary Figure S1).

The growth rate markedly impacted on the GFP-PriA_{BS} foci pattern. In rich LB medium, several foci per cell were generally observed (Figure 1B), with a majority of cells containing three foci. In this case, foci were located close to the 1/4, 1/2 and 3/4 points of the long axis of the cells (as pointed for one cell in Figure 1B, lower panel). By contrast, cells grown in minimal medium mainly exhibited a single GFP-PriA_{BS} focus at mid-cell position (82.1% of cells exhibited a single focus, whereas 15.9, 1.5 and 0.5% exhibited 2, 3 and 4 foci, respectively; *n* = 177 cells). This distribution was highly reminiscent of that of GFP fusions to proteins of the replisome that have highlighted the DNA replication factory (Lemon and Grossman, 1998). We demonstrated that GFP-PriA_{BS} was targeted to the chromosomal replication center of all growing cells. First, the average number of GFP-PriA_{BS} foci per nucleoid in cells grown in rich medium (2.1) was identical to that of a GFP fusion to the replisomal DnaX protein (2.2; Supplementary Table S3). Second, the distribution of GFP-PriA_{BS} foci relative to cell length in cells grown in minimal medium was indistinguishable from that of GFP-tagged DnaX and PolC proteins of the replisome (Supplementary Figure S1). Third, a YFP-PriA_{BS} fusion was found to colocalize mainly with a DnaX-CFP fusion expressed in the same cell grown in minimal medium (Figure 1E; more than 85% of the DnaX-CFP foci colocalized with YFP-PriA_{BS} foci). Fourth, the formation of the GFP-PriA_{BS} foci at mid-cell depended on active DNA replication. A block of the initiation of chromosomal DNA replication led to the disappearance of the GFP-PriA_{BS} foci in the cells (Supplementary Figure S1).

Figure 1 Localization of GFP-PriA_{BS} and GFP-PriA_{Ec} at the *B. subtilis* replication factory. (A, C) Western blots of protein extracts prepared from cells grown in LB with 0.2% (+) or without (–) D-xylose, probed with anti-PriA_{BS} (A) or anti-GFP (C) antibodies. The relevant genotypes of the strains are indicated above the blots. Immunodetected PriA_{BS}, GFP-PriA_{BS} and GFP-PriA_{Ec} proteins are indicated. The * in (C) refers to a crossreacting protein with the anti-GFP. (B, D) GFP (green), DAPI (blue), FM5-95 membrane dye (red) and overlay of those three fluorescent signals in wild-type (PriA⁺) cells carrying *gfp-priA_{BS}* (B) or *gfp-priA_{Ec}* (D) and grown in LB supplemented or not with 0.2% D-xylose. (E) Right panels: YFP (yellow), CFP (blue), DAPI staining (red) and overlay of those three fluorescent signals in PriA⁺ cells carrying *yfp-priA_{BS}* and *dnaX-cfp*. Cells were grown in MMX with 1% D-xylose, which is the condition needed to correctly detect the foci on the nucleoid of the YFP-PriA_{BS} and DnaX-CFP fusions in the same cell. Left panel: statistical analysis of the colocalization of DnaX-CFP and YFP-PriA_{BS} foci. The percentage of colocalized and individual foci of the DnaX-CFP and YFP-PriA fusions has been calculated from 269 scored foci. GFP or YFP foci visible in two representative cells were pointed by white arrows.

Finally, GFP-PriA_{BS} foci were observed in nearly all the exponentially growing cells of an asynchronous population, indicating that GFP-PriA_{BS} localizes to the replication factory during most of the cell cycle.

The heterologous PriA_{Ec} localizes continuously in the *B. subtilis* replication factory

Given the functional equivalence of PriA_{BS} and PriA_{Ec} for their individual activities, we investigated PriA_{Ec} localization

in *B. subtilis*. A GFP-PriA_{Ec} fusion was constructed and expressed exactly as was the GFP-PriA_{BS} fusion. It was synthesized at the same level as GFP-PriA_{BS} in response to D-xylose induction (Figure 1C, lanes d and b; the uninduced levels of both GFP-PriA fusions were not detectable with the anti-GFP antibodies; lanes c and a). The GFP-PriA_{Ec} fusion did not complement the growth defects of the *B. subtilis* *priA1* null mutant strain; it was even found to be toxic when overexpressed in this genetic background but not

in a wild-type context (data not shown; see Figure 4F). This mirrored our previous results with PriA_{Bs} expressed in *E. coli* (Polard *et al*, 2002). Remarkably, however, wild-type *B. subtilis* cells carrying the *gfp-priA_{Ec}* fusion exhibited the same GFP focus pattern as cells carrying the *gfp-priA_{Bs}* construct (Figure 1D and Supplementary Table S3).

Thus, localization of the GFP-PriA_{Bs} fusion in the *B. subtilis* replication factory mainly reflects its continuous anchoring in the vicinity of the two active chromosomal forks present in this compartment, rather than its sporadic recruitment when fork arrest occurs. That the GFP-PriA_{Ec} fusion localized similarly in *B. subtilis* without complementing the defects of *priA_{Bs}* null mutants supports this proposal. When GFP-PriA_{Bs} or GFP-PriA_{Ec} was expressed at higher levels, the fluorescent foci were markedly brighter. This suggests an accumulation of the fusion proteins in the replication factory, presumably resulting from their binding to a target site present in multiple copies. Several lines of evidence presented below lead to the conclusion that this site is the SSB protein.

In vitro interaction of PriA_{Bs} and PriA_{Ec} with *B. subtilis* SSB

PriA_{Ec} is known to interact *in vitro* with its cognate SSB, via the C-terminal end of SSB (Cadman and McGlynn, 2004). Although PriA_{Bs} is known to act with different replication restart partners than does PriA_{Ec}, we tested whether this interaction was conserved for *B. subtilis* proteins. As shown in Figure 2B, a mixture of purified PriA_{Bs} and *B. subtilis* SSB formed a complex in solution that elutes from a gel-filtration column before either of the proteins assayed individually. We reproduced this experiment with an SSB derivative, designated SSBΔCter, deleted for its Cter domain (Figure 2A). SSBΔCter proved to be as efficient as wild-type SSB for ssDNA interactions, as well as for stimulating DNA replication and recombination (unpublished). However, in gel filtration experiments, SSBΔCter was unable to produce a complex with PriA_{Bs} (Figure 2C). Also, PriA_{Bs} could be retained on large circular ssDNA molecules pre-coated with SSB, but not pre-coated with SSBΔCter (Supplementary Figure S2). Thus, like PriA_{Ec}, PriA_{Bs} interacts via the Cter domain of its cognate SSB, either in solution or when pre-bound to ssDNA. Purified PriA_{Ec} also interacted with *B. subtilis* SSB in solution (Figure 2D, panels 1, 2 and 3) or when bound to M13 ssDNA (data not shown). Mixing equal amounts of PriA_{Ec} and PriA_{Bs} with SSB produced a similar amount of each complex (Figure 2D, panel 4), indicating that the two PriA proteins have a similar affinity for *B. subtilis* SSB.

SSB-directed recruitment of PriA_{Bs} onto ssDNA

We then investigated the functional consequences of the interaction between PriA_{Bs} and its cognate SSB. A functional interplay between the PriA_{Ec} and SSB_{Ec} proteins was previously reported to rely on PriA_{Ec} helicase activity onto a small synthetic forked DNA substrate, with a single-stranded leading-strand arm and the two others arms double-stranded (Jones and Nakai, 2001): the PriA-directed unwinding of the parental duplex arm observed with the naked fork was found to be reoriented toward the lagging duplex arm in the presence of SSB_{Ec}. The same SSB-mediated reorientation of PriA helicase activity with the *B. subtilis* proteins was repro-

Figure 2 PriA_{Bs} and PriA_{Ec} interact with *B. subtilis* SSB *in vitro*. (A) Alignment of SSB with the SSBΔCter. (B–D) Purified PriA_{Bs} and PriA_{Ec} (10 μM), SSB (40 μM) and SSBΔCter (40 μM) were mixed on ice and loaded onto a gel-filtration column (Superdex 200HR 10/30). Fractions (0.5 ml, numbered on the top of the stained gels) were analyzed by 12.5% SDS-PAGE and Coomassie blue staining. The arrow heads indicate the molecular weight (in kDa) of standard proteins. Final concentrations are in monomers.

duced on a similar fork substrate (Supplementary Figure S3). Next, we found that the SSBΔCter derivative was as efficient as SSB in channeling PriA_{Bs} helicase activity toward the lagging arm of the fork, indicating that the ssDNA binding activity of SSB is the determinant in this interplay with PriA_{Bs} rather than its physical interaction with PriA_{Bs} (see additional discussion in the Supplementary data about the interplay between PriA_{Bs} and SSB in helicase assays on synthetic forked DNA).

We next used an electrophoretic-mobility shift assay (EMSA) to further analyze the interaction of PriA_{BS} with SSB or with SSBΔCter pre-bound to various linear and forked DNA substrates *in vitro*. We found that PriA_{BS} binds more efficiently to any linear DNA substrate pre-bound by SSB but not by SSBΔCter, as shown in Figure 3A and B with a linear, 5' tailed, DNA substrate. PriA_{BS} affinity for the naked DNA substrate was even higher with DNA if pre-coated with SSBΔCter (Figure 3A and B), showing that SSBΔCter impedes PriA_{BS} accessibility to the ssDNA and indicating that the upper-shifted bands in the experiment with SSB reflect PriA_{BS} binding to SSB sitting on the ssDNA portion of the DNA substrate. The barrier created by SSBΔCter appears alleviated at high PriA_{BS} concentration, as judged by the appearance of poorly resolved shifted bands exhibiting the same mobility as those observed with the naked DNA. The PriA_{BS}-directed super-shift of SSB-bound DNA substrates was observed in the presence of a vast excess of unbound SSB relative to PriA_{BS} (up to 100-fold more; Figure 3B), indicating that PriA_{BS} prefers to interact with SSB bound on ssDNA rather than with SSB in solution. The amount of PriA_{BS} needed to super-shift a ssDNA template pre-bound by SSB

was similar to that of PriA_{BS} needed to shift naked forked DNA substrates (not shown). SSB was unable to shift a naked fully double-stranded fork (with a nick at the junction between leading- and lagging-strand arms) even if bound by PriA_{BS} (Supplementary Figure S4). Thus, it appears that, once PriA_{BS} is engaged in a specific interaction with this particular forked DNA substrate, the SSB-interacting domain of PriA_{BS} is no longer accessible to SSB. Finally, competition experiments indicated that the interaction between PriA_{BS} and SSB (bound to ssDNA) is less stable than the interaction between PriA_{BS} and its dedicated replication restart forked DNA substrate (drawn on the left of the EMSA of Figure 3D). Preformed complexes of SSB with ssDNA or with such a synthetic fork, revealed as shifted bands of radiolabeled DNA substrates (named S_S and F_S; lane 3 in Figure 3C and D, respectively), were fully super-shifted by the addition of the same amount of PriA_{BS} (named S_{SP}, F_{SP1} and F_{SP2}; lane 7 in Figure 3C and D, respectively). The resulting nucleoprotein complexes were then incubated with increasing amounts of unlabeled fork. With a labeled ssDNA substrate, the complex S_{SP} was efficiently chased into a complex of the same mobility as S_S (Figure 3C; lanes 8–10), suggesting that

Figure 3 *B. subtilis* SSB tethers PriA_{BS} dynamically on ssDNA. (A) Increasing amounts of PriA_{BS} (indicated above the gels, lanes 2–5) were incubated for 10 min at 30°C with 0.4 nM of a ³²P-labeled 5' tailed dsDNA (lane 1). (B) The 5' tailed dsDNA (lane 1) was pre-bound for 10 min at 30°C with 100 nM of SSB (lanes 2–6) or SSBΔCter (lanes 7–11) and used as in (A). The apparent binding constants (K_d ; referring to the protein amount in nM needed to shift 50% of the labeled DNA) were determined from three independent experiments. (C, D) SSB, PriA_{BS} and the cold F_{lead} were successively added to ³²P-labeled ssDNA (C) or F_{lead} fork (drawn on the left part of D), with 10 min incubation at 30°C at each step. Final protein concentrations (in monomers) are indicated on top, and the identities of the distinct shifted bands are indicated to the right and left of the gels. The * on the DNA substrate cartoons indicate the position of the ³²P label. (E) PriA_{BS} ATPase activity. ΦX174 or M13 ssDNA were pre-incubated, or not, for 10 min at 37°C, with 1 μM (in monomers) of SSB or SSBΔCter. Assays were reproduced three times independently and led to identical results.

most of the PriA_{BS} had left the complex S_{SP} and been sequestered. With the labeled fork substrate, the complex of lower mobility F_{SP2} was chased by the unlabeled fork concentrations (Figure 3D; lanes 8–10), whereas the complex F_{SP1} was stable and was not displaced into the F_P complex. Control experiments showed that SSB–DNA complexes (S_S and F_S; Figure 3C and D, lane 3) were stable, and that PriA_{BS}–DNA complexes (S_P and F_P; Figure 3C and D, lane 5) were only slightly displaced when challenged with the unlabeled fork (Figure 3C and D, lane 6). Thus, the S_{SP} and F_{SP2} complexes appear highly sensitive to the unlabeled fork, and likely correspond to ternary nucleoprotein complexes formed by the protein–protein interaction of PriA_{BS} with SSB (bound to ssDNA). The F_{SP1} ternary complex could correspond to the independent binding of SSB and PriA_{BS} to two distinct parts of the fork: ssDNA for SSB and at the junction of the two duplex arms for PriA_{BS}. Overall, the PriA_{BS}–SSB interaction appears less stable than the PriA_{BS}–DNA interactions, especially with the forked DNA substrate specific for PriA-mediated replication restart.

SSB helps PriA_{BS} to reach its replication restart DNA-binding site

Given the above results, how could interaction with SSB modulate the accessibility of PriA_{BS} to its specific DNA-binding site when embedded in a large ssDNA molecule coated with many SSB tetramers (a situation that should mimic an arrested fork with a long stretch of ssDNA occupied by SSB in the cell)? To address this, we used the ssDNA circular forms of M13 and ΦX174 phages. Only the ΦX174 ssDNA substrate carries the so-called *pas* sequence, a particular folded ssDNA site onto which PriA_{Ec} directs the assembly of the replisome, and triggers its own ATPase activity, even in the presence of SSB (Marians, 1999). We found that PriA_{BS} ATPase activity was also triggered on the ΦX174 *pas* sequence (Figure 3E), providing an indirect measure of its binding efficiency to *pas*. Next, we measured and compared the ATPase activity of PriA_{BS} induced on M13 and ΦX174 ssDNA substrates, either naked or pre-coated with *B. subtilis* SSB or SSBΔCter.

PriA_{BS} ATPase activity was slightly higher on naked ΦX174 ssDNA than on M13 ssDNA (Figure 3E), a difference that could be explained by the presence of *pas* on the ΦX174 template. In contrast, PriA_{BS} ATPase activity was almost abolished when M13 ssDNA was coated with SSB, even though PriA_{BS} interacted with the M13 ssDNA–SSB nucleofilament (Supplementary Figure S2). A similar inhibition occurred when the M13 ssDNA was coated with SSBΔCter (Figure 3E). As expected, PriA_{BS} ATPase activity was detected on SSB-coated ΦX174 ssDNA because of the presence of the *pas*. Strikingly, however, the ATPase activity was even higher on SSB-coated ΦX174 ssDNA than on the naked template, especially at low concentrations of PriA_{BS} (Figure 3E). This apparent stimulation of PriA_{BS} ATPase activity was not observed when the ΦX174 ssDNA was coated with SSBΔCter. Thus, PriA_{BS} accessibility to *pas* appears to be facilitated by its interaction with SSB. When *pas* is embedded in a SSBΔCter ssDNA-filament incapable of retaining PriA_{BS} (see Supplementary Figure S2), access of PriA_{BS} to *pas* would be unaided, leading to a lower ATPase activity at low protein concentration.

A PriA_{BS} ATPase and helicase mutant still interacts with SSB and localizes continuously to the replication factory

We next wondered whether the PriA_{BS} ATPase (and helicase) activity(ies) influence(s) PriA_{BS} interaction with SSB and PriA_{BS} property to localize in the replication factory. First, the PriA_{BS}–SSB physical interaction observed by gel filtration was not modified by the presence of ATP (data not shown). Second, we constructed and analyzed a PriA_{BS} point mutant of the conserved lysine of the helicase motif I, to give the PriA^{K301A}. This mutation has been previously made in PriA_{Ec} and shown to abolish its ATPase and helicase activities, but not its replication restart activity (Zavitz and Marians, 1992). Purified PriA^{K301A} was also shown to be inactive as an ATPase and a helicase (Supplementary Figure S5 and data not shown). However, PriA^{K301A} was still capable of physically interacting with SSB as efficiently as wild-type PriA_{BS} (Supplementary Figure S5). Furthermore, PriA^{K301A} was still found to localize continuously in the replication factory of living *B. subtilis* cells (Supplementary Figure S5 and Supplementary Table S3), showing that PriA_{BS} ability to hydrolyze ATP is not needed for its specific colocalization with the active chromosomal DNA replication forks in the cell.

Swapping the ‘8-cystein motif’ of PriA_{BS} with that of PriA_{Ec} abolishes the interaction with SSB

A unique feature shared by all PriA proteins is a 44 amino-acid long ‘8-cystein motif’ (‘8-C motif’) located between the helicase domains IV and V, and proposed to fold into two small consecutive zinc fingers. PriA_{Ec} mutants affected in some of the cystein residues in this motif are deficient in helicase activity and in their interplay with PriB, the first PriA_{Ec} replication restart partner. However, they remain capable of binding forked DNA and to promote replisome assembly *in vitro* in the presence of an excess of DnaT, the second PriA_{Ec} partner (Liu *et al*, 1996).

We precisely exchanged the 8-C motif in *B. subtilis* PriA with that of PriA_{Ec}, (from C-1 to C-8) to give a ‘PriA_{ZF}’ chimera. Purified PriA_{ZF} still displayed ssDNA-dependent ATPase activity (Figure 4A), and specific but slightly lower helicase activity on a forked substrate (Supplementary Figure S6). However, PriA_{ZF} bound forked DNA substrates with a similar affinity as did PriA_{BS} (Figure 4B). PriA_{ZF} also displayed a prominent affinity for naked ssDNA in EMSA (data not shown), a distinctive property of PriA_{BS} relative to PriA_{Ec} (Polard *et al*, 2002). Thus, replacing the 8-C motif of PriA_{BS} with that of PriA_{Ec} did not alter drastically the various interactions of PriA_{BS} with DNA.

Surprisingly, we found that PriA_{ZF} was unable to interact with SSB, either in solution (data not shown) or when pre-bound to ssDNA (Figure 4C). In keeping with this, the PriA_{ZF} ATPase activity on ΦX174 ssDNA was not stimulated by SSB (Figure 4A), supporting the notion that the *pas*-dependent stimulation of the ATPase activity relies on PriA_{BS} interaction with SSB. Despite this lack of interaction, the orientation of the helicase activity of PriA_{ZF} on an SSB-bound fork was as for PriA_{BS} (Supplementary Figure S6), further indicating that the SSB-directed orientation of PriA_{BS} helicase activity on forked DNA is independent of the PriA_{BS}–SSB interaction.

In living cells of *B. subtilis*, the GFP–PriA_{ZF} fusion accumulated to similar levels as the GFP–PriA_{BS} (Figure 4D), but

Figure 4 Functional analysis of PriA_{ZF}, a chimera obtained by swapping the '8-C motif' of PriA_{BS} with that of PriA_{EC}. (A) ATPase activity of PriA_{BS} and PriA_{ZF} (50 nM) was measured with Φ X174 ssDNA pre-bound (+SSB), or not (–SSB), with 1 μ M of SSB as in Figure 3E. (B) PriA_{ZF} binding on an F_{lead} fork was analyzed by EMSA as in Figure 3A. (C) PriA_{ZF} interaction with SSB-bound ssDNA (80 nts) was tested by EMSA as in Figure 3B. (D) Immunodetection of GFP-PriA_{ZF} in *B. subtilis* cells (as in Figure 1A). (E) Fluorescence signals in cells with the gfp-priA_{ZF} construct grown in LB with 0.2% D-xylose. (F) Spotting assay for cell viability. The relevant genotype of the strains is given at the top of the figure. Cells were propagated up to mid-log phase at 37°C in LB with 0.2 mM IPTG and without D-xylose. Serial dilutions (10 $\times$ step) of these cultures ($\sim 1 \times 10^8$ cells/ml) were generated and 2.5 μ l were spotted onto plates with (+) or without (–) 0.2% D-xylose and/or 1 mM IPTG, and incubated O/N at 37°C. Cell viability was expressed by growth observed over that range of dilution, ending with a few colonies at the 10 $^{-6}$ dilution for a PriA⁺ background. Black rectangles represent wild-type viability. Gray rectangles represent the diminished viability of cells that no longer express PriA_{BS} (–IPTG). Assays in (G) were as in (F), except that 20 ng/ml of MMC was included in the plates. n.d., not determined. Experiments have been reproduced at least three times with independent clones and have led to identical results.

did not generate foci (over a wide range of D-xylose concentrations; Figure 4E and Supplementary Table S3; data not shown). Most of the GFP-PriA_{ZF} fluorescence in the cell appeared associated with the nucleoid. These results provide strong evidence that PriA_{BS} is recruited to the replication factory via its interaction with SSB.

Importance of the PriA–SSB interaction for replication restart efficiency in vivo

We compared the ability of PriA_{ZF} (as a fusion to GFP) to promote replication restart *in vivo* with that of GFP-PriA_{BS} and GFP-PriA_{EC}. The three GFP fusions were expressed under D-xylose control from the ectopic *amyE* locus, in a strain background in which endogenous PriA_{BS} expression was under IPTG control (the *priA_{ind}* strain; Polard *et al.*, 2002). Control experiments with a *def_{ind}* strain (the *def* orf follows *priA* on the *B. subtilis* chromosome; Polard *et al.*, 2002) showed that none of the effects described below could be ascribed to polar effects on genes downstream of *priA*. The

ability to form colonies on rich medium (LB) supplemented with or without IPTG and D-xylose is a measure of PriA_{BS} function (Figure 4F). As reported previously (Polard *et al.*, 2002), repression of *priA_{ind}* (i.e., without IPTG) resulted in reduced cell viability (Figure 4F, left). This was completely restored by ectopic expression of PriA_{BS}, even in the absence of the D-xylose inducer, and by overexpression of PriA_{ZF} in the presence of D-xylose. In sharp contrast, PriA_{EC} failed to complement the lack of endogenous PriA_{BS} and was even toxic when induced (Figure 4F).

The growth deficiencies of *priA* null mutant cells are thought to be due to their failure to restart 'spontaneously' arrested chromosomal replication forks. If so, DNA-damaging agents, such as MMC, which increase the replication fork arrest frequency, should have a severe impact on the viability of *priA* mutant cells. As expected, with no GFP-PriA construct, the *priA_{ind}* strain was severely inhibited on MMC plates lacking IPTG (Figure 4G). Growth was completely rescued by GFP-PriA_{BS}, either in the presence or absence

of inducer. In contrast, the GFP-PriA_{ZF} chimera slightly restored viability in the presence, but not in the absence, of D-xylose. Thus, the failure of PriA_{ZF} to localize at the replication factory is accompanied by a reduction of its ability to support multiple replication restart events. This failure could be compensated to some extent by overexpression in the cell.

The Cter domain of SSB is required for targeting PriA_{Bs} to the replication factory

We constructed a viable *B. subtilis* mutant strain, *ssbΔ35*, which expresses SSB truncated of its last 35 amino-acid

residues (Figure 5A). The presence of a shortened SSB protein in *ssbΔ35* cells was demonstrated by Western blot analysis (Figure 5B). As a control, we derived an isogenic strain, *ssb3⁺*, encoding a wild-type SSB protein (Figure 5A and B). *ssbΔ35* cells had a slightly reduced viability and were temperature-sensitive for growth above 47°C in rich medium (data not shown; the *ssb3⁺* strain could grow up to 51°C).

The *ssbΔ35* strain permitted testing whether the GFP-PriA_{Bs} foci were dependent upon the Cter region of SSB. Constructs expressing fusions of PriA_{Bs} and DnaX (as a known replication factory marker) to GFP were introduced into the *ssbΔ35* and *ssb3⁺* strains. GFP-PriA_{Bs} foci were not

Figure 5 The formation of GFP-PriA_{Bs} foci requires the Cter domain of SSB. (A) Representation of the *ssb* operon in the wild-type, *ssbΔ35* and *ssb3⁺* strains. *ery*, erythromycin resistance gene; *P_{spac}*, IPTG-inducible promoter; *rpsR*, ribosomal gene. (B) Immunodetection of SSB and SSBΔ35 by Western blotting with anti-SSB antibodies. Protein extracts were prepared from *ssbΔ35* or *ssb3⁺* cells carrying *amyE::gfp-priA_{Bs}* or *amyE::dnaX-gfp*. Lane 1: proteins prepared from a strain into which the plasmid is integrated at a distinct locus from *ssb*. The * refers to a crossreacting protein with the anti-SSB antibodies. (C-F). GFP (green), DAPI (blue), FM5-95 membrane dye (red) and an overlay of those three fluorescent signals in the same strains as in (B). Cells were grown in LB with 0.2% D-xylose and 1 mM IPTG. GFP-foci visible in two representative cells were pointed by white arrows.

detected in *ssbΔ35* cells, whereas they were still clearly visible in *ssb3⁺* cells (compare the cells in Figure 5C and D grown with a D-xylose concentration giving maximal synthesis of the fusions; for a statistical analysis of the number of foci per nucleoid in these genetic backgrounds see Supplementary Table S3). The GFP-PriA_{BS} fusion was still expressed in the *ssbΔ35* background, as demonstrated by Western blot analysis (data not shown). GFP-PriA_{BS} fusion localized over the entire nucleoid (Figure 5C and Supplementary Table S3), as did GFP-PriA_{ZF} in wild-type cells (Figure 4E). A similar failure to generate foci in *ssbΔ35* cells was observed for GFP-PriA_{EC} (data not shown). Importantly, DnaX-GFP generated foci in both strains (with or without D-xylose, Figure 5E and F and data not shown; Supplementary Table S3), showing that the replication factory was still present in *ssbΔ35* cells. In addition, we observed that GFP-PriA_{BS} did not form foci in a strain expressing an SSB protein missing its last six residues. We also found that the purified SSBΔ6 mutant protein fails to interact with PriA_{BS} *in vitro* (data not shown). Therefore, the very last Cter residues of SSB appear crucial for interaction with PriA and for recruitment of PriA (but not DnaX) to the replication factory.

SSB-directed localization of RecG_{BS} and RecQ_{BS} DNA helicases to the *B. subtilis* replication factory

Like PriA, RecQ and RecG are DNA helicases of the SF2 family, displaying binding specificity to forked DNA and involved in rescue of arrested replication forks in *E. coli* (McGlynn and Lloyd, 2002; Hishida *et al.*, 2004). We studied the subcellular localization of RecG_{BS} and RecQ_{BS} by fusing GFP to their N termini. The resulting fusion proteins were expressed from the *amyE* locus under the control of a D-xylose-inducible promoter. GFP-RecG_{BS} was functional, as judged by its sensitivity to UV irradiation when expressed at basal levels (data not shown). *recQ_{BS}* null mutant cells are hardly sensitive to DNA-damaging agents (Sanchez *et al.*, 2006; our unpublished results), precluding from testing reliably the functionality of the GFP-RecQ_{BS} fusion. Wild-type cells (data not shown) and *ssb3⁺* cells carrying *gfp-recG_{BS}* and *gfp-recQ_{BS}* and grown in rich medium to mid-log phase exhibited a similar multifocal pattern as cells carrying *gfp-priA_{BS}* (Figure 6A). The GFP-RecG_{BS} and GFP-RecQ_{BS} foci were detected in a majority of cells without induction (data not shown), and they became much more brilliant and present in virtually all cells upon induction by D-xylose (Figure 6A). The average number of these GFP-fusions foci per nucleoid (1.7 and 2.1 for GFP-RecG_{BS} and GFP-RecQ_{BS} respectively, Supplementary Table S3) was nearly identical to the number observed for DnaX-GFP foci per nucleoid (2.3) in the *ssb3⁺* cells. Moreover, the RecG_{BS} and RecQ_{BS} foci mainly colocalized with DnaX (~80% of the YFP-RecG_{BS} and YFP-RecQ_{BS} colocalized with DnaX-CFP foci, Figure 6C and D), indicating that, as PriA_{BS}, RecG_{BS} and RecQ_{BS} are continuously localized in the replication factory. Remarkably, both GFP-RecG_{BS} and GFP-RecQ_{BS} were not localized in foci in *ssbΔ35* cells (Figure 6B). Thus, localization of RecG_{BS} and RecQ_{BS} to the replication factory requires the Cter of SSB, suggesting that these two helicases also interact with SSB. Strong support for this proposal was obtained by purifying RecQ_{BS} from *B. subtilis* cells: SSB was found to be the main

Figure 6 Localization of the GFP-RecG_{BS} and GFP-RecQ_{BS} fusions to the replication factory of *B. subtilis*. (A, B) Fluorescence signals of GFP (green) and DAPI (blue) in cells carrying *gfp-recG_{BS}* or *gfp-recQ_{BS}*, together with the *ssbΔ35* or *ssb3⁺* alleles. Cells were grown in LB with 0.2% D-xylose and 1 mM IPTG. (C) YFP (yellow), CFP (blue), DAPI (red) and overlay of those three fluorescent signals in cells carrying *dnaX-cfp* and the *yfp-recG_{BS}* or *yfp-recQ_{BS}*. Cells were grown in MMX with 1% D-xylose. The foci in at least two representative cells were pointed by white arrows. (D) Statistical analysis of the colocalization of DnaX-CFP and YFP-RecG_{BS} (left panel) or YFP-RecQ_{BS} (right panel) foci. The percentage of colocalized and individual foci of the DnaX-CFP and YFP-RecG_{BS} or YFP-RecQ_{BS} fusions has been calculated from 252 (DnaX/RecG) and 332 (DnaX/RecQ) foci, respectively.

protein partner co-purifying with affinity-tagged RecQ_{BS} (Supplementary Figure S7). The same result was obtained with affinity-tagged RecG_{BS}, while giving rise to a lesser amount of co-purified SSB molecules (Supplementary Figure S7). Further evidence for a direct interaction between SSB and RecG_{BS} came from a gel-filtration assay, in which

RecG_{BS} co-purified with a ssDNA molecule coated by SSB but not by SSBΔCter (Supplementary Figure S2).

Discussion

We report that PriA, the primary replication restart protein in bacteria, as well as the recombination proteins RecG and RecQ localize at the replication factory in living *B. subtilis* cells during active duplication of the chromosome. We demonstrate that the association of these proteins with the replication factory depends on their interaction with the C-terminal end of SSB, which is a central protein of the DNA replication fork. Thus, in addition to protecting and facilitating replication of the lagging-strand ssDNA template, SSB also equips the active replication forks with specific DNA repair proteins. Furthermore, we provide evidence that the SSB-mediated targeting of PriA to the replication factory is crucial for replication restart activity. These findings have important implications for coordination and execution of the different repair events that could come into play after accidental chromosome fork arrest (Cox *et al*, 2000; McGlynn and Lloyd, 2002; Michel *et al*, 2004; Sherratt *et al*, 2004; Heller and Mariani, 2006).

We propose that the localization of PriA, RecG and RecQ at the replication factory in most growing cells reflects a surveillance mechanism of the active chromosomal forks, rather than the constitutive involvement of these proteins in the repair or reactivation of arrested forks (Figure 7). Although it cannot formally be ruled out that fork arrests requiring the action of these three repair proteins might occur at a very high frequency during chromosome replication in *B. subtilis*, this hypothesis seems unlikely as null mutants in *recG*, *recQ* and under some conditions *priA* are viable. Rather, the constitutive presence of PriA, RecG and RecQ at the replication factory could maintain a rescue potential in the vicinity of active forks in 'anticipation' of accidental fork arrest during chromosome replication. This surveillance role would ensure a smooth progression of the cell cycle, and maintain the stability of the genome.

Accumulation of PriA, RecQ and RecG at the replication factory also increases their concentration close to active chromosomal forks. As demonstrated by the PriA_{ZF} mutant (Figure 4), the low cellular concentration of PriA_{BS} is compensated by SSB-dependent targeting to the replication factory, which also facilitates binding onto arrested DNA forks and thus replication restart. Quantification of the levels of RecG_{BS} and RecQ_{BS} indicates that these are also low abundance proteins (data not shown). Thus, their action at replication forks could also be similarly favored by their SSB-dependent targeting and accumulation near the active forks.

The key action of PriA on arrested forks is to trigger replisome assembly on forked DNA (Liu and Mariani, 1999). The forked DNA substrate could simply be the arrested fork directly accessible to PriA, or could result from the repair and/or the remodeling of the arrested fork by specific proteins (such as RecG or RecQ). In this case, the action of PriA would take place at a later step (Heller and Mariani, 2006). The colocalization of PriA with active replication forks explains how PriA can be used efficiently by all these rescue

Figure 7 Model for an SSB-based targeting mechanism of PriA, RecG and RecQ to the *B. subtilis* DNA replication factory. The upper part of the figure illustrates a growing *B. subtilis* cell with a DNA replication factory located at mid-cell. The factory contains multiple molecules of the low-abundance PriA, RecG and RecQ proteins. (A) The targeting or retention of these three repair helicases is based on dynamic interactions (symbolized by the thin arrows) with Cter domains of SSB molecules, which are polymerized along the lagging-strand template of active forks in the replication factory (only one fork is pictured). (B) Upon inactivation of the replication fork provoked, for instance, by damage to the replisome, the retained helicases can immediately act on the fork to attempt its rescue. The multiple opportunities to find an appropriate solution are symbolized by the larger arrows.

pathways, without the need to develop specific individual PriA recruitment mechanisms.

SSB tetramers polymerize at active bacterial replication forks along ~1–2 kb of the lagging strand template (Lohman and Ferrari, 1994). As a result, hundreds of copies of the C termini of SSB are concentrated in the replication factory. Detailed analysis of the interaction of PriA with SSB *in vitro* has shown an apparent preference of PriA for SSB bound to ssDNA (see Figure 3A and B). This would favor continual contact of PriA with the fork in interaction with the lagging strand of the active forks along which SSB is constantly cycling. In addition, when SSB was present in excess with respect to PriA and its specific DNA-binding site, SSB did not

sequester PriA, but instead favored PriA accession to its binding site. Thus, the PriA-SSB interaction is not static, but seems to alternate between bound and unbound state. We propose that this dynamic mode of protein-protein interaction and the enrichment of SSB molecules of replication forks account for the retention of PriA in the replication factory, as well as for driving PriA to its replisome assembly site. As RecG and RecQ copurify with SSB (Supplementary Figure S7), we suggest that they interact with SSB in a similar way to PriA (Figure 7). We propose that PriA, RecG and RecQ are dynamically retained in the replication factory by interaction with the pool of SSB molecules (Figure 7A). When replication arrest occurs, their forked DNA substrate is generated and the SSB-driven enrichment of the recombination/repair proteins leads to efficient fork repair (Figure 7B). A central feature of the model is the high local concentration of these three repair helicases in the replication factory due to their dynamic retention by SSB, which would favor their action on stalled forks. As a result, they (the repair helicases) are already present to initiate a rescue action on the many structurally distinct types of arrested forks resulting from distinct causes of arrest. In such a scenario, the stalled forks that present long stretches of ssDNA bound by SSB (as drawn in Figure 7B) could be more favorable targets than fully double-stranded fork that would be accessed less efficiently.

Deletion of C-terminal amino acids from *E. coli* SSB leads to cell death (Curth *et al*, 1996), in contrast to the viability of the *B. subtilis* *ssb*Δ35 strain. Nonetheless, this mutant strain has a reduced viability (~threefold less than the wild type) and appears moderately temperature sensitive for growth. In addition, *ssb*Δ35 cells are highly defective in repair of DNA damage, as judged by extreme sensitivity to UV and MMC (F Lecoïnte, A Costes and P Polard, unpublished data). These later defects could be due to the failure to efficiently repair and restart the replication forks blocked at damaged DNA, because repair proteins do not accumulate in the vicinity of the forks. Nonetheless, the viability of the *ssb*Δ35 strain in rich medium contrasts with the inviability of *priA* null strains in the same medium. The death of *priA* null cells is explained by the failure to efficiently restart 'spontaneously' arrested chromosomal forks that occur at a higher frequency in fast-growing cells (because of a higher numbers of forks per cell). How can *ssb*Δ35 cells, which are deficient for anchoring PriA in the replication factory, survive spontaneous replication fork arrest when grown in rich medium? First, PriA is still present in the cell and may still be able to restart some arrested forks, albeit in an inefficient SSB-independent manner. Second, a PriA-independent replication restart pathway has been characterized, in both *E. coli* and *B. subtilis* (Sandler and Marians, 2000; Bruand *et al*, 2001), which is essential for growth of *priA* null strains in minimal medium. This secondary replication restart pathway could be more efficient in *ssb*Δ35 cells than in wild-type cells. Third, RecG and RecQ and possibly many other proteins (see below) are no longer present in the replication factory of *ssb*Δ35 cells. Although these proteins are viewed as positive effectors of the repair-arrested forks, they could also act undesirably on active forks and provoke their arrest. This would not be deleterious for PriA⁺ cells, as such 'accidentally' arrested forks could be efficiently reactivated by PriA present in the replication factory. This could also be tolerated by the *ssb*Δ35 cells,

because the reduced concentration of error-prone repair proteins in the replication factory would reduce the level of spontaneous replication arrests.

Constitutive localization at the replication factory is not a general feature of all proteins involved in rescue of arrested forks. Thus, the replication restart partners of PriA_{BS} (i.e., DnaD, DnaB and DnaI) exhibit distinctive subcellular localizations (Imai *et al*, 2000; Meile *et al*, 2006). The RecA recombinase is another example of a protein that does not localize constitutively in foci during cell growth, even though it is central to many pathways of replication reactivation (Kreuzer, 2005). However, RecA does localize in foci in *B. subtilis* and *E. coli* cells treated with DNA-damaging agents (Kidane and Graumann, 2005; Renzette *et al*, 2005; Simmons *et al*, 2007). In *B. subtilis*, these RecA_{BS} foci localize to the replication factory, and their formation depends on replisome activity (Simmons *et al*, 2007). Thus, the many repair proteins that can potentially act on arrested DNA chromosomal forks could be distinguished by their property to associate or not with active forks. We propose that proteins of the former group provide a first line of rescue responses, such as the direct PriA-mediated replication restart process, to solve the problem that has provoked the arrest. The solution developed by the proteins of this first group might be the recruitment of effectors of the second group, which can then provide another set of responses. Such a scenario could apply to RecQ_{EC}, which localizes constitutively at the *E. coli* replication factory (Sherratt *et al*, 2004), and mediates recruitment of RecA_{EC} on forks stalled by means of a temperature sensitive replicative DNA polymerase: RecA_{EC} bound to ssDNA at stalled forks could then trigger the SOS response (Hishida *et al*, 2004).

In conclusion, SSB acts as a protein platform at active DNA replication forks to retain protein tools that could provide immediate solutions to accidental fork arrest. The next challenge will be to identify the complete repertoire of proteins in this SSB-based toolbox of the replication forks. A first clue is given by the many conserved proteins that can interact with *E. coli* SSB (Butland *et al*, 2005), including the PriA, RecG and RecQ helicases.

Materials and methods

Bacterial strains and plasmid constructions

B. subtilis strains were based on the 168 strain (Supplementary Table S1). The plasmids used are listed in Supplementary Table S2 along with the strategies used for their construction. Strains were propagated in LB (except for microscopy analysis, see below) supplemented with appropriate antibiotics (ery, 0.6 μg/ml; spec, 60 μg/ml and/or cm, 5 μg/ml). Unless indicated, *priA*_{ind}, *def*_{ind}, *ssb*Δ35 and *ssb*3⁺ strains were maintained with IPTG (1 mM).

Western blot analysis

Whole-protein extracts from *B. subtilis* cells and Western blot analysis were performed as described previously (Bruand *et al*, 2005), using rabbit sera containing anti-PriA_{BS}, -SSB_{BS} or -GFP antibodies.

Microscopy and analysis of the localization patterns

Strains containing a GFP fusion were grown in LB at 30°C supplemented or not with 0.2% D-xylose to an A₆₅₀ ~0.5. Cells were stained with FM5-95 (Molecular Probes) to visualize the cell membrane and with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes) to visualize the nucleoid. For strains containing YFP and the DnaX-CFP fusions, cells were grown overnight at 37°C in 5 ml minimal medium glucose (MMG) supplemented with 20 μl of LB. MMG was a modification of MSM medium (Meile *et al*, 2006)

containing 0.5% glucose, 6 mM MgSO₄ and 0.005% Tryptophan. Each culture was then diluted to an A₆₅₀ = 0.02 in fresh Minimal Medium Xylose (MMX, i.e. MMG with 1% D-xylose and without glucose) and cells were grown at 30°C until A₆₅₀ ~ 0.2. Cells were deposited on slides covered with 1.2% agarose in minimal medium (Meile *et al*, 2006). Coverslips were positioned and slides were examined with a Leica DMRA2 microscope equipped with a × 100 magnification oil-immersion objective and a COOLSNAP HQ camera (Roper Scientific, USA). Images were captured and processed with METAMORPH V6.3r5.

Protein purification

PriA_{BS} and PriA_{EC} have been purified as monomers and SSB_{BS} as a tetramer as described previously (Polard *et al*, 2002; Bruand *et al*, 2005). PriA_{ZF} and PriA^{K301A} were purified by the same procedure. SSB_{Acter} was purified as soluble untagged protein expressed in *E. coli* as described in the Supplementary Data.

Gel filtration assays

The high-resolution assays on Superdex 200 HR10/30 columns (GE) in Figure 2B–D were carried out as described previously (Velten *et al*, 2003); the buffer used was 50 mM Tris–HCl, pH 8, 0.4 M NaCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT.

EMSA and ATPase assays

EMSA were performed with 0.4 nM of DNA substrates prepared as described previously (Marsin *et al*, 2001) with the use of

oligonucleotides listed in the Supplementary Data. Reactions were incubated 10 min at 30°C in buffer B (20 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.1 mg/ml BSA, 1 mM DTT, 2.5% glycerol) and loaded on 5% native polyacrylamide gel (29:1) and run at 4°C for 3 h either in TAE 1 × (Figure 3A–B) or in TAME buffer (6 mM Tris–HCl, pH 7.8, 4 mM MgOAc, 10 mM NaOAc, 1 mM EDTA) (Figure 3C–D). ATPase assays were carried out as described previously (Velten *et al*, 2003) except that the 10 µl reactions contained 3.5 nM of [³²P]γATP (added to 1 mM ATP) and 2.5 µM in nucleotides of M13mp18 or Φ × 174 circular ssDNA (before PriA_{BS} addition, all components were incubated for 10 min at 37°C). After 90 min at 37°C, the reactions were stopped by addition of 5 µl of buffer S (3 mM ATP, 3 mM ADP, 50 mM EDTA).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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