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Bacillus subtilis RecO and SsbA are crucial for RecA-mediated recombinational DNA repair

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ABSTRACT

Genetic data have revealed that the absence of *Bacillus subtilis* RecO and one of the end-processing avenues (AddAB or RecJ) renders cells as sensitive to DNA damaging agents as the null *recA*, suggesting that both end-resection pathways require RecO for recombination. RecA, in the rATP·Mg^{2+} bound form (RecA·ATP), is inactive to catalyze DNA recombination between linear double-stranded (ds) DNA and naked complementary circular single-stranded (ss) DNA. We showed that RecA·ATP could not nucleate and/or polymerize on SsbA·ssDNA or SsbB·ssDNA complexes. RecA·ATP nucleates and polymerizes on RecO·ssDNA·SsbA complexes more efficiently than on RecO·ssDNA·SsbB complexes. Limiting SsbA concentrations were sufficient to stimulate RecA·ATP assembly on the RecO·ssDNA·SsbB complexes. RecO and SsbA are necessary and sufficient to 'activate' RecA·ATP to catalyze DNA strand exchange, whereas the AddAB complex, RecO alone or in concert with SsbB was not sufficient. In presence of AddAB, RecO and SsbA are still necessary for efficient RecA·ATP-mediated three-strand exchange recombination. Based on genetic and biochemical data, we proposed that SsbA and RecO (or SsbA, RecO and RecR *in vivo*) are crucial for RecA activation for both, AddAB and RecJ–RecQ (RecS) recombinational repair pathways.

INTRODUCTION

The recombinases of the RecA family (e.g. bacterial RecA, archaeal RadA and eukaryotic RAD51 and DMC1), in the presence of accessory factors, efficiently promote DNA strand exchange in a reaction that requires nucleotide cofactor binding and hydrolysis *in vivo*, but only nucleotide binding *in vitro* (1,2). The use of dATP or rATP as a nucleotide cofactor has also unraveled differences between the different bacterial RecA proteins identified so far. *Escherichia coli* RecA, in the rATP·Mg^{2+}-bound form (denoted as RecA·ATP), nucleates and polymerizes onto single-stranded (ss) DNA and catalyzes DNA strand exchange (1,3–5). Conversely, RecA proteins from naturally competent bacteria (e.g. *Bacillus subtilis*, *Streptococcus pneumoniae*, *Deinococcus radiodurans*) nucleate onto protein-free ssDNA, but do not catalyze DNA strand exchange in the presence of ATP (6–10). When *E. coli* RecA activities were compared with those of distantly related RecA protein from naturally competent bacteria, it was assumed that the latter proteins have evolved to accommodate new activities (e.g. natural chromosomal transformation) absent in the former (11).

All RecAs are likely regulated at multiple levels through different factors, to ensure that the dynamic nucleoprotein filaments (NPFs) are assembled onto ssDNA only where homologous recombination is required. Accessory factors can inhibit or stimulate RecA activities at the pre-synaptic, synaptic or post-synaptic stage. The factors that act at the pre-synaptic stage, known as mediators, can be divided into two groups: (i) single-stranded binding (SSB) proteins (the essential SsbA and the competence-specific SsbB) that protect ssDNA, eliminate DNA secondary structures to facilitate NPF formation, but limit or inhibit RecA nucleation; and (ii) the 'facilitators' that mediate RecA assembly onto protein-free ssDNA (such as the *E. coli* RecBCD complex) during double-strand break (DSB) repair or that facilitate the RecA loading onto SSB-coated ssDNA during the repair of single-strand gaps (SSGs) or DSBs (e.g. RecO, RecR and RecF) (reviewed in 5,12,13).

The mechanism by which the recombinase is delivered to the 3′-tailed duplex or at SSGs is of universal importance. In wild-type (wt) *E. coli*, ∼99% of the recombination events occurring at DSBs require the RecBCD (functional counterpart of Firmicutes AddAB or Actinobacteria AdnAB) complex for the resection of the broken ends. The *E. coli* RecBCD complex, through a RecB·RecA interaction, directly loads RecA onto newly generated 3′-ended naked ssDNA during continuous unwinding (14–17). Then, *E. coli* RecA forms a pre-synaptic filament that efficiently

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displaces SSB bound to other regions of ssDNA (5,13). This species-specific RecA loading mechanism might be conserved among bacteria of the Proteobacteria phylum. Indeed, the co-expression of *recBCD* and *recA* genes of *Serratia marcescens, Proteus mirabilis* or *Helicobacter pylori* was necessary to suppress the DNA repair defect in *E. coli recBCD* cells (18,19). However, the expression of only *B. subtilis* AddAB or the *Streptomyces ambofaciens* AdnAB complex was sufficient to partially restore the recombination deficiency of the distantly related *E. coli* Δ*recB* or *recBCD* strain (20,21). In *E. coli*, in the absence of the RecBCD complex, end-processing requires the 'activation' of the RecJ–RecQ complex to promote end resection in the *recBC sbcA* or *recBC sbcB sbcCD* context. In this bacterium, the RecOR or the RecORF complex loads RecA onto SSB-coated SSGs and RecA NPF growth displaces the SSB protein to catalyze DNA recombination to repair SSGs (22–27).

Are these RecA loading mechanisms conserved among bacteria? The distribution of genes among different bacteria suggests that at least in some cases different end-processing systems or facilitators might be used. Indeed, among free-living bacteria >99% of the 782 genomes examined contained *recA* and *ssbA/ssb* genes, >95% of the genomes contained *recO* and >87% of the genomes contained the *recR* and *recJ* genes. In contrast, only ∼50 and ∼30% of the genomes examined contained *recBCD* (*addAB* or *adnAB*) and *recF* genes, respectively (28). It is likely, therefore, that a substantially large number of bacterial species might use the RecO or RecOR loading system instead of RecBCD or RecORF 'facilitators'. (Note that, unless stated otherwise, the indicated genes and products are of *B. subtilis* origin. The nomenclature used to denote the origin of proteins from other bacteria is based on the bacterial genus and species [e.g. *E. coli* RecA is referred as $RecA_{Eco}$]).

The RecA loading step is poorly understood in Firmicutes and has been partially characterized in *B. subtilis*, being the best-characterized member of this phylum. Genetic data have shown that (i) the over-expression of a heterologous SSB protein, the absence of HelD (also termed Helicase IV) or the presence of the RecA73 mutation partially suppressed mutations in the *recO*, *recR* and *recF* genes (29,30); and (ii) *recO*, *recR* or *recF* mutations suppress the lethality of the *prcA* mutation (31). Altogether, these data suggest that RecO, although epistatic to RecF, might play an independent role (32,33). Two pathways, AddAB or RecJ (in concert with a RecQ-like DNA helicase [RecQ or RecS] and SsbA), are required for long-range end processing (34). A null mutation in *recJ* (Δ*recJ*), in *addA* (*addA*5) and *addB* (*addB*72), or in *recF* (*recF*15) renders cells moderately, sensitive and very sensitive, respectively, to the killing action of DNA damaging agents, whereas a null mutant in *recO* (Δ*recO*) or *recA* (Δ*recA*) renders cells extremely sensitive (34–37). Furthermore, cytological analyses revealed that (i) plasmid establishment is marginally impaired in *recF*15 competent cells (two-fold), but reduced ∼25-fold in Δ*recO* natural competent cells (38); (ii) RecO and RecR are required for damage-induced RecA foci formation rather than RecF (39,40); and (iii) RecO (or RecOR) targets RecA onto DNA damage-induced RecN foci, whereas RecF subsequently and concomitantly localizes with RecX (34,39,41,42).

Previous studies have shown that RecA·ATP is not competent to promote strand exchange between linear dsDNA (*lds*) and SsbA- or SsbB-coated circular ssDNA (*css*) (8,9). However, like $RecA_{Eco}$·ATP (1,3,4), RecA·dATP efficiently polymerizes and catalyzes DNA recombination onto SsbA-coated ssDNA *in vitro* (43,44). Since RecA binds dATP and ATP with similar affinity, but the ATP pool is 100- to 500-fold higher than that of dATP (45,46), it was assumed that ATP is a genuine substrate and accessory factors are required to activate RecA·ATP to catalyze DNA recombination (9,47). Indeed, RecA·dATP catalyzes DNA recombination in the absence of accessory factors (43,44), but the addition of ATP at ATP:dATP ratios of 4:1 or higher, blocked RecA·dATP-mediated DNA strand exchange (9). Previous studies have shown that competence-specific DprA in concert with SsbA activates RecA·ATP to catalyze DNA strand exchange during chromosomal transformation (9), but it remains unknown which factor(s) are required for RecA·ATP activation during recombinational DNA repair.

To understand how RecA·ATP might be activated to catalyze DNA recombination, the molecular mechanism of RecA·ATP nucleation and NPF extension on SsbA- or SsbB-coated ssDNA in the presence of different facilitators was studied *in vitro*. Here, we showed that RecO facilitates RecA·ATP nucleation onto protein-free ssDNA, but these NPFs are inactive to catalyze DNA strand exchange *in vitro*. RecA·ATP could not displace SsbA or SsbB from ssDNA, but the addition of RecO overcomes the negative effect exerted through SsbA or SsbB to a different extent. RecA·ATP nucleates and polymerizes on RecO·ssDNA·SsbA complexes more efficiently than on RecO·ssDNA·SsbB complexes. RecO, upon interacting with SsbA, facilitates the activation of RecA·ATP to catalyze a three-strand exchange reaction. However, RecO and SsbB did not activate RecA·ATP to catalyze DNA recombination. AddAB cannot activate RecA·ATP to catalyze DNA recombination between a tailed duplex and complementary circular ssDNA, containing single or an array of crossover hotspot instigator (χ) sites. In the presence of AddAB, RecA·ATP-mediated DNA strand exchange still required RecO and SsbA. Taken together, these results provided the first evidence that RecO and SsbA might work as a two-component mediator of RecA-mediated DNA repair. Furthermore, the contribution of SsbA and RecO (probably in concert with RecR *in vivo*) to the dynamics of RecA–ssDNA NPF extension onto ssDNA is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids and mutant survival

Escherichia coli BL21(DE3)[pLysS] cells bearing pCB722 *ssbA*, pCB777 *ssbB* or pCB669 *recO* were used to overproduce the SsbA, SsbB and RecO proteins, respectively, as described (8,43,44,48). *B. subtilis* BG214 cells bearing pBT61 *recA* was used to overproduce RecA (49). The 3199-bp pGEM3 Zf(+) was used as a source of ss- and ds-DNA (Promega Biotech, Spain). A 91-bp AlfIII-cleaved DNA segment containing ten

closely spaced χ sequences (χ10, underlined) (5′-cggaacatgtTCAGCGGCAGCGGATAGCGGGAAAGCGGATAGCGGCAAGCGGAAAGCGGATAGCGGTAAGCGGAAGCGGTTacatgtggca-3′) or a variant with these ten χ sequences mutated (χ0) (5′-cggaacatgtTCAGTGGCAGTGGATAGTGGGAAAGTGGATAGTGGCAAGTGGAAAGTGGATAGTGGTAAGTGGAAGTGGTTacatgtggca-3′) (50,51) was joined to *Afl*II-cleaved pGEM3 Zf(+) to render a 3,290-bp pGEM3 Zf(+) derivatives containing ten (χ10) in the forward (χ10F or χ0F) or in the reverse (χ10R or χ0R) orientation. These DNA substrates described as pGEMχ10F, pGEMχ0F, pGEMχ10R or pGEMχ0R). Another set of plasmids were constructed where the 91-bp DNA segment containing χ10 was also joined to EcoRI-cleaved pGEMχ10F to render the 3381-bp pGEM3 Zf(+) derivative containing a χ10F (at position 424) and χ10R (at position 3275) to generate the pGEMχ10F χ10R or pGEMχ0F χ10R substrates.

All *B. subtilis* strains listed in Supplemental Table S1 are isogenic to BG214 (34,52,53). Survival assays were performed using *B. subtilis* strains grown to an OD_{560} of 0.4 at 37°C in LB broth (~5 × 10^7 cells/ml in the wt or Δ*recJ* context). For chronic studies, 10 μl of serial 10-fold dilutions (1 × 10^{-3} to 1 × 10^{-6}) were spotted onto freshly prepared LB agar plates supplemented with the indicated concentrations of MMS or H_2O_2 and the plates were incubated overnight at 37°C as described. For acute survival studies, *B. subtilis* strains exponentially grown to an OD_{560} of 0.4 at 37°C in LB broth were exposed for 15 min to different concentrations of MMS or H_2O_2 and subsequently diluted and plated onto LB agar plates. In a large majority of *B. subtilis* cells with no affected growth rate (e.g. wt or Δ*recJ* cells) there were one and two non-separated cells with an average of ~1.6 cell/colony forming units (CFU), suggesting that the proportion of cells is similar to the CFUs. In cells with diminished growth rates (e.g. Δ*recO*, Δ*recO* Δ*recJ*, Δ*recO* Δ*addAB* and Δ*recA* cells), the majority showed doublet cells with an average of ~1.9 cell/CFU.

Enzymes, reagents, protein and DNA purification

All chemicals used in this study are of analytical grade. IPTG was from Calbiochem; DNA restriction enzymes, DNA ligase, etc. were supplied by Roche; and polyethyleneimine, DTT, ATP, dATP, ATPγS and AMP–PNP were from Sigma. The ATPγS nucleotide is contaminated with 5–8% of ATP (54). DEAE, Q- and SP-sepharose were from GE healthcare, hydroxyapatite from BioRad and phosphocellulose was from Whatman.

Untagged SsbA (18.7 kDa), SsbB (12.4 kDa), RecO (29.3 kDa) and RecA (38.0 kDa) proteins were expressed and purified as described (43,44,55). The corresponding molar extinction coefficients for SsbA, SsbB, RecO and RecA were calculated as 11 400; 13 000; 19 600 and 15 200 M^{-1} cm^{-1}, respectively, at 280 nm, as previously described (55). The protein concentrations were determined using the above molar extinction coefficients. RecA is expressed as moles of monomeric, RecO as dimeric and SsbA and SsbB as tetrameric proteins. However, under optimal RecA conditions (10 mM Mg^{2+}) the SSB proteins occlude 65-nt, with the ssDNA wrapping around all four subunits of the tetramer (SSB_{65}) (56,57). In this study, the experiments were performed under optimal RecA conditions (10 mM MgOAc), hence the SSB proteins were expected to be in the SSB_{65} binding mode (see above). A RecO dimer should bind 30- to 40-nt (58) and a RecA monomer should bind 3-nt (59). AddAB and its nuclease-dead mutant ($AddA^N addB^N$, which contains two mutations ($AddA_{D1172A}$ and $AddB_{D961A}$) that effectively eliminate the nuclease activity of the AddAB complex were a gift of M. S. Dillingham (Univ. of Bristol, UK) and expressed as moles of heterodimers.

Duplex DNA from pGEM3 Zf(+), pGEMχ10F, pGEMχ0F, pGEMχ10R or pGEMχ0R and ssDNA from pGEM3 Zf(+), pGEMχ10F, pGEMχ0F, pGEMχ10R or pGEMχ0R were purified as described (55). DNA concentrations were established using the molar extinction coefficients of 8780 and 6500 M^{-1} cm^{-1} at 260 nm for ssDNA and dsDNA, respectively, and are expressed as moles of nt. In the text the protein concentrations are expressed as stoichiometric ratios with respect to ssDNA, which is expressed as moles of nt, whereas in the corresponding Figure legends the molar concentrations of proteins and ssDNA/dsDNA are presented.

RecA (d)ATP hydrolysis assays

The ssDNA-dependent dATP or ATP [(d)ATP] hydrolysis activity of RecA protein was assayed via a coupled spectrophotometric enzyme assay as previously described (24,44). The rate of ssDNA-dependent RecA-mediated (d)ATP hydrolysis and the lag times were measured in buffer A (50 mM Tris–HCl [pH 7.5], 1 mM DTT, 80 mM NaCl, 10 mM MgOAc [magnesium acetate], 50 μg/ml BSA, and 5% glycerol) containing 5 mM (d)ATP for 25 min at 37°C as described (44).

The orders of addition of 3199-nt pGEM3 Zf(+) ssDNA (10 μM in nt) and purified proteins and the concentrations of these molecules are indicated in the text. The data obtained from (d)ATP hydrolysis were converted to [(d)ADP] and plotted as a function of time as described (44).

As previously reported (24,44), the lag time, which represents the delay in reaction progress relative to a theoretically reaction curve lacking it, was derived from the time intercept of a linear regression line fit to the steady state portion of data in (d)ATP hydrolysis assays

RecA-mediated DNA strand exchange

Standard reactions containing 3199-bp KpnI-cleaved pGEM3 Zf(+) dsDNA (20 μM in nt) and the homologous circular 3199-nt ssDNA (10 μM in nt) were pre-incubated with the indicated protein or protein combination in buffer A containing 5 mM dATP, ATP, ATPγS or AMP-PNP for 5 min at 37°C. Then a fixed RecA concentration was added and the reaction incubated for fixed or variable times at 37°C. A (d)ATP regeneration system (8 units/ml creatine phosphokinase and 8 mM phosphocreatine) was included in the recombination reaction.

When indicated KpnI-cleaved 3290-bp dsDNA (pGEMχ10F, pGEMχ0F, pGEMχ10R or pGEMχ0R) and the homologous circular ssDNA (+ strand) were

pre-incubated in the presence of the indicated proteins. After the recombination reaction took place, samples were deproteinized as described (48,60) and fractionated through 0.8% agarose gel electrophoresis (AGE) with ethidium bromide. The signal of the different DNA substrates and products was quantified from the gels using a Geldoc (BioRad) system as described (43).

RESULTS AND DISCUSSION

The *ΔrecO ΔrecJ* or *ΔrecO ΔaddAB* strain renders cells extremely sensitive to DNA damaging agents

The accessory factors that act prior to RecA during DSB repair can be divided into three discrete classes (Figure 1A): (i) the proteins required for DNA damage recognition and basal end processing, such as RecN and PNPase (38,41,54,61,62); (ii) the proteins involved in long-range end-resection to generate a 3′-tailed complex, such as the AddAB complex or RecJ, in concert with a RecQ-like DNA helicase (RecQ and/or RecS) and SsbA (63,64); and (iii) the facilitators, such as RecO, RecOR or RecORF, which assist in the recovery of RecA from the inhibitory effect of the SsbA protein and are necessary for DNA damage-induced RecA foci formation (39–41). During end-processing, the $RecBCD_{Eco}$ enzyme facilitates the loading of $RecA_{Eco}$ onto ssDNA (14–17). If this feature is universally conserved, then in a coupled reaction, AddAB should resect the DNA ends and directly load RecA onto newly generated 3′-end naked ssDNA during continuous unwinding. This hypothetical loading process was depicted herein, but at present, the coupled reaction (RecA loading during AddAB end-resection) is not understood in *B. subtilis* (see Figure 1A). During the repair of SSGs, the requirement of DSB recognition functions and one of the end processing pathways (AddAB complex) is not obvious (Figure 1A) (33,64). Here, end resection and RecA loading are two discrete reactions linked by SsbA, which physically interacts with RecJ, RecQ, RecS and RecO (65).

Previously, it was shown that the Δ*recJ recF*15 or the *addA*5 *addB*72 *recF*15 strain renders cells very sensitive, but the Δ*recO* or Δ*recA* strain renders cells extremely sensitive to different DNA damaging agents (34–37), suggesting that RecO, although epistatic to RecF, might play another role (32,33). To gain insight into this hypothetical RecO function and determine whether long-range end processing contributes to RecA activation to catalyze DNA strand exchange, a genetic analysis was performed. Double (Δ*recJ* Δ*recO*) and triple (Δ*addAB* Δ*recO*) mutant strains were constructed (see supplementary Table S1) and challenged with methylmethane sulfonate (MMS) and H_2O_2 to assess cell survival (see Supplementary Table S1). Both DNA damaging agents are specifically removed through base excision repair (32,33) and generally repaired through homologous recombination. Unrepaired MMS-damage, which might not compromise nucleoid integrity, stalls replication fork progression and generates singled-stranded DNA regions. In contrast, H_2O_2 collapses replication fork progression and promotes, among other types of DNA lesions, single-strand nicks and/or DSBs leading to one- or two-ended DSBs (66).

Serial dilutions of Δ*addAB*, Δ*recJ*, Δ*recO*, Δ*addAB* Δ*recO* or Δ*recJ* Δ*recO* cells, grown to a similar OD_{560} at 37°C in LB broth, were exposed to increasing MMS or H_2O_2 concentrations and the rate of survival was observed after chronic exposure to these two agents. The Δ*recA* strain was used as a control in these experiments. Except Δ*recJ*, untreated cultures rendered a reduced number of CFUs (Figure 1B, No drug), suggesting that these genes are important for normal growth. Under the drug concentrations used (50–90 μM MMS and 60–250 μM H_2O_2) the survival rate of the Δ*recJ* strain was marginally affected if at all (Figure 1B and C), but the survival of Δ*recJ* cells was significantly reduced compared with the wt *rec*$^+$ strain upon chronic exposure to 2.2 mM MMS or 1 mM H_2O_2 (data not shown) (34). As expected, Δ*addAB* cells were not affected when chronically exposed to 90 μM MMS; but the cells were sensitive to H_2O_2-generated DSBs (Figure 1B and C). The Δ*recO* strain rendered cells extremely sensitive to 50 μM MMS and 60 μM H_2O_2 at levels comparable to the Δ*recA* strain (Figure 1B and C). Similarly, a *recO* mutation decreased resistance to UV or ionizing radiation, respectively, to levels similar to the *recA* mutant in naturally competent *Neisseria gonorrhoeae* or *D. radiodurans* cells (67,68). Similarly, the clastogen sensitivity of the *Mycobacterium smegmatis* Δ*recO* strain was indistinguishable from that of the Δ*recA* (69).

Previous results have shown that RecA is epistatic to RecJ, AddAB or RecO (34,42). The double (Δ*recJ* Δ*recO*) or triple (Δ*addAB* Δ*recO*) mutant strains were also extremely sensitive to both DNA damaging agents at levels comparable to Δ*recO* (or Δ*recA*) (Figure 1B and C). To quantitate these DNA repair defects, cells exponentially grown to a similar OD_{560} at 37°C in LB broth were acutely exposed to 50 to 90 μM MMS, 60 to 250 μM H_2O_2 or no drug for 15 min (Supplemental material Supplementary Figure S1A and B). Except Δ*recJ*, untreated cultures showed approximately one in seven to ten inviable cells (S1A and B). As observed during chronic exposure (Figure 1B and C), the Δ*recJ* Δ*recO* or Δ*addAB* Δ*recO* strain rendered cells as sensitive to MMS or H_2O_2, similar to Δ*recO* or Δ*recA* (Supplementary Figure S1A and B).

These results suggest that *recO* is epistatic to *recJ* and *addAB*. At least two alternatives can be envisioned: (i) RecO and RecJ or RecO and AddAB might work in similar RecA-dependent sub-pathways to promote homologous recombination; or (ii) RecO might also work in a RecA-independent pathway. We favored the first alternative because (i) MMS-mediated SSGs or one-ended DSBs cannot be repaired through specific two-ended DSB repair pathways (e.g. single-strand annealing or non-homologous end-joining); (ii) RecA-dependent survival of *B. subtilis* spores was unaffected in end-processing mutants (*addAB* or *recJ*), but dependent on RecO, RecR, RecF and RecA functions (70); (iii) deletion between repeated sequences occurs at high frequency in the *recO*16 (formerly *recL*16) or *recA*4 (formerly *recE*4) context (71); (iv) the Δ*recO*, Δ*recJ* Δ*recO* or Δ*addAB* Δ*recO* strain exposed to 90 μM MMS led to mutation rates (measured as rifampicin-resistant) within the error for the wt strain (data not shown); and (v) in the absence of DprA, RecO plays an active role in RecA-dependent natural chromosomal transformation between internalized linear ssDNA coated with SsbA and the homologous recipient duplex (44).

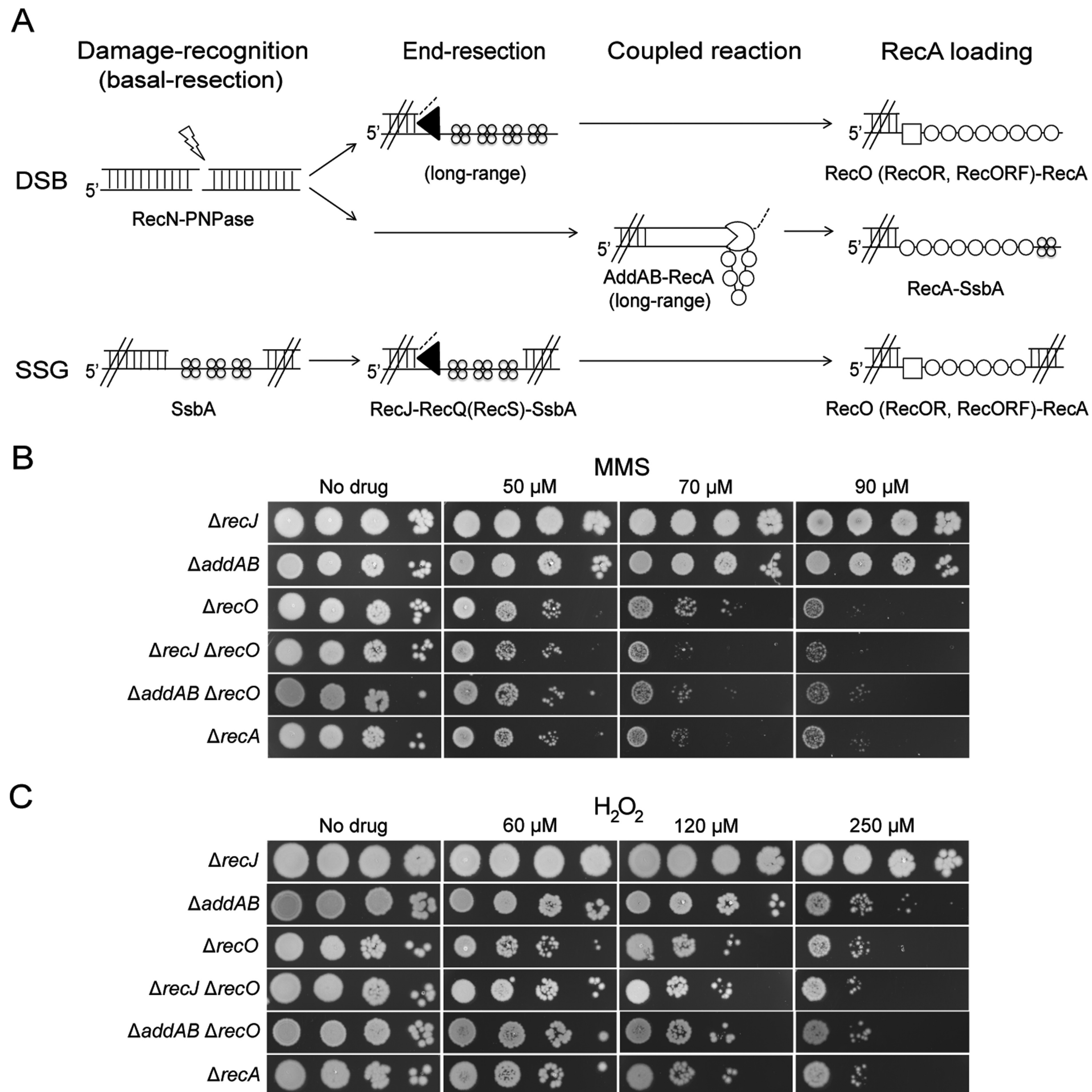

Figure 1. General model of substrate processing at the presynaptic stage during recombinational DNA repair. (**A**) A DSB is first recognized by RecN and processed by PNPase or other uncharacterized nucleases to generate a blunt or nearly blunt DNA end. Then, the ends are resected by one of the long-range end-processing complexes: the RecJ–RecQ (RecS) complex (denoted by as filled triangle) in concert with SsbA and the resulting ssDNA is then coated by SsbA (denoted as four joined circles) or by the AddAB complex (denoted as a pacman). Both end-processing complexes generate the 3′-terminated ssDNA overhang (χ- or random terminated) used by a mediator(s) to load RecA·ATP (denoted as a circle). It is unknown whether the AddAB complex contributes to RecA loading (coupled reaction). At a SSG, the RecJ-RecQ (RecS)–SsbA complex resects the end and RecA requires mediators (RecO, RecOR or RecORF, denoted as a square) for loading onto SsbA bound to ssDNA. (**B** and **C**), Survival of Δ*addAB*, Δ*recJ*, Δ*recO*, Δ*addAB* Δ*recO*, Δ*recJ* Δ*recO* or Δ*recA* cells after chronic exposure to the indicated concentrations of MMS (B) and H_2O_2 (C). The strains used are identified according to the indicated relevant genotype. Serial dilutions of a culture of each strain were plated onto LB medium containing the indicated MMS or H_2O_2 concentrations or lacking them (No drug). The results obtained are representative from more than four independent experiments.

Unlike *E. coli* where ~99% of the recombination events occurring at one- or two-ended DSBs require the RecBCD complex (1,4), in *B. subtilis, N. gonorrhoeae*, *D. radiodurans* and *M. smegmatis* ~99% of the recombination events occurring at stalled or collapsed forks require RecO (Figure 1C and Supplementary Figure S1B) (67–69).

SSBA OR SSBB BLOCKS RECA-MEDIATED ATP HYDROLYSIS *in vitro*

To understand why RecA·ATP poorly polymerizes onto naked ssDNA, we measured ssDNA-dependent dATP or ATP (generically termed (d)ATP) hydrolysis as an indirect readout for the nucleation and polymerization of RecA onto ssDNA coated with SsbA or SsbB. For comparison,

the experiments were also performed in the presence of dATP. SsbA or SsbB (1 SSB /33-nt) did not exhibit (d)ATP hydrolysis activity compared with the mock reaction in the absence of the SSB protein (data not shown). Therefore, the hydrolysis of (d)ATP observed in these experiments can solely be attributed to the presence of RecA protein.

As previously documented, the rate of (d)ATP hydrolysis is generally correlated with the amount of RecA bound to the ssDNA (9,43). RecA·dATP (1 RecA /12-nt) showed a biphasic shape with an initial lag of 4–5 min corresponding to the nucleation time, followed by robust dATP hydrolysis, at a rate of K_{cat} of 17.8 ± 0.3 min^{-1} as previously observed (Supplementary Figure S2A, Table 1) (44). The lag time (nucleation) observed in achieving the steady state rate of RecA-mediated dATP hydrolysis was significantly shorter than the rate observed for $RecA_{Eco}$·ATP, but both proteins showed a similar hydrolysis rate (K_{cat}) (24,44,47,72,73).

When dATP was replaced with ATP, RecA nucleation onto ssDNA showed a monophasic shape (i.e. no apparent delay in the nucleation time), and at maximal ATP hydrolysis, a K_{cat} of 8.9 ± 0.2 min^{-1} was observed (Supplementary Figure S2B and 2, Table 1). This result is consistent with previous studies showing that RecA has a higher rate of dATP than ATP hydrolysis when bound to ssDNA, and changes in reaction conditions, including the pH, do not overcome the observed differences (8,9,43,74). Conversely, $RecA_{Eco}$ filament extension is broadly insensitive (<1.4-fold) to the nucleotide cofactor used (75).

SsbA and SsbB (1 SSB/33-nt) bound to ssDNA and blocked the RecA-mediated hydrolysis of ATP and nucleated RecA·ATP could not compete with SsbB or SsbA (1 SSB/33-nt) when added 5 min after RecA (Supplementary Figure S2B and Table 1) (9). The interaction of RecA with SsbB *in vivo* (76), however, did not contribute to RecA·ATP nucleation or RecA·ATP NPF formation.

When dATP replaced ATP as the nucleotide cofactor, however, SsbA or SsbB prebound to ssDNA increased the lag phase of the nucleation step of RecA·dATP, but after nucleating onto ssDNA, the SSB protein no longer presented a barrier to RecA NPF extension (Supplementary Figure S2A and Table 1) (43,44). The results obtained with RecA·dATP were similar to those observed when $RecA_{Eco}$·ATP nucleation was analyzed (77–79). Conversely, RecA·ATP requires '*activation*' to nucleate or filament onto SsbA- or SsbB-coated ssDNA (Supplementary Figure S2A and B).

RecO facilitates RecA-mediated ATP hydrolysis *in vitro*

To characterize potential differences in the effect of RecO in RecA loading and filament growth onto naked ssDNA, using ATP or dATP as a cofactor, the same type of experiments was performed in the presence of RecO. The control reaction with RecO alone (1 RecO/50-nt) did not exhibit (d)ATP hydrolysis activity compared with the mock reaction in the absence of the RecO protein (data not shown).

The addition of RecO (100 nM; 1 RecO/100-nt) neither reduced the nucleation time nor stimulated RecA·dATP filament growth compared with protein-free ssDNA (Figure 2, Table 1). Similar results were observed when higher RecO concentrations were used (44). However, in the presence of ATP and RecO (1 RecO/100-nt), RecA NPF formation was stimulated (K_{cat} of 10.9 ± 0.2 min^{-1}) when compared with protein-free ssDNA (Table 1). An increase in the RecO concentration (1 RecO/50-nt) further stimulated the final rate of ATP hydrolysis (16.1 ± 0.5 min^{-1}) (Figure 2). From these and previous data, it can be concluded that RecO facilitates nucleation and RecA·ATP NPF formation to levels comparable with RecA·dATP in the absence of RecO (Figure 2).

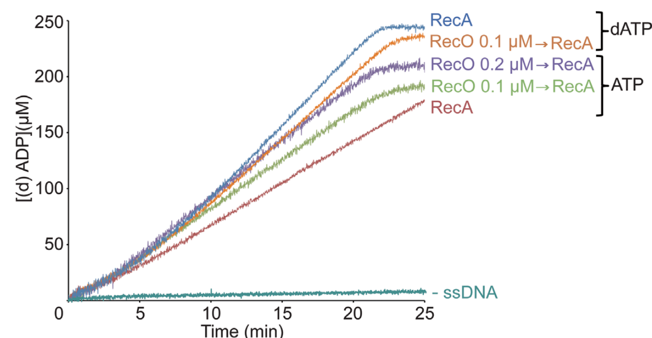

Figure 2. Effect of RecO on RecA nucleation and polymerization. Circular pGEM3 Zf (+) ssDNA (10 μM in nt) was pre-incubated with the indicated concentration of RecO for 5 min at 37°C in buffer A containing 5 mM ATP or dATP. Then RecA (0.8 μM) was added and the (d)ATPase activity measured for 25 min. All reactions were repeated three or more times with similar results. The amount of ATP or dATP hydrolyzed was calculated as described in 'Materials and Methods' section.

Sequence alignments revealed that the full-length RecO has 22% sequence identity to *M. smegmatis* RecO ($RecO_{Msm}$) and 19% identity to $RecO_{Eco}$. Unlike RecO (Figure 2), $RecO_{Eco}$ or *M. smegmatis* RecO ($RecO_{Msm}$) facilitates $RecA_{Eco}$- or $RecA_{Msm}$-filament growth only as part of the $RecOR_{Eco}$ or $RecOR_{Msm}$ complex (24,80). The biochemical differences between these proteins distantly related with RecO remain poorly understood.

RecO nucleates RecA·ATP onto SsbA- or SsbB-coated ssDNA

Previously it has been shown that (i) RecO facilitates RecA·dATP nucleation onto SsbA- or SsbB-coated ssDNA (44); and (ii) RecA·ATP cannot nucleate on the SsbA·ssDNA or SsbB·ssDNA complexes (Supplementary Figure S2) (9). To investigate the effect of RecO on RecA·ATP nucleation and NPF extension onto SsbA- or SsbB-coated ssDNA, the hydrolysis of ATP was monitored in the presence of these three proteins (Figure 3A and B).

The addition of RecO overcame the SsbA or SsbB blockage in RecA-mediated ATP hydrolysis, albeit to different extents (Figure 3A and B, Table 1). At low ratios (1 RecO/100-nt), RecO stimulated RecA·ATP polymerization onto SsbA-coated ssDNA (Figure 3A). Here, RecA nucleation and subsequent filament formation was biphasic, with a slow nucleation step (∼5 min lag phase) preceding the establishment of the maximal hydrolysis rate of 15.5 ± 0.2 min^{-1} (Figure 3A, Table 1). At higher ratios (1 RecO/50-nt), RecO further stimulated RecA filament formation (17.1 ± 0.2 min^{-1}) (Figure 3A). RecA·ATP polymerization rate onto SsbA-coated ssDNA in the presence of RecO was com-

Table 1. Rates of ssDNA-dependent (d)ATP hydrolysis and lag time measurements

Proteins[a]	Condition	Lag time[a] (in min)	k_{cat} min^{-1a}
RecA	dATP[b]	4 ± 0.5	17.8 ± 0.3
(1 RecA/12-nt)	ATP[b]	<1	8.9 ± 0.2
RecA + SsbA	dATP[b]	~9	13.2 ± 0.3
(1 SsbA/33-nt)	ATP[b]	ND	1.5 ± 0.3
RecA + SsbB	dATP[b]	~7	15.4 ± 0.2
(1 SsbB/33-nt)	ATP[b]	ND	1.9 ± 0.2
RecO + RecA	dATP[b]	3 ± 0.3	16.3 ± 0.2
(1 RecO/100-nt)	ATP	<2	10.9 ± 0.3
RecO + RecA	dATP	-	-
(1 RecO/50-nt)	ATP	4 ± 0.2	12.1 ± 0.3
SsbA + RecO + RecA	dATP	-	-
(1 SsbA/33-nt + RecO/100-nt)	ATP	~5	15.5 ± 0.2
SsbA + RecO + RecA	dATP	-	-
(1 SsbA/33-nt + RecO/50-nt)	ATP	~4	17.1 ± 0.2
SsbB + RecO + RecA	dATP	-	-
(1 SsbB/33-nt + RecO/100-nt)	ATP	5–6	7.1 ± 0.2
SsbB + RecO + RecA	dATP	-	-
(1 SsbB/33-nt + RecO/50-nt)	ATP	5–6	7.5 ± 0.2

[a]Rates of RecA-mediated (d)ATP hydrolysis and the nucleation lag times were measured as indicated in 'Materials and Methods' section.
[b]RecA-mediated (d)ATP hydrolysis and the lag time were reported elsewhere (8,43,44) and determined here again for a direct comparison. The steady state kinetic parameters for RecA (1 RecA/12-nt) were derived from the data presented in Figures 2 and 3 and Supplementary Figure S2. The average rate of (d)ATP hydrolysis were obtained from more than three independent experiments. ND, not detected; -, not done.

parable with that of RecA·dATP alone (compared Figures 2 and 3A and Table 1) or $RecA_{Eco}$·ATP NPF formation onto protein-free ssDNA (77–79).

A different outcome was observed when SsbB was used in place of SsbA. The presence of RecO (1 RecO/100 to 50-nt) only partially reversed the negative effect exerted through SsbB on RecA·ATP nucleation (Figure 3B). Here, RecA nucleation onto SsbB-coated ssDNA and subsequent filament formation was also biphasic, with a slow nucleation step preceding the establishment of the maximal rates of hydrolysis of 7.1 and 7.5 min^{-1}, which were slower than the rate of RecA-mediated ATP hydrolysis on protein-free ssDNA (Figure 3B, Table 1). This result is consistent with the observation that SsbB does not interact with RecO, but rather RecO physically interacts with SsbA in solution (43,44).

RecA efficiently nucleates onto RecO·SsbA·ssDNA·SsbB complexes

The results shown in the previous sections suggested that (i) RecA·ATP cannot compete with SsbA or SsbB for binding to ssDNA (Supplementary Figure S2B); and (ii) RecA·ATP nucleates and polymerizes on RecO·ssDNA·SsbA complexes more efficiently than on RecO·ssDNA·SsbB complexes (Figure 3A and B). Furthermore, during natural competence development SsbB might be more abundant that SsbA (11). To analyze these conditions, the ssDNA was pre-incubated with stoichiometric amounts of SsbB (1 SsbB/33-nt) and increasing amounts of SsbA (1 SsbA/333- to 33-nt). RecO and subsequently RecA were added and RecA-mediated ssDNA-dependent ATP hydrolysis was measured (Figure 3C). Saturating (1 SsbB/16-nt) or stoichiometric amounts of SsbB blocked ATP hydrolysis (K_{cat} of <2 min^{-1}) (Figure 3C, Table 1). In the presence of RecO, the rate of RecA-mediated ATP hydrolysis increased with increasing SsbA in the presence of constant amounts of SsbB (Figure 3C). It is likely that RecO limits the destabilization of both SsbA·ssDNA and SsbB·ssDNA complexes, and the RecO and SsbA interaction is necessary to activate RecA·ATP. After RecO and SsbA facilitate functional transitions, the levels of RecA·ATP NPF formation are comparable with those of RecA·dATP (Figure 3B and C, Table 1).

RecO and SsbA are necessary for RecA·ATP-mediated recombination

RecA from naturally competent bacteria (e.g. RecA, $RecA_{Spn}$ or $RecA_{Dra}$) cannot promote DNA strand exchange between protein-free circular ssDNA (*css*) and a homologous linear dsDNA (*lds*) in the presence of ATP (6–10,74). This inability cannot be attributed to RecA because when dATP was provided in place of ATP as a nucleotide cofactor, RecA·dATP (1 RecA/8-nt) initiated DNA recombination by pairing the 3199-bp *lds* with the homologous 3199-nt *css* substrate, leading to the formation of joint molecules (*jm*) between the free end of *lds* and *css*, followed by DNA strand exchange to generate the nicked-circular (*nc*) and the linear ssDNA (*lss*) products in about 50 to 60% of the total substrate, even in the absence of mediators (Figure 4A, lane 13 and 4B, lane 2). This finding is consistent with previously published results (9,43,44). In contrast, and as previously observed, under similar experimental conditions used for RecA·dATP, RecA·ATP rendered low levels of DNA pairing intermediates (denoted as *jm*, <10%) and only a trace of the final *nc* product (<4%) (Figure 4A, lane 2) (6,8,9,74). Similar results were observed when the incubation time (Supplementary Figure S3, lanes 2–3) or the RecA concentration (1 RecA/6- to 3-nt) was increased (8). When ssDNA was pre-incubated with SsbA or SsbB, RecA·ATP (1 RecA/8-nt) did not catalyze DNA recombination (Figure 4A, lanes 3–6). Similar results were previously observed (8,9).

RecO has two activities: to recruit RecA·ATP onto SsbA-coated ssDNA complex (Figure 3) and to mediate the an-

Figure 3. RecO facilitates RecA assembly on SsbA- or SsbB-coated ssDNA. (**A**) Circular pGEM3 Zf (+) ssDNA (10 μM in nt) was pre-incubated with SsbA (0.3 μM) or SsbA and RecO for 5 min at 37°C in buffer A containing 5 mM ATP. Then RecA (0.8 μM) was added and the ATPase activity measured for 25 min. (**B**) Circular ssDNA was pre-incubated with SsbB (0.3 μM) (or both SsbA [0.3 μM] and SsbB [0.3 μM]) and when indicated with RecO (0.1 and 0.2 μM) for 5 min at 37°C in buffer A containing 5 mM ATP or dATP. Then RecA (0.8 μM) was added and the (d)ATPase activity measured for 25 min. (**C**) The circular ssDNA was pre-incubated with a fixed amount of SsbB (0.6 μM), SsbA (0.3 μM) plus SsbB (0.3 μM) or a fixed amount of SsbB (0.3 μM) and increasing SsbA (0.03 to 0.3 μM) concentrations for 5 min at 37°C in buffer A containing 5 mM ATP. Then RecO (0.1 μM) was added to the preformed SSB·ssDNA complexes and incubated for 5 min at 37°C. Finally RecA (0.8 μM) was added and the ATPase activity measured for 25 min. All reactions were repeated three or more times with similar results.

Figure 4. SsbA and RecO contribute to RecA-promoted DNA strand exchange. (**A**) Circular pGEM3 Zf (+) ssDNA (10 μM in nt) and homologous linear dsDNA (20 μM in nt) were pre-incubated with increasing concentrations of SsbA or SsbB (0.15 and 0.3 μM) or RecO (0.2 and 0.4 μM) for 5 min at 37°C in buffer A containing 5 mM ATP or dATP. In the indicated reactions, RecO (0.2 and 0.4 μM) was added to the preformed SsbA·ssDNA or SsbB·ssDNA (SSB 0.3 μM) complexes and incubated for 5 min at 37°C. Finally, RecA (1.2 μM) was added and the reaction was incubated for 60 min at 37°C. (**B**) The circular ssDNA and homologous linear dsDNA were pre-incubated with SsbA or SsbB (0.3 μM) for 5 min at 37°C in buffer A containing 5 mM dATP, ATP or ATPγS, and RecO (0.2 μM) was added to the preformed SsbA·ssDNA or SsbB·ssDNA complexes and incubated for 10 min at 37°C. Finally, RecA (1.2 μM) was added and the reaction was incubated for 60 min at 37°C. The products of the reactions were deproteinized, separated on a 0.8% AGE with ethidium bromide and quantified as described in 'Materials and Methods' section. The positions of the bands corresponding to *css*, *cds*, *lds*, *jm, nc* and ATPγS-generated *prd* products are indicated. Symbols + and − denote the presence or absence, respectively, of the indicated protein. In A (lane 14) and in B (lane 17) nicked circular pGEM3 Zf (+) plasmid DNA was added as a mobility control (**C**). The amounts of *jm*s and products (*nc* and *prd*) are indicated and expressed as the percentage of total substrate added. The results are the average value obtained from more than three independent experiments (the results given stand within a 5% standard error).

nealing of two complementary ssDNA molecules (58). To determine whether RecO alone activates RecA·ATP to catalyze DNA recombination, the ssDNA was pre-incubated with RecO and then RecA·ATP was added. RecO did not activate RecA·ATP to catalyze DNA strand exchange (Figure 4A, lane 7). Similar results were observed when saturating RecO concentrations (1 RecO/25-nt) were added (Figure 4A, lane 8). Since RecO stimulated the ATPase activity of RecA (Figure 2) but did not facilitate RecA·ATP-mediated DNA strand exchange (Figure 4A, lanes 7 and 8), we hypothesized that RecA·ATP poorly displaces the *lss* product used by RecO to promote strand annealing, thereby reversing the recombination reaction. We reasoned that the SsbB protein would compete for the binding to the displaced ssDNA to facilitate the visualization of RecA-mediated recombination in the presence of RecO. The ssDNA was pre-incubated with SsbB and RecO prior to the

addition of RecA·ATP, and then DNA strand exchange was analysed. The presence of RecO (1 RecO/25-nt) and SsbB (1 SsbB/33-nt) did not facilitate the catalysis of DNA strand exchange by RecA·ATP (Figure 4A, lanes 11–12). The same results were observed when the reaction was incubated for a variable time (Supplementary Figure S3). It is likely, therefore, that i) the displaced strand does not reverse the recombination reaction because sequestration through SsbB does not lead to the accumulation of recombinant products; (ii) RecO cannot catalyze DNA recombination; and (iii) RecO, SsbB or both do not activate RecA·ATP to catalyze DNA recombination.

To determine whether RecO in concert with SsbA activates RecA·ATP to promote DNA strand exchange, the reactions were performed after pre-incubating the ssDNA with SsbA and RecO; and then RecA was added. In the presence of SsbA (1 SsbA/33-nt) and RecO (1 RecO/100-nt), 18 and 45% of the *lds* substrate was converted into *jm* and *nc*, respectively, through RecA activity (Figure 4A, lane 9). Similar results were observed when the incubation time was varied (Supplementary Figure S3, lanes 7–10). A higher RecO (1 RecO/50-nt) concentration further increased the amount of final recombinant product (Figure 4A, lane 10), suggesting that RecO and SsbA are necessary and sufficient to facilitate RecA·ATP-mediated recombination *in vitro*. Furthermore, SsbA fulfills an essential role during natural chromosomal transformation that cannot be compensated by SsbB in the presence of RecO. This finding is consistent with the observation that (i) SsbB is not ubiquitous in naturally transformable bacteria (reviewed in 11); (ii) the absence of SsbB marginally reduced (two- to four-fold) the transformation efficiency of competent *B. subtilis* or *S. pneumoniae* cells (11,81); (iii) SsbB creates a reservoir of ssDNA to facilitate successive recombination events (82); and (iv) RecO physically interacts with SsbA, rather than with SsbB, in solution (43,44).

SsbA and RecO facilitate three-strand exchange in the absence of ATP hydrolysis

To determine whether RecA competes with SSB proteins for DNA strand exchange in the absence of ATP hydrolysis through RecO, the three-stranded exchange reaction was performed in the presence of a non-hydrolysable ATP analog, ATPγS or AMP-PNP. As controls, the reaction was performed in the presence of dATP or ATP.

As previously documented, RecA·dATP (1 RecA/8-nt) efficiently catalyzed DNA strand exchange, even in the absence of accessory factors, but ~90% of the substrate was converted to intermediates and products in the presence of both RecO and SsbA (Figure 4B, lanes 3 and 5) (44). The presence of SsbB or both SsbB and RecO did not significantly stimulate RecA·dATP-mediated recombination. As shown in the previous figure, RecA·ATP produced low levels of DNA pairing intermediates and only a trace of the final *nc* product was observed when SsbA, SsbB, RecO or RecO and SsbB were added. SsbA and RecO were necessary for RecA·ATP-mediated DNA strand exchange (Figure 4B, lanes 7–11).

RecA·ATPγS produced low levels of DNA pairing intermediates and only a trace of the final *nc* product (<4%) was observed (Figure 4B, lane 12) (8). The addition of RecO did not contribute to RecA·ATPγS-mediated DNA strand exchange (data not shown). Similar results were observed upon the addition of SsbB, or both RecO and SsbB (Figure 4B, lanes 14–16). It is likely that the slow dissociation of RecA·ATPγS from the NPF, the interaction of RecA with SsbB, as observed *in vivo* (76), and the facilitation of RecA NPF formation on the RecO·ssDNA·SsbB complexes were not sufficient to activate RecA to catalyze DNA recombination. However, RecA·ATPγS led to the accumulation of *jm* in the presence of SsbA, and the addition of RecO further stimulated the accumulation of a novel recombination intermediate (Figure 4B, lanes 13 and 15), suggesting that in the absence of ATP hydrolysis, SsbA partially activates RecA to catalyze DNA recombination; however, full activation also requires RecO. When ATPγS was replaced with AMP–PNP as a nucleotide cofactor, RecA did not catalyze DNA recombination even in the presence of RecO and SsbA (data not shown).

In the presence of ATPγS, the mobility of the recombinant intermediate/products (*prd*), which were resistant to deproteinisation, differed from that of 'classical' *jm* intermediates, which showed characteristic diffuse mobility, and from that of *nc*, which migrates faster than *jm*s in 0.8% AGE (see Figure 4B, lanes 3 and 5 versus lanes 13 and 15). Analysis of this intermediate using restriction enzymes that cut at either end of the linear duplex DNA revealed nicked circular products and bands of more complex structure (*prd*). It is likely that re-invasion of the *lss* DNA could account for the anomalous mobility. Consistent with this hypothesis, *nc* products were accumulated when heterology at the 5′-end of the DNA end was provided (Carrasco, B. and Serrano, E., unpublished results).

AddAB did not activate RecA to catalyze DNA strand exchange

Heterodimer AddAB catalyzes the end-resection of both strands of a linear substrate, and upon cleavage at the pentameric χ site, the non-functional helicase domain of AddB protects the χ terminated 3′-end from cleavage through the AddB nuclease domain. However, degradation of the 5′-strand continues as the AddA motor unwinds the duplex DNA in a 3′→5′ direction (50,51,83–86). This mechanism produces χ terminated 3′-ssDNA ends (63,64).

The RecBCD$_{Eco}$ complex facilitates the direct loading of RecA onto naked 3′-tailed χ-terminated ssDNA ends through physical interactions between RecA and the RecB subunit during continuous unwinding (13–15,17,87). The C-terminal nuclease domain of the RecB$_{Eco}$ subunit (RecB^{nuc}), which forms stable complexes with RecA$_{Eco}$, could load a non-cognate RecA protein onto ssDNA in response to χ activation (16,17). However, whether AddAB facilitates the recruitment of RecA·ATP onto ssDNA and whether AddAB might activate RecA·ATP to catalyze DNA recombination remain unknown. To determine whether the AddAB activates RecA·ATP to promote DNA strand exchange, we analyzed a conventional substrate (3199-bp pGEM3 Zf[+] DNA) for the presence of χ sites. Unlike the octameric χ$_{Eco}$ (5′-GCTGGTGG-3′), present once every 4500-bp, the *B. subtilis* pentameric χ

(5′-AGCGG-3′) is present once every ∼350-bp (83). The pGEM3 Zf(+) dsDNA substrate contains 16 individual χ sites (seven in one orientation and nine sites in the other orientation). In the presence of AddAB, the random χ sites in the KnpI-cleaved pGEM3 Zf(+) DNA substrate were not sufficient to activate RecA·ATP to catalyze DNA recombination under the *in vitro* conditions used (data not shown). This impairment did not reflect a lack of ATP because a regeneration system was included in the recombination reaction.

The density of χ sites was artificially increased to favor AddAB pausing at the χ-terminated 3′-ssDNA end (85). An array of 10 χ sites, in the forward or reverse orientation, were integrated at the AflIII site (at position 424 of the KnpI-cleaved *lds* substrate) to produce a 3290-bp DNA substrate. AddAB-mediated resection of these substrates is expected to generate a long χ-terminated 3′-end (−strand, arbitrarily termed χ10F) at position 424 of the KnpI-cleaved dsDNA substrate (Supplementary Figure S4). Circular 3290-nt ssDNA (*css*, +strand) and *Kpn*I-linearised 3290-bp dsDNA (*lds*), containing (χ10F or χ10R) or lacking (arbitrarily termed χ0F or χ0R) an array of χ sites at position 424 (Supplementary Figure S4), were incubated with various concentrations of AddAB or $AddA^{N}addB^{N}$ (a nuclease deficient mutant that unwinds dsDNA to ssDNA (84)), in the presence or absence of SsbA. Then, subsaturating RecA was added, and RecA·ATP-mediated recombination was measured.

In the absence of RecA, limiting $AddA^{N}AddB^{N}$·ATP concentrations (1 protein complex/0.06- to 0.25-dsDNA end) facilitated the accumulation of an intermediate/product between the *css* (+strand) and the linear dsDNA. The intermediate/product was likely generated through $AddA^{N}AddB^{N}$-mediated unwinding of the KpnI *lds* substrate containing χ10F or χ10R followed by spontaneous annealing (Figure 5B and D, lanes 2–4). Similar results were observed when the dsDNA substrate lacked the array of χ sites (χ0F or χ0F) (data not shown). When wt AddAB·ATP was provided in place of $AddA^{N}AddB^{N}$·ATP, an intermediate/product with similar mobility than a *nc* product was formed, but the reaction was less efficient, perhaps reflecting the partial degradation of the substrate, followed by the accumulation of self-annealing gapped circular heteroduplex DNA accumulated (<10% of total substrate) (Figure 5A and C, lanes 2–4). However, the addition of SsbA (1 SsbA tetramer/33-nt) blocked the accumulation of this new intermediate/product (termed *prd*) (Figure 5A–D, lane 5). The *prd* product was formed with the χ10F (Figure 5A and B) or the χ10R (Figure 5C and D) substrate. Since *prd* was observed in the absence of RecA·ATP and either with AddAB or $AddA^{N}AddB^{N}$ and it was inhibited in the presence of SsbA, we assumed that AddAB·ATP-mediated *lds* + strand degradation or $AddA^{N}AddB^{N}$·ATP-mediated strand unwinding leads to the formation of an heteroduplex with the complementary *css* (+strand), generated by spontaneous strand annealing.

AddAB, upon encountering a locus containing 10 χ-sequences, briefly pauses, sequesters the χ 3′-terminated end and forms a ssDNA loop (50,51,83–86). It has been suggested that similarly to $RecBCD_{Eco}$, AddAB might load RecA onto the sDNA loop (17). To examine whether AddAB might activate RecA·ATP to catalyze DNA recombination, RecA-mediated DNA strand exchange was assayed in the presence of AddAB. In the presence of limiting AddAB concentrations, RecA·ATP rendered low levels of *jm* and the accumulation of the new recombinant intermediate/product was observed independently of the substrate used (χ10F or χ10R) (Figure 5A and C, lanes 6–8). Similar results were observed in the presence of limiting $AddA^{N}addB^{N}$ concentrations (Figure 5B and D, lanes 6–8). Consistently, the addition of SsbA (1 SsbA/33-nt) to the above reaction inhibited the accumulation of this new intermediate/product (Figure 5A to D, lanes 9–11).

RecA-mediated recombination requires RecO and SsbA, even in the presence of AddAB

Similar to $RecBCD_{Eco}$ (13,17), AddAB might recruit RecA·ATP onto χ terminated 3′-ssDNA. However, AddAB bound to the χ 3′-ssDNA might not activate RecA to catalyze DNA strand exchange. This is consistent with the observation that RecO is still required to repair H_2O_2-induced DNA DSBs in the *addAB*$^{+}$ *recJ*$^{-}$ context *in vivo* (Figure 1B and C). To examine whether RecA catalyzes DNA strand exchange in the presence of AddAB (or $AddA^{N}addB^{N}$), the SsbA and RecO mediators were also included in the recombination reaction (Figure 5A–D, lanes 12–14). In the presence of variable amounts of AddAB (or $AddA^{N}addB^{N}$) and SsbA, RecO (1 RecO/100-nt) was required to activate RecA·ATP to initiate DNA recombination through pairing the *lds,* χ10F or χ10R, with the complementary *css* (+strand) substrate, leading to the formation of *jm* intermediates and *nc* products (Figure 5A–D, lanes 12–13). At a 4:1 ratio of AddAB or $AddA^{N}addB^{N}$ complex/DNA, the accumulation of RecA-mediated recombinant products in the presence of SsbA and RecO was significantly reduced compared with the absence of AddAB or $AddA^{N}addB^{N}$ (Figure 5A–D, lane 14 versus 15), suggesting that the degradation or unwinding of the *lds* substrate decreased the accumulation of *nc* products. Alternatively, the exonuclease activity of AddAB in concert with the annealing of RecO might lead to the accumulation of gapped circular heteroduplex DNA (reviewed in 88). This possibility was considered unlikely because the digestion of dsDNA ends or DNA unwinding through increasing AddAB or $AddA^{N}addB^{N}$ concentrations, respectively, inhibited the accumulation of recombinant products irrespective of the presence or absence of an active χ site. Moreover, in the presence or absence of AddAB (or $AddA^{N}addB^{N}$), SsbA and RecO activate RecA to catalyze DNA strand exchange. Furthermore, when SsbA was omitted, only spontaneous annealing to the *prd* intermediate/product (<10% of total substrate) was observed in the presence of AddAB, RecO and RecA (data not shown).

Taken together, these results suggest that (i) AddAB cannot activate RecA·ATP to mediate DNA strand exchange between the *lds* (−strand) and the *css* (+strand) under the experimental conditions used; (ii) AddAB bound to the χ-terminated end cannot block RecA·ATP-mediated DNA recombination in the presence of RecO and SsbA; and (iii)

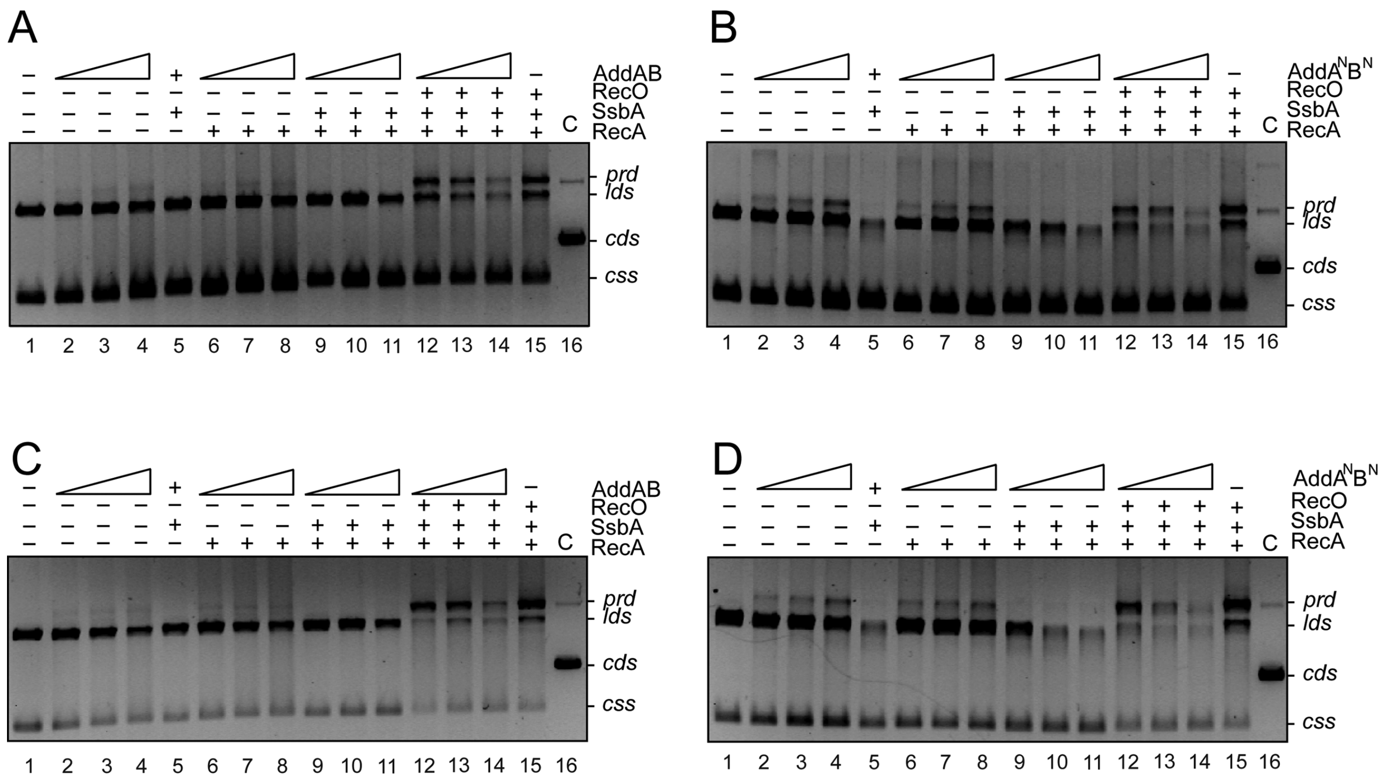

Figure 5. AddAB does not contribute to RecA·ATP activation to catalyze DNA recombination. Circular pGEMχ10F or pGEMχ10R ssDNA (10 μM in nt) and linear pGEMχ10F (χ10F, **A** and **B**) or pGEMχ10R (χ10R, **C** and **D**) dsDNA (20 μM in nt) were incubated with increasing AddAB or $AddA^N AddB^N$ (0.35, 0.75 and 1.5 nM) concentrations for 60 min at 37°C in buffer A containing 5 mM ATP. The circular ssDNA and linear dsDNA substrate were pre-incubated with increasing AddAB or $AddA^N AddB^N$ in the presence or absence of SsbA (0.3 μM) for 5 min at 37°C in buffer A containing 5 mM ATP. Then RecA (1.2 μM) was added and the reactions were incubated for 60 min at 37°C. In reactions where RecO was also added, the circular ssDNA and linear dsDNA substrate were pre-incubated with SsbA (0.3 μM), RecO (0.2 μM) and increasing AddAB or $AddA^N AddB^N$ for 5 min at 37°C in buffer A containing 5 mM ATP. Then RecA (1.2 μM) was added and the reactions were incubated for 60 min at 37°C. The separation of the products and the symbols are the same as those described in Figure 4. The results are representative of more than three independent experiments.

addition of AddAB cannot overcome the need of RecO and SsbA for activation of RecA·ATP to mediate DNA strand.

CONCLUSIONS

Bacilli (e.g. *Bacillus, Streptococcus*) are homogeneous and evolutionarily separated by more than 1500 million years from the γ-Proteobacteria Class (e.g. *E. coli*). This divergence, which is larger than that between humans and plants, is also encompasses differences in DNA repair and genetic recombination (natural chromosomal transformation) at the genetic, biochemical and biophysical level.

We showed here that in the absence of one of the two end-resection pathways (defined by the *recJ*$^-$ *addAB*$^+$ or *addAB*$^-$ *recJ*$^+$ strain), RecO is required for efficient SSG and DSB repair (Figure 1). Indeed, in *B. subtilis, N. gonorrhoeae*, *D. radiodurans* and *M. smegmatis* ∼99% of the recombination events occurring at SSGs or DSBs require RecO (Figure 1B and C and Supplementary Figure S1A and S1B) (67–69). In *M. smegmatis recO adnAB* or *recO adnAB recBCD* mutant strains showed decreased resistance to UV, ionizing radiation or MMS at levels comparable to the *recO* or *recA* mutant strain (69). Although *Mycobacterium* differs from *Bacillus*, bacteria of the former genus encode for two (RecBCD and AdnAB) rather than one exonuclease–helicase complex (AddAB) and do not encode RecJ (69,89). However, it will be of significant interest to determine whether *B. subtilis* RecO also contributes to error-prone single-strand annealing during two-ended DSB repair, as described for *M. smegmatis* RecO (69). Conversely, in *E. coli* cells, ∼99% of the recombination events occurring at DSBs require RecBCD and RecA, and the events occurring at SSGs require RecJ, RecQ, SSB, RecO, RecR and RecA (5,12,13).

Similar to $RecA_{Spn}$·ATP and $RecA_{Dra}$·ATP (7,10), RecA·ATP cannot catalyze DNA strand exchange between circular ssDNA and its homologous linear dsDNA (Figure 4) (6,8), suggesting that RecA·ATP from naturally competent bacteria requires activation to catalyze DNA recombination. We proposed that (i) RecA from natural competent bacteria has evolved to accommodate new activities missing for example in $RecA_{Eco}$; (ii) SsbA or SsbB blocks RecA·ATP nucleation and/or polymerization onto ssDNA to a similar extent and also blocks RecA-mediated recombination (8,9); (iii) the addition of sub- or stoichiometric amounts of RecO cannot activate RecA·ATP to catalyze DNA strand exchange between protein-free ssDNA and its homologous linear duplexes; (iv) RecO and SsbB cannot activate RecA to catalyze DNA strand exchange in the presence of ATP; (v) RecO and SsbA, which might function as a two-component mediator, are necessary and suf-

ficient to activate RecA·ATP to catalyze DNA strand exchange *in vitro*; (vi) RecO *in vitro* (or RecOR *in vivo*) recruit(s) and activate(s) RecA·ATP to nucleate and polymerize onto SsbA-coated ssDNA; (vii) RecA·ATPγS catalyzes DNA strand exchange in the presence of SsbA, although the addition of RecO is required to fully activate RecA to catalyze strand exchange; and (viii) the addition of limiting to sub-stoichiometric amounts of AddAB cannot activate RecA·ATP to catalyze three-strand DNA exchange. This result is consistent with the observation that RecO, RecR and to a lesser extent RecF, are necessary for DNA damaged-induced RecA:GFP foci at stalled or collapsed forks (34,39,41,42).

Herein, we presented evidence that under these experimental conditions, AddAB might not 'activate' RecA·ATP to promote DNA strand exchange in the presence or absence of SsbA. In the presence of AddAB, the addition of SsbA and RecO facilitated the activation of RecA·ATP to catalyze DNA strand exchange. It will be of significant interest to determine whether AddAB recruits RecA·ATP onto χ-terminated 3′-tailed duplex DNA to catalyze four-strand DNA exchange and identify the other factor(s) are required to activate RecA·ATP to promote DNA recombination.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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