The RecD2 helicase balances RecA activities

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ABSTRACT

DNA helicases of the RecD2 family are ubiquitous. *Bacillus subtilis* RecD2 in association with the single-stranded binding protein SsbA may contribute to replication fork progression, but its detailed action remains unknown. In this work, we explore the role of RecD2 during DNA replication and its interaction with the RecA recombinase. RecD2 inhibits replication restart, but this effect is not observed in the absence of SsbA. RecD2 slightly affects replication elongation. RecA inhibits leading and lagging strand synthesis, and RecD2, which physically interacts with RecA, counteracts this negative effect. In vivo results show that recD2 inactivation promotes RecA–ssDNA accumulation at low mitomycin C levels, and that RecA threads persist for a longer time after induction of DNA damage. In vitro, RecD2 modulates RecA-mediated DNA strand-exchange and catalyzes branch migration. These findings contribute to our understanding of how RecD2 may contribute to overcome a replicative stress, removing RecA from the ssDNA and, thus, it may act as a negative modulator of RecA filament growth.

INTRODUCTION

Accurate genome duplication is essential for life, and all organisms have multiple mechanisms to protect, repair and reactivate DNA replication forks. In addition to proteins directly involved in DNA replication, like the replicative helicase, the polymerase, primase, etc., active replication forks are pre-equipped with several accessory proteins by their interaction with replisome proteins (1–3). Our current knowledge shows that these accessory effectors may contribute to the progression of replication, and they include repair proteins, error prone polymerases and homologous recombination proteins.

The intimate links between homologous recombination and DNA replication were early noted in some lytic (e.g. *Escherichia coli* T4 and *Bacillus subtilis* SPP1) as well as temperate (*Staphylococcus aureus* 80α and φ11) dsDNA bacteriophages. These phages strictly require homologous recombination proteins for their replication (4–6). Growing evidences show that these two DNA transactions are tightly linked in all organisms (7,8). In bacteria, several recombination proteins are associated with active replication forks by its interaction with the single-stranded DNA binding protein (SSB in *E. coli* and SsbA in *B. subtilis*) (2,3). In both cases, the number of DNA helicases targeted to active replication forks in the absence of DNA damage is remarkably high, and in the case of *B. subtilis* these helicases are: PriA, RecG, the RecQ-like enzymes (RecQ and RecS), and RecD2 (2).

RecD enzymes, which belong to superfamily 1B (SF1B) of DNA helicases (9), are divided into two sub-groups: RecD1 (known as RecD) and RecD2 (10,11). RecD2 helicases have a longer N-terminal region prior to the first helicase motif (Supplementary Figure S1). The role of RecD2 in the cell is poorly known, whereas that of RecD is known. *E. coli* RecD (RecD*Eco*) forms a part of the well-studied RecBCD complex, crucial for double-strand break repair. Bacteria that have RecD2 lack the RecBCD complex (10–12) The best biochemically characterized member of this group is *Deinococcus radiodurans* RecD2 (RecD2*DrDu*). RecD2*DrDu* is a monomeric DNA helicase with 5′→3′ polarity (13–16). RecD2 helicases also show homology with human DNA Helicase B (named HDHB, or HELB, Supplementary Figure S1), a conserved helicase present in higher eukaryotes with a poorly understood role in initiation of DNA replication and genome maintenance (17–19). Amino acid sequence identity and similarity between mouse HELB and *B. subtilis* RecD2 are 16.5 and 26%, respectively (20). Both proteins interact with their respective SSB (2,19,21,22).

The *in vivo* role of *B. subtilis* RecD2 is poorly understood (unless stated otherwise, indicated genes and products are of *B. subtilis* origin). Inactivation of *recD2* confers a modest increase in the spontaneous mutation rate (21,23). A role of RecD2 in homologous recombination is inferred from its contribution to acquire DNA from related species during natural chromosomal transformation (23). *recD2* mutants

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are sensitive to DNA damaging agents that stall or collapse replication forks such as the alkylating agent methyl methane sulfonate (MMS) and the intra- and inter-strand crosslinking agent mitomycin C (MMC) (21,24,25). RecD2 is important for replication fork progression (21). However, different results were obtained when a recD2 deletion was analyzed in *D. radiodurans*. The *recD2*Δ strain mutants were not sensitive to MMS and MMC, and showed greater efficiency in chromosomal transformation (26), obscuring the role of this protein in the cell.

In this work, using a combination of genetic, cytological, and biochemical techniques, we show that RecD2 is a modulator of replication restart and of the RecA recombinase. RecD2 interacts with RecA and inhibits the initiation of strand-exchange, because it inhibits the formation of the presynaptic filament. Consistent with this, RecA threads, which are formed after MMC treatment, persist for a longer period in the *recD2*Δ mutants. In the replisome context, RecD2 may inhibit unwanted recombination when replication forks stall, displacing RecA and allowing the advance of the replication fork.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

*B. subtilis* BG214 is the wild type (*wt*) strain. The Δ*recX* and Δ*recO* strains were previously constructed (27). A Δ*recD2* deletion mutant was constructed replacing by homologous recombination 2193-bp of the BG214 chromosome by a *six-cm-six* cassette (28) and chloramphenicol selection (Cm, 5 μg/ml). Briefly, the region of the *recD2* gene (781 amino acids long) has two BseRI restriction sites, one located 10-bp before the start codon and the other one overlapping codon 721. These restriction sites were used to replace this region by the *six-cm-six* cassette. The resulting Δ*recD2* deletion strain lacks from codon 1 to 721, so that almost the full gene is deleted. In a second step, the antibiotic resistance gene was deleted by the β site-specific recombinase, which deletes the region between the two *six* sites (29). The final strain (ΔΔ*recD2*) was sequenced to confirm accuracy. The construction of his-tagged *recD2* on the *B. subtilis* chromosome was as follows. The C-ter of *recD2* gene (500 bp) without the stop codon was cloned in the integration vector pSG1164-His. The resulting plasmid, pSG1164-*C-ter-recD2*-His, was integrated by homologous recombination into the *B. subtilis* genome at the *recD2* locus by Cm selection, and the accuracy of the recombination reaction was confirmed by PCR and DNA sequencing. This allows the replacement of RecD2 by RecD2-His, a fusion protein with a linker (GPGLSG) and 6xHis, that is expressed from its natural promoter. The resulting strain was named recD2Δ6His.

The pET-*recD2*-His expression vector was constructed in two steps. First, the *recD2* gene with its stop codon (2397-bp) was cloned into the pET-3b vector. PCR primers to amplify the recD2 gene with flanking NdeI and BamHI restriction sites were designed. The accuracy of the resultant plasmid (pET-3b-*recD2*) was confirmed by sequencing. In a second step, the region in pET-3b-*recD2* corresponding to the C-ter of the *recD2* gene was replaced by the one of the pSG1164-*C-ter-recD2*-His plasmid by BseRI and BamHI digestion. This resulted in the pET-*recD2*-His expression vector, that allows the expression in *E. coli* of RecD2-His, a fusion protein with linker (GPGLSG) and 6xHis. The pET-*recD2*-K373A-His expression vector was constructed by site-directed mutagenesis using the Quickchange kit (Stratagene) with corresponding primers and pET-recD2-His as template. Plasmids to overproduce SsbA and RecO, respectively, have been described previously (30). *Escherichia coli* XL1 Blue was used for plasmid construction, and *E. coli* BL21 (DE3) pLysS was used for protein expression. *B. subtilis* BG214 cells bearing pBT61-*recA* were used to overexpress RecA as described (31). Note that the nomenclature used to denote the origin of proteins from bacteria other than *B. subtilis* is based on the bacterial genus and species (e.g., *D. radiodurans* RecD2 is referred as RecD2Δ6His).

**In vivo assays**

Viability tests in the presence of MMS were performed to analyze the functionality of the RecD2-His protein. *B. subtilis* *wt*, Δ*recD2* and Δ*recD2*Δ6His cells were grown to an OD$_{560}$ = 0.5 and appropriate dilutions were spotted on LB plates containing 1.5 mM of MMS. Plates were incubated O/N at 37°C and photographed.

To measure RecD2 levels in vivo, a 250 ml culture of recD2Δ6His cells was grown to OD$_{560}$ = 0.7 at 37°C in LB. Then, cells (0.2 g wet weight) were collected and resuspended in 1 ml of buffer A (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 1 mM DTT, 10% v/v glycerol) containing lysozyme 0.2 mg/ml. After 60 min at 4°C, cells were sonicated and cell debris were separated by centrifugation (30 min, 12,000 rpm, 4°C). RecD2-His that was present in the soluble fraction was concentrated by binding to Ni-Sepharose (20 μl). RecD2 was eluted from the column with twice 100 μl of buffer A containing 200 mM imidazole. 30 μl of each fraction were loaded on a 12% SDS-polyacrylamide gel, and the amount of RecD2 was deduced from a standard curve obtained loading different amounts of purified RecD2. The number of RecD2 molecules per colony forming units (CFUs) was calculated considering that for an OD$_{560}$ = 0.7 there are ~1.4 × 10$^8$ CFUs/ml.

RecA protein accumulation upon SOS induction was analyzed in *wt* and ΔΔ*recD2* cells as previously described (27). Briefly, cells were grown to OD$_{560}$ = 0.4 in LB, and exposed to increasing concentrations of MMC for 30 min. Then, cells were lysed, and proteins separated by 10% SDS-PAGE, followed by immunoblot transfer. Blots were developed with rabbit polyclonal anti-RecA antibodies and quantified with the ImageJ software. The amount of RecA protein in each sample was interpolated from the standard curve obtained loading different amounts of purified protein in the same gel.

**Fluorescence microscopy**

A functional RecA-mVenus fusion for the visualization of fluorescent RecA in live cells was used. This C-terminal fusion is integrated at the original gene locus, so that the fusion is the sole source of RecA expressed in cells, and under the control of the original promoter (32). The fusion
RecD2-His (89 kDa, hereafter named RecD2), was overexpressed and purified as follows: E. coli BL21(DE3) pLysS cells carrying pET3b-RecD2-His plasmid were grown in LB medium supplemented with 100 μg/ml ampicillin and 15 μg/ml chloramphenicol at 30°C to OD_{600} = 0.2, and then at 18°C to OD_{600} = 0.4. Overexpression of the protein was induced by 0.25 mM IPTG addition. After overnight growth, the cell mass was recovered by centrifugation at 9,000 rpm for 10 min at 4°C. Pellets were resuspended in buffer B (50 mM Tris–HCl pH 7.5, 1 M NaCl, 1 mM DTT and 10% v/v glycerol) supplemented with 5 mM imidazole and sonicated. After centrifugation at 18,000 rpm for 45 min, the pellet was resuspended in buffer A with 5 mM imidazole and centrifugated at the same conditions. The resultant supernatant of both centrifugations was loaded onto a Ni-Sepharose column equilibrated in the same buffer. Proteins were eluted using a gradient of increasing concentrations of imidazole (5–100 mM), and analyzed by 12.5% SDS-PAGE. Fractions containing RecD2 were precipitated by the addition of 70% ammonium sulfate for 30 min. The protein pellet was resuspended in buffer B with 1 mM EDTA and 10 mM sodium phosphate pH 7.5 and then loaded onto a hydroxyapatite column. Protein elution was performed with a gradient of sodium phosphate (10–100 mM) in buffer B. Purified RecD2 was aliquoted and stored at -80°C with 5% (v/v) skim milk powder and 0.1% (v/v) Triton X-100. The membrane was then incubated overnight at 4°C with 6× His monoclonal antibody (Clontech Ref: 631212, dilution 1:6000) and subsequently with the secondary antibody anti-mouse IgG conjugated with peroxidase (Cytiva, Ref: NXA931 dilution 1:5000) for 1 h at room temperature. The interactions were visualized by staining the membrane with the Clarity™ Western ECL Substrate kit (Bio-Rad). The images were obtained and processed with the ChemiDoc Imaging System and the Image Lab software (Bio-Rad).

**Purification of B. subtilis RecD2**

RecD2-His (89 kDa, hereafter named RecD2), was overexpressed and purified as follows: E. coli BL21(DE3) pLysS cells carrying pET3b-RecD2-His plasmid were grown in LB medium supplemented with 100 μg/ml ampicillin and 15 μg/ml chloramphenicol at 30°C to OD_{600} = 0.2, and then at 18°C to OD_{600} = 0.4. Overexpression of the protein was induced by 0.25 mM IPTG addition. After overnight growth, the cell mass was recovered by centrifugation at 9,000 rpm for 10 min at 4°C. Pellets were resuspended in buffer B (50 mM Tris–HCl pH 7.5, 1 M NaCl, 1 mM DTT and 10% v/v glycerol) supplemented with 5 mM imidazole and sonicated. After centrifugation at 18,000 rpm for 45 min, the pellet was resuspended in buffer A with 5 mM imidazole and centrifugated at the same conditions. The resultant supernatant of both centrifugations was loaded onto a Ni-Sepharose column equilibrated in the same buffer. Proteins were eluted using a gradient of increasing concentrations of imidazole (5–100 mM), and analyzed by 12.5% SDS-PAGE. Fractions containing RecD2 were precipitated by the addition of 70% ammonium sulfate for 30 min. The protein pellet was resuspended in buffer B with 1 mM EDTA and 10 mM sodium phosphate pH 7.5 and then loaded onto a hydroxyapatite column. Protein elution was performed with a gradient of sodium phosphate (10–100 mM) in buffer B. Purified RecD2 was aliquoted and stored at -80°C with 5% (v/v) skim milk powder and 0.1% (v/v) Triton X-100. The membrane was then incubated overnight at 4°C with 6× His monoclonal antibody (Clontech Ref: 631212, dilution 1:6000) and subsequently with the secondary antibody anti-mouse IgG conjugated with peroxidase (Cytiva, Ref: NXA931 dilution 1:5000) for 1 h at room temperature. The interactions were visualized by staining the membrane with the Clarity™ Western ECL Substrate kit (Bio-Rad). The images were obtained and processed with the ChemiDoc Imaging System and the Image Lab software (Bio-Rad).

**DNA strand exchange**

 Strand exchange reactions were performed as described (37) using KpnI-linearised pGEM-3Zf (+) dsDNA (3197-bp, 20 μM in nt, 3 nM in molecules) and its homologous circular ssDNA (10 μM in nt, 3 nM in molecules) in a buffer C (50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT, 0.05 mg/ml BSA, 5% glycerol) containing 2 mM ATP, 8 U/ml creatine phosphokinase, 8 mM phosphocreatine, RecA (1.5 μM), RecO₂ (200 nM), SsbA (300 nM) and variable concentrations of RecD2 (1.5–50 nM) were added as indicated. Samples were deproteinized after 20 or 40 min incubation at 37°C by the addition of stop buffer to a final concentration of 25 mM EDTA, 0.5% v/v SDS, and 0.5 mg/ml proteinase K. DNA species were fractionated by 0.8% w/v agarose gel electrophoresis run in 1× TBE. Gels were stained with ethidium bromide and analyzed using a GelDoc system (Bio-Rad) and densitometric analysis was performed with the Image Lab software (Bio-Rad). The percentage of the three-stranded recombination intermediates with various lengths of heteroduplex DNA (joint molecules, jm) and full exchanged products (nicked circular dsDNA, nc) was calculated dividing each signal by the sum of the signals of the three bands (dsDNA substrate, nc and jm).

**Branch migration of recombination intermediates**

A large-scale standard strand exchange reaction was set up at 37°C and stopped after 10 min by addition of EDTA to 45 mM, and SDS to 0.5%. Then, the mixture was loaded into a 1 ml Sephadex-G50 spin column to remove all components except proteins and DNA, that were recovered in the Flow through fraction. DNA was further purified by ethanol precipitation. The pellet containing remaining DNA substrates and recombination intermediates was resuspended in water and incubated with increasing concentrations of RecD2 (3–24 nM) during 30 min at 37°C in buffer C containing 2 mM ATP, 8 U/ml creatine phosphokinase and 8 mM phosphocreatine, 5% glycerol. Reactions were stopped and analyzed as stated above.

**ATPase assays**

ATPase activity of RecD2 or RecD2 K373A was analyzed using an ATP/NADH-coupled spectrophotometric assay. PolydT₈₀ (3 μM in nt) was preincubated 5 min at 37°C in buffer C containing 0.5 mM phosphoenolpyruvate, 0.3 mM NADH, 10 U/ml pyruvate kinase and 10 U/ml lactate dehydrogenase) with 5 nM RecD2 or RecD2 K373A. Then,
DNA replication assays

Reactions were carried out using a reconstituted PriA-dependent rolling circle DNA replication system as described (34,38). Protein concentrations used were: 20 nM PriA, 20 nM PolC, 25 nM HolA (δ), 25 nM HolB (δ′), 25 nM DnaX12(τ), 25 nM DnaN2 (β), 25 nM DnaI6, 30 nM DnaC6, 30 nM DnaG, 15 nM DnaE, 50 nM DnaB4, 50 nM DnaD4, 60 nM SsbA4, RecD2 (5-40 nM), RecO2 (50 nM) and RecA (500 nM). The minicircle DNA template used allows quantification of leading and lagging strands synthesis, because the circular leading strand template is deficient in dCMP residues (G:C ratio 50:1), and the flapped lagging strand template is deficient in dGMP residues. This permits quantification of leading and lagging strand synthesis by measuring radioactive dCMP and dGMP incorporation. The B. subtilis replisome was first preassembled incubating proteins with the DNA substrate (5 nM) in buffer BsRec (40 mM Tris-acetate pH 7.8, 12 mM MgOAc, 200 mM potassium glutamate, 3 μM ZnSO4, 1% w/v polyethylene glycol MW ~8000, 0.02% Pluronic F68 and 1 mM DTT) for 5 min at 37°C in the presence of 5 μM ATP. The reactions were initiated by the addition of 1 mM ATP and 24 μM dNTPs with radiolabelled dCTP or dGTP, and aliquots were stopped at certain times. Unbound nucleotides were removed by gel filtration through Sephadex G-25 mini-columns and quantification of DNA synthesis was performed by scintillation counting. For visualization of replication products, aliquots were mixed with loading dye buffer (30 mM NaOH, 2 mM EDTA, 5% v/v glycerol and 0.1% v/v bromophenol blue) and fractionated by alkaline 0.8% (w/v) agarose gel electrophoresis.

Protein-displacement assays

Streptavidin displacement assays were done according to (39). 0.5 nM of biotinylated oligo bioT-45 (5'-GTACGTATTCGATCGCTATGATCTGTA-3') was incubated with streptavidin (2.5 nM) for 5 min at room temperature in buffer D (50 mM Tris-HCl pH 7.5, 2 mM MgCl2, 2 mM DTT, 50 mM NaCl, 0.05 mg/ml BSA and 2 mM ATP). An equal volume of a solution containing increasing concentrations of RecD2 and 50 nM biotin as a trap for the displaced streptavidin was added. The reactions were incubated for 20 min at 37°C and stopped with stop buffer (25 mM EDTA and 0.5% w/v SDS) for 10 min at 37°C. The samples were separated on 10% PAGE in 1 x TBE. Gels were dried and analyzed with the Personal Molecular Imager and the Image Lab software (Bio-Rad).

Statistical methods

Graphs were performed with the Excel and Prism v6.0 (GraphPad) software, and data are represented as mean ± SD (standard deviation). Statistical differences between samples were assessed with the non-parametric Mann-Whitney test.

RESULTS

Purification of RecD2 and protein levels in B. subtilis

So far, all RecD2 proteins have been purified as a His-tagged protein, with the tag fused at the C-terminus (13,14,21), but if the tag affects protein activity was not analyzed. We attempted to purify a RecD2 non-tagged protein from E. coli pET-3b-recD2 cells, but the protein was poorly soluble, whereas RecD2-His was soluble. Then, we analyzed in vivo the functionality of the tagged protein. A B. subtilis strain expressing RecD2-His from its natural promoter, and as sole source of the protein in the cell was constructed. The recD2Δhis cells were grown until mid-exponential phase and then dilutions were spotted on plates having 1.5 mM MMS. RecD2-His expressing cells were as resistant as wt cells to this drug (Supplementary Figure S2A) while the recD2 deletion mutant (∆recD2) was sensitive to this DNA damaging agent, as previously observed (25). We concluded that the C-terminal His-tag does not affect protein activity and used the purified protein without tag removal. The His-tagged protein is thereafter named RecD2.

The expression of the recD2 gene has been analyzed in global transcriptome analysis showing that gene expression is low and comparable to the level of expression of the low abundant essential replication proteins, as polC, dnaX or priA (40). RecD2 protein levels have not been determined so far. We used the recD2Δhis strain that expresses RecD2 from its own promoter to quantify levels of the protein in the cell during exponential growth in LB. RecD2 was not detected when a protein extract from 1 ml of an exponential growing culture (i.e. ~1.4 x 10⁸ cells) was analyzed by Western blot, suggesting that RecD2 levels are low. Thus, recD2Δhis cells from a 250 ml culture grown to exponential phase (~3.5 x 10¹⁰ cells) were lysed, and the RecD2 protein, which was present in the soluble fraction, was concentrated by binding to a Ni-sepharose column. The amount of RecD2 eluted from the Ni-column was interpolated from the standard curve obtained with increasing amounts of purified RecD2 protein. These assays showed that RecD2 is low abundant in the cell, with approximate values of ~25 RecD2 monomers/CFUs (Supplementary Figure S2B).

RecD2 may regulate replication restart

Previously it has been shown that: (i) inactivation of recD2 renders cells sensitive to DNA-damaging agents that block or impair replication fork movement, as MMS or the UV mimetic 4-nitroquinoline-1-oxide (21,25) and (ii) RecD2 is not required to copy or impair replication fork movement, as MMS or the UV mimetic 4-nitroquinoline-1-oxide (21,25). It has been shown that RecD2 affects replication restart or fork progression when performed in vitro DNA replication assays with purified B. subtilis proteins. First, we have confirmed that RecD2 physically interacts with SsbA (Figure 1A). For replication of a DNA substrate that mimics a blocked or stalled fork with a gap in the lagging strand (Figure 1B), the essential pre-primosomal Pri-A-DnaD-DnaB interactions were restored.
Figure 1. RecD2 action on replication restart. (A) RecD2 interaction with SsbA and RecA is detected by immuno-dot blot. Increasing concentrations of RecA, SsbA and BSA (from 100–400 ng) were bound to a nitrocellulose membrane, which was subsequently incubated with a buffer containing 200 ng/ml of purified RecD2. After washing, the membrane was incubated with a mouse monoclonal anti-His antibody. (B) The mini-circular DNA template mimics a blocked replication fork with a 396-nt gap in the lagging strand. Diagram of the DNA template and *B. subtilis* replisome assembly scheme. Replisomes were assembled in the presence of RecD2 helicase incubating the minicircle DNA replication template in the presence of 5 μM ATP γS. Reactions were started by the addition of dNTPs and ATP, and stopped after the indicated times. (C) Leading strand synthesis in the presence of increasing amounts of RecD2. (D) Lagging strand synthesis in the presence of increasing amounts of RecD2. Replication products were analyzed by alkaline gel electrophoresis and autoradiography. Ld, leading; Lg, lagging; M, molecular weight marker (radiolabeled λ-HindIII DNA). (E) Quantification of leading strand synthesis obtained after 2 min with increasing amounts of RecD2 (0–20 nM). (F) Quantification of lagging strand synthesis obtained after 2 min with increasing amounts of RecD2 (0–20 nM). Results are plotted as the mean ± SD of five independent experiments.

components, DnaI loader, DnaC helicase, PolC and DnaE polymerases, the clamp loader complex (τ δε'), the β-sliding clamp (DnaN), and DnaG primase are required (35). SsbA, as *E. coli* SSB, is not essential, but strongly stimulates *in vitro* DNA synthesis (38,41).

When the replisome was assembled in the presence of RecD2, and then dNTPs and ATP were added to initiate DNA synthesis, we found that a concentration of one RecD2/DNA molecule (5 nM RecD2), barely affected synthesis, but in the presence of two RecD2/DNA molecules or more, both leading and lagging strand synthesis were significantly inhibited (*P*-values < 0.01; Figure 1 and Supplementary Figure S4A), suggesting that the inhibitory effect was dependent on protein concentrations.

Having observed an interaction of RecD2 with SsbA (Figure 1A), we checked if the inhibitory effect of RecD2 is due to some action on SsbA. When DNA replication was performed with all replisome components except SsbA, DNA replication was less efficient (Supplementary Figure S3), as earlier described (35,38). In the absence of SsbA, we did not observe the inhibitory effect conferred by RecD2 on leading strand synthesis (Supplementary Figures S3 and S4B). Lagging strand synthesis was inhibited at 40 nM RecD2 (Supplementary Figure S4B). It is likely that the
inhibitory effect of RecD2 on DNA replication restart is conferred over SsbA, either promoting SsbA disassembly, or preventing it to properly interact with other binding partners (2,42). To test the first hypothesis, we purified the RecD2-K373A variant (21) that has no ATPase activity (Supplementary Figure S5A). No inhibition of DNA replication was observed at 40 nM RecD2-K373A, whereas at this protein concentration the wt protein strongly inhibited the reaction (Supplementary Figure S5B). This result suggests that RecD2 uses the energy from ATP hydrolysis to promote SsbA disassembly from the ssDNA.

To analyze if the effect of RecD2 occurs only at the initiation step of DNA replication, RecD2 was added during ongoing DNA replication. The complete replisome was first assembled on this DNA substrate and replication was allowed to start for 1 min, then increasing concentrations of RecD2 were added and DNA replication continued for 5 min. No inhibitory effect was observed, even having an excess of RecD2 over the DNA template (Figure 2). However, we noticed that increased RecD2 concentrations led to an increase in the length of the Okazaki fragments, an effect that has been observed before in reconstituted systems lowering the concentration of DnaG primase (35,38). These results suggest that RecD2 could affect the elongation step during unperturbed replication, as well as modulate replication restart.

**RecD2 interacts with RecA and reverses RecA inhibition of replication restart**

Previously it has been shown that: (i) bacterial RecD2 and human HELB interact with their cognate SSB protein (2,21,22) and (ii) GFP-tagged HELB colocalizes with RPA and also with the Rad51 recombinase (43). Since RecD2 shows homology with HELB, we analyzed whether RecD2 interacts with RecA by immuno-dot blot assays. As revealed in (Figure 1A), RecD2 physically interacted with RecA.

Measurement of replication fork progression in vivo showed that forks arrest more frequently in ΔrecD2 cells (21). We reasoned that a role for RecD2 could be to remove protein obstacles on the DNA, and thereby facilitate replisome loading or replication fork progression. In the presence of SsbA, RecA slightly affects DNA replication restart, and addition of the RecO mediator enhances RecA inhibition of DNA synthesis, facilitating the binding of RecA to the ssDNA regions, and preventing replication restart thereby (44). We tested if RecD2 could counteract RecA negative action on replication restart. The DNA template was mixed with SsbA, RecA, RecD2, RecO and RecA in buffer BsRC to allow protein assembly. Then, the replisome proteins were added, and reactions were initiated by the addition of dNTPs and ATP. We confirmed that RecO and RecA inhibit leading and lagging strand synthesis (Figure 3A, B and Supplementary Figure S6A, B) as earlier reported (44). Remarkably, a catalytic amount of RecD2 was sufficient to bypass the inhibitory effect exerted by RecO and RecA on DNA synthesis (Figure 3A, B and Supplementary Figure S6A, B). For the removal of RecA from the stalled fork substrate ATP hydrolysis was needed, because the RecD2 K373A variant could not bypass RecA inhibitory action on replication restart (Supplementary Figure S5C).

We then tested if the interaction of RecD2 with SsbA is necessary to reverse the negative effect that RecA exerts on the resumption of DNA replication. In the absence of SsbA, RecA does not need its mediator RecO, and efficiently loads on the ssDNA (37). A RecA nucleoprotein filament assembled on the DNA template blocked both, leading and lagging strand synthesis (Figure 3C, D) as previously observed (44). RecD2 was still able to counteract the inhibitory effect exerted by RecA on DNA synthesis, but to a lesser extent (P-values < 0.06, Figure 3C, D and Supplementary Figure S5C, D). Then we tested if RecD2 can displace any other proteins from the ssDNA by its translocase activity. We detected some displacement of streptavidin from a biotinylated oligonucleotide only at high RecD2 concentrations (Supplementary Figure S7).

**RecD2 modulates DNA strand exchange**

Since RecD2 seemed to displace RecA from the blocked replication fork to allow restart, we analyzed whether RecD2 also inhibits the strand exchange reaction catalyzed by RecA. We examined RecA-mediated three-strand-exchange reactions with circular ssDNA (css) and its homologous linear duplex DNA (lds) in the presence of ATP and an ATP-regeneration system. In the presence of ATP, *B. subtilis* RecA requires the two-component RecO-SsbA mediator for efficient loading and filamentation onto the ssDNA (37). Reactions were performed in two different conditions: adding RecD2 at the beginning of the reaction, or after DNA strand-exchange has been initiated, and the outcome was monitored by agarose gel electrophoresis.

The lds and css DNA substrates were incubated with RecA at 37°C. In a 20 min reaction, RecA catalyzed mainly the formation of joint molecule (jm) intermediates and some nicked circular (nc) products, and these were the major species after 40 min (Figure 4A, lanes 3 and 4). When RecD2 was added at the same time as RecA, the effect observed was concentration and time dependent. After 20 min of incubation, at low RecD2 concentrations (3 and 6 nM, 1–2 RecD2/ssDNA molecule), the percentage of jm decreased and we observed an increase in nc formation (3 nM RecD2, P-value = 0.023; 6 nM, P-value = 0.026) (Figures 4A, B). However, no significant difference in the amount of nc products was detectable at these protein concentrations in a 40 min reaction (Figure 4A, lanes 10 and 12, and 4C). The presence of 25 nM RecD2, which corresponds to ~8 RecD2/ssDNA molecule and a RecA:RecD2 molar ratio of 60:1, was sufficient to strongly inhibit the accumulation of jm intermediates and nc products at the two times of incubation tested (jm, P-value = 0.024; nc, P-value = 0.063). When RecD2 concentrations further increased (~16 RecD2 monomers/ssDNA molecule, RecA:RecD2 30:1), the accumulation of both intermediates (jm) and products (nc) was completely blocked (Figure 4A–C). A similar result was obtained when first RecA and RecD2 were initially incubated with the ssDNA substrate and then the homologous linear dsDNA was added (Supplementary Figure S8).

We then analyzed if this inhibition is also observed when strand exchange has initiated. RecA was pre-incubated with css and lds DNA in the presence of the two-component mediator RecO-SsbA, and strand exchange started by the
addition of ATP. After 20 min, increasing concentrations of RecD2 were added and the reaction continued for another 20 min (total incubation time 40 min, Figure 4D, E). An aliquot was taken at the time of RecD2 addition to see the amount of strand exchange intermediates and products formed at this time point (Figure 4D, lane 3). Increasing concentrations of RecD2 reduced the accumulation of jm (Figure 4D, E, P-values < 0.01). We observed that the amount of nc products slightly increased at low RecD2 concentrations (P-value 0.033) and was not significantly affected (P-values > 0.05) at higher RecD2 concentrations, so that most of the jm intermediates that were present at the time of RecD2 addition were converted to nc products. These results showed that RecD2 does not freeze the reaction once added, even having an excess of RecD2 over the DNA molecules (50 nM RecD2).

In summary, the strand exchange assays showed a different outcome depending on the order of protein addition. If RecD2 was added at the same time as RecA, low RecD2 concentrations (1 helicase/ ssDNA molecule) slightly stimulated the production of nc product and concentrations over 50 nM blocked the reaction, whereas this effect was not observed if 50 nM RecD2 was added after strand exchange had initiated.

RecD2 catalyzes branch migration

The former results suggested that low concentrations of RecD2 can stimulate nc formation because RecD2 may branch migrate the recombination intermediate. This hypothesis is consistent with in vivo results that showed that a mutation in RecD2 is synthetically lethal when combined with mutations in RecG or RuvAB, which branch migrate recombination intermediates (25). To directly test this hypothesis, we performed standard RecA strand-exchange reactions for 10 min and purified the DNA from such reactions after EDTA/SDS treatment and ethanol precipitation to remove RecA, RecO and SsbA. This DNA, which is enriched in jm intermediates (Figure 5B, lane 8), was then incubated with RecD2 alone in the presence of ATP. The accumulation of the nc product was observed at concentrations as low as 3 nM RecD2 (Figure 5B, lane 5), and this was not observed in the reaction having the substrate incubated without RecD2 (Figure 5B, lane 4). Interestingly, under these conditions, we observed neither the accumulation of lds that could result from the disruption of the jm intermediate by the 5′→3′ translocation of RecD2 on the circular ssDNA, nor the appearance of a new jm intermediate with the displaced strand removed, that could be the product when RecD2 binds to the displaced strand and unwinds the DNA in the 5′→3′ direction.

RecD2 promotes the disassembly of RecA filaments in vivo

Given that our biochemical data are consistent with RecD2 acting as a negative regulator of RecA–ssDNA filament formation, we investigated whether this effect can be observed in vivo. Previously it has been shown that: i) in concert with mediators that act before homology search, RecA-ATP nucleates on the ssDNA produced by MMC-induced DNA damage, to form the activated RecA* (45); ii) RecA*, regulated by modulators that act during filament growth, facilitates the auto-proteolysis of the transcriptional repressor LexA to induce expression of ~40 genuine SOS genes, being recA one of them (46,47). Previous assays have shown that in the absence of positive mediators (e.g. RecO) the maximal levels of RecA are obtained at higher MMC concentrations when compared to the wt control, whereas in the absence of negative modulators (e.g. RecX) maximal levels of RecA are obtained at lower MMC concentrations when compared to the wt strain (Supplementary Figure S9, (27,32)). Therefore, the quantification of RecA protein lev-
for 2 min at 37 °C. RecD2 helicase overcomes the inhibition of replication restart by RecA. In A and B: Reactions in the presence of SsbA. An enzyme mix having B. subtilis replisome proteins was added to a substrate mix containing the DNA template, SsbA 90 nM, RecD2 5 nM, RecO 50 nM and RecA 500 nM, and DNA replication was allowed for 2 min at 37 °C. (A) Leading strand synthesis. (B) Lagging strand synthesis. In C and D: Reactions without SsbA. An enzyme mix having B. subtilis replisome proteins, but no SsbA was added to a substrate mix having the DNA template, RecD2 5 nM, and RecA 500 nM, and DNA replication was allowed for 2 min at 37 °C. (C) Leading strand synthesis. (D) Lagging strand synthesis. In Supplementary Figure S6, the quantifications of DNA synthesis from three independent experiments are shown.

Figure 3. The RecD2 helicase overcomes the inhibition of replication restart by RecA. In A and B: Reactions without SsbA. An enzyme mix having B. subtilis replisome proteins was added to a substrate mix having the DNA template, RecD2 5 nM, RecO 50 nM and RecA 500 nM, and DNA replication was allowed for 2 min at 37 °C. 

DISCUSSION

RecD2 helicases are proteins widely conserved in bacteria that lack the RecBCD complex. Previous in vivo studies in B. subtilis cells revealed that this DNA helicase is associated with the replisome (2), and that forks arrest more frequently in ΔrecD2 cells (21), supporting the hypothesis that RecD2 is important for normal replication fork progression. RecD2 depleted cells are synthetically lethal in the ruvAB or recG context and also show a synergistic loss of viability when combined with PcrA depletion (counterpart of UvrD_Eco and homologous to Rep_Eco) (25). In contrast, the RecD2_Psa enzyme in an heterologous system (E. coli) inhibited the resumption of DNA replication, and RecD2_Psa expression was toxic in E. coli strains lacking Rep, but not in strains lacking RuvAB (50). All these results highlight the relevance to analyze the activity of this protein in a homologous system.

In this work we purified the B. subtilis RecD2 helicase and characterized its mode of action on replication restart, using a reconstituted in vitro DNA replication system with purified B. subtilis proteins. We found that the effect of RecD2 on PriA-dependent replication restart depends on the protein concentration used. No significant effect in replication restart was observed when one RecD2 per DNA molecule was present, but in the presence of two or more RecD2 /DNA molecule an inhibitory effect was observed. RecD2_Psa also inhibited the resumption of DNA replication when a heterologous in vitro replication system and ~50 RecD2_Psa /DNA molecule were used in the assay (50). Taking into account the low levels of RecD2 in the cell, and that these protein levels are lower than the ones calculated for replisome components (51), we favour the hypothesis that the number of RecD2 helicases/replisome will be low, and suggest that RecD2 might modulate DNA replication restart in vivo. We demonstrate that this inhibition is done mostly over SsbA, because we do not observe this negative effect on DNA synthesis when SsbA is absent, unless high concentrations of RecD2 are used. The negative effect could be because during translocation, RecD2 may displace SsbA from the ssDNA. Consistent with this, the ATPase-defective mutant did not inhibit replication restart. In the absence of SsbA, several replication proteins are catalytically less efficient, such as the polymerases PolC and
Figure 4. RecD2 modulates strand exchange. (A) Strand exchange reactions with RecD2 added at time 0. KpnI-linearized pGEM-3Zf(+) dsDNA (lds, 3 nM) and its homologous circular ssDNA (css, 3 nM) were incubated with RecA (1.5 μM), RecO (200 nM), SsbA (300 nM) and variable concentrations of RecD2 (1.5 to 50 nM) for 5 min at 37°C with all buffer components except ATP. Then, ATP was added and reactions continued for 20 or 40 min. DNA species were separated by 0.8% w/v agarose gel electrophoresis. RecD2 concentration in lanes 7 and 8: 1.5 nM; lanes 9 and 10: 3 nM, lanes 11 and 12: 6 nM, lanes 13 and 14: 12.5 nM, lanes 15–16: 25 nM, lanes 17–18: 50 nM. In lane 1 a control with the running position of supercoiled and nicked DNA is shown. (B) Quantification of percentage of joint molecules (jm) intermediates and nicked products (nc) after 20 min. Results are the mean of three independent experiments ± SD. (C) Quantification of jm and nc after 40 min. Results are the mean of three independent experiments ± SD. (D) Strand exchange reactions with RecD2 added after strand exchange initiates at 20 min. lds and css were incubated with RecA, RecO, and SsbA for 20 min at 37°C with all buffer components. Then, variable concentrations of RecD2 (3 to 50 nM) were added and reactions continued for another 20 min (total time 40 min). Lane 3 is a control of the extent of the strand exchange reaction when RecD2 is added (i.e. 20 min reaction). In lanes 4 and 5, two controls of 40 min reactions without RecD2. (E) Quantification of jm and nc observed when RecD2 is added after strand-exchange has been initiated. Plotted are the results from three independent experiments ± SD.
DnaE (38,42), and the replicative helicase (52,53). We cannot discard a direct effect of RecD2 in the activity of one of these replisome proteins, because RecD2 slightly affected the length of Okazaki fragments during replication elongation, which strictly requires PolC, DnaC and DnaE. A role in initiation of DNA replication has been proposed for the human HELB protein (18), which belongs to the same helicase superfamily.

Our results suggest that in vivo, RecD2 may favor replication restart preventing RecA from binding to the blocked fork, in order to avoid unnecessary recombination during replication restart (Figure 7, panel A). Using a DNA substrate that mimics a blocked fork with a gap in the lagging strand and in vitro replication we show that RecD2 displaces RecA from the ssDNA, and for that ATP hydrolysis is needed. Our results are consistent with a catalytic mechanism of RecA filament disassembly by RecD2 in the replisome context, and that the activity is not the result of stoichiometric binding to RecA, because RecD2:RecA at a molar ratio of 1:100, which corresponded to 1 RecD2 helicase per DNA molecule was sufficient to overcome the inhibitory effect of having RecA during replication restart.

RecA filamentation on the ssDNA is the first step in the strand exchange reaction and this is a highly regulated process (54,55). In Gram-negative bacteria, many proteins have been identified that modulate RecA activity (RecFOR, DinD, DinI, RecX, RdgC, PsiB and UvrD (54,56), but many of these proteins are not present in B. subtilis. In B. subtilis RecX, RecU, and PcrA have been identified as negative regulators of RecA filament growth. All of them directly interact with RecA (2,57–60). PerA, like UvrD_Eco, that also dismantles RecA-ssDNA filaments, is a SF1A DNA helicase with a 3′→5′ polarity (9,61). In contrast, RecD2 is a SF1B DNA helicase that moves in a 5′→3′ DNA direction. Remarkably, eukaryotic Srs2, FBH1 and RecQ5 helicases also disassemble pre-synaptic filaments and interact with Rad51 (62–65). We propose that the human HELB helicase may also interact with Rad51.

We found that RecD2 impairs RecA nucleation and filament growth on the SsbA–ssDNA–RecO complexes, but it can also accelerate the strand exchange reaction (Figure 7, panel B). When RecD2 is added at the same time as RecA, we found that catalytic RecD2 concentrations (1 helicase/DNA substrate) stimulated the appearance of final strand-exchange products at early times of incubation, suggesting that RecD2 could branch migrate this recombination intermediate, and our experiments confirmed this activity. This has been also observed with the related Dda helicase (66). In summary, RecD2 may have both a negative and a positive action on strand exchange (Figure 7, panel B). HELB stimulated nc product formation with dsDNA with 3′-overhanging termini, but inhibited the formation of nc products when dsDNA with 5′-overhanging termini were used (43). In humans, there appear to be other helicases that also antagonize and stimulate recombination protein Rad51, as human BLM helicase (67–70). In our assays, the formation of nc products was almost unaffected when RecD2 was added after strand exchange had initiated, even in the presence of a high excess of RecD2. Similarly, ongoing DNA strand exchange was not affected by the presence of the DinI negative regulator (71). It could be a general feature that once RecA is engaged in the strand exchange reaction, it becomes more resistant to the negative action of filament regulators, but this needs to be analyzed.

As an important control, we found that RecD2 acts as a negative modulator of RecA in vivo. In response to DNA damage induced by MMC, RecA first forms visible foci, followed by filamentous structures, termed threads, which arise during repair via homologous recombination and dissipate when the DNA damage has been repaired and growth resumes. In the absence of RecD2, the threads formed by RecA-mVenus persisted for much longer time. Previous assays that analyzed the formation of RecA-GFP foci as an in vivo marker for replicative stress in B. subtilis found a nearly 2-fold elevation in the percentage of cells with RecA-GFP foci in the absence of RecD2 in untreated cells (21). Our results seem to be different, we did not observe more foci, or threads, in non-treated recD2 mutant cells, but found that filaments are longer living, as previously described in the absence of the RecX negative regulator (26). Consistent with this, we found that in ΔrecD2 mutants, a lower dose of MMC was required to achieve maximal levels of SOS induction, that requires the formation of RecA* (a RecA–ssDNA filament) to facilitate autocleavage of the LexA SOS repressor. MMC-induced replicative stress triggers the SOS response necessary for cell survival. Cells may require longer living or longer filaments to ensure proper signaling.
Figure 6. Epifluorescence microscopy showing that RecA assembly into threads is affected by RecD2. Quantitative analysis of RecA being diffuse, forming spots or threads in wt (A) or ΔrecD2 (B) cells after treatment with MMC at the indicated times. NT, not treated cells. The results are the average of three independent experiments (n = 400 cells). The corresponding movies are displayed in the Supplementary data (movie S1 and S2, respectively).

Figure 7. Model depicting how RecD2 helicase may modulate RecA during replication restart and strand exchange. (A) On a stalled fork, RecA may bind to the ssDNA regions inhibiting replication restart. RecD2 removes RecA from the stalled fork and thereby, facilitates replisome loading and replication restart. (B) During homologous recombination the invading strand is coated with RecA. RecD2 may translocate 5'→3' along the ssDNA and remove RecA. However, once RecA initiates strand exchange it may accelerate heteroduplex extension.
so that the SOS response is initiated. So maybe the activity of RecD2 must be overcome for the RecA–ssDNA filament to properly signal the SOS response. Taken together, our data show an important role for RecD2 in replication restart via counteracting RecA, and an additional role in controlling proper SOS induction. We suggest that these functions are widely conserved in bacteria.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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