Bacillus subtilis DprA Recruits RecA onto Single-stranded DNA and Mediates Annealing of Complementary Strands Coated by SsbB and SsBA*

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Tribhuwan Yadav†1, Begoña Carrasco†, James Hejna‡, Yuki Suzuki§2, Kunio Takeyasu‡, and Juan C. Alonso§1,3

From the †Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, 28049 Madrid, Spain and the §Graduate School of Biostudies, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

Background: Different mediators assist RecA to catalyze genetic recombination.
Results: DprA facilitates the displacement of both SSBs (SsbB and SsbA), increases RecA nucleation onto SSB-coated ssDNA, and mediates DNA strand annealing.
Conclusion: DprA facilitates RecA-mediated strand exchange and anneals complementary strands coated by an SSB protein.
Significance: RecA-dependent chromosomal transformation and RecA-independent plasmid transformation require the competence-induced DprA mediator.

Naturally transformable bacteria recombine internalized ssDNA with a homologous resident duplex (chromosomal transformation) or complementary internalized ssDNAs (plasmid or viral transformation). Bacillus subtilis competence-induced DprA, RecA, SsbB, and SsBA proteins are involved in the early processing of the internalized ssDNA, with DprA physically interacting with RecA. SsbB and SsBA bind and melt secondary structures in ssDNA but limit RecA loading onto ssDNA. DprA binds to ssDNA and facilitates partial dislodging of both single-stranded binding (SSB) proteins from ssDNA. In the absence of homologous duplex DNA, DprA does not significantly increase RecA nucleation onto protein-free ssDNA. DprA facilitates RecA nucleation and filament extension onto SSB-coated or SsbB plus SsBA-coated ssDNA. DprA facilitates RecA-mediated DNA strand exchange in the presence of both SSB proteins. DprA, which plays a crucial role in plasmid transformation, anneals complementary strands preferentially coated by SsbB to form duplex circular plasmid molecules. Our results provide a mechanistic framework for conceptualizing the coordinated events modulated by SsbB in concert with SsBA and DprA that are crucial for RecA-dependent chromosomal transformation and RecA-independent plasmid transformation.

Natural transformation and virus-mediated transduction are the main routes of horizontal transfer of chromosomal DNA segments among bacteria (1, 2). With few exceptions, natural transformation, which is a widely distributed mechanism for genetic recombination (GR) in many bacterial genera, is transiently induced (competence state) (1, 3). The competence machinery actively processes exogenous dsDNA and takes up the internalized ssDNA to replace homologous (or partially homologous) chromosomal sequences in a mechanism catalyzed by RecA with the help of accessory factors (2, 3). The GR mechanism, which entails unidirectional recombination of genes between different lineages, represents a form of bacterial sex that contributes to diversity, adaptation, and also the emergence of pathogens and antibiotic resistance variants (2). In Bacillus subtilis, only a small fraction of cells differentiate and become naturally competent (1). These genetically programmed cells have distinct physiological characteristics that include the transient inability to synthesize DNA of its single genome or undergo cell division (1, 2). A common core of at least 20 pole-localized proteins, which are either associated with the membrane or cytosolic, are temporarily expressed and collectively dedicated to the internalization, processing, and recombination of the incoming ssDNA with the resident chromosome or with itself to promote plasmid establishment (1, 2). The membrane-associated proteins, which form the DNA uptake machinery, bind environmental dsDNA, degrade one of the strands to produce ssDNA, and internalize it into the cytosol (1). The functional DNA uptake machinery is required for the localization of the cytosolic recombination proteins to the cell pole (4).

The cytosolic competence proteins that form the recombination machinery can be divided into those that localize at the entry pole even in the absence of exogenous dsDNA (e.g. SsbB, DprA (also termed Smf or CilB), RecA, CoiA, RecU, and RecX) and those that form a discrete focus at the pole-localized DNA

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†Ph.D. Fellow of the International Fellowship Program of La Caixa Foundation (La Caixa/CNB).
‡Present address: Dept. of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan.
§To whom correspondence should be addressed. Tel.: 34-91585-4546; Fax: 34-91585-4506; E-mail: jcalonso@cnb.cisc.es.

The abbreviations used are: GR, genetic recombination; AFM, atomic force microscopy; jm, joint molecules; lds, linear dsDNA; lss, linear ssDNA; nc, nicked circular; css, circular ssDNA; nt, nucleotide(s); SSA, single strand annealing; SSB, single-stranded binding.
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uptake machinery when dsDNA is added (e.g. RecN and RecO) (4–7). A third category comprises proteins (e.g. SsbA and Sms) whose polar location has not been studied but whose role is assumed because their expression is induced during competence in some bacterial species (2). In B. subtilis, RecA, SsbA, RecU, RecO, RecN, RecX, and Sms are expressed during exponential growth, with RecA and SsbA also transiently up-regulated during competence. In contrast, SsbB, DprA, and CoiA are specifically induced during competence development (reviewed in Ref. 2). 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. Escherichia coli RecA is referred to as RecA_Eco).

With few exceptions, competent bacteria express two ssb proteins (SsbA and SsbB) (2). SsbA is an essential 172-residue polypeptide that shares a significant degree of identity with SSB_Eco (8). SsbB is a 113-residue polypeptide that shares 63% identity with the N-terminal DNA-binding domain of SsbA (amino acids 1–106), but it has a different C-terminal domain. Like SSB_Eco (reviewed in 8, 9), SsaB and SsbB are tetramers in solution and bind ssDNA as tetramers (10, 11). The crystal structure of two poly(dT) 35-nt oligomers (dT35) bound to the surface of tetrameric SsbB was determined (11). The overall arrangement of monomers within the SsbB-ssDNA complex strongly resembles that of SSB_Eco and Helicobacter pylori SsbA (SsbA Hp) bound to ssDNA (11–13), suggesting that the structure of SsbA will probably resemble SsbB (11). The primary ssDNA binding modes of a bacterial SSB protein are denoted as the SSBss and SSB35 modes, where the subscript reflects the average number of nt occluded by each tetramer in the SSB-ssDNA complex (reviewed in Refs. 8, 9, and 14). SsbA and SsbB, which are stable homotetramers, formed mixed complexes on ssDNA (11), but the formation of heterotetramers was not detected when SsbA and SsbB were co-assembled on the same ssDNA molecule (11). SsbA binds homopolymeric dT30 in a concentration-dependent manner, with a K_D(app) of ~0.2 nM in the presence or absence of Mg2+, whereas a 6–8-fold excess of SsbB was needed to reach its respective K_D(app) under similar experimental conditions (11).

Recombination reactions catalyzed by the RecA family of proteins form an integral part of DNA metabolism in all free-living bacteria, archaea, and eukaryotes (15, 16). During the natural transformation process, the major role of RecA is to form a helical nucleoprotein filament on the internalized ssDNA and to catalyze integration of the homologous ssDNA into the genome of the competent cell (2, 17). The assembly of the first subunits of RecA (nucleation) onto a ssDNA tract coated by a SSB protein is relatively infrequent and thus rate-limiting (15, 18–20). A set of RecA accessory factors is required to stimulate RecA nucleation and filament extension. The accessory factors that act before RecA nucleation can be subdivided into two classes: the SSB proteins (SsbA and SsbB) that limit RecA nucleation, thereby suppressing unwanted RecA nucleoprotein filaments, and the RecA mediators that facilitate RecA assembly (11, 15, 19, 20).

Depending on the particular substrate onto which RecA is to be loaded, different mediators are required to overcome the inhibition exerted by the SSB protein(s). During double-strand break repair, RecBCD_Eco (counterpart of AddAB) resects the broken ends and loads RecA_Eco directly onto the ssDNA as it is generated, whereas with the RecOR_Eco or RecFOR_Eco mediators, end processing and recombinate loading are uncoupled. In that case, RecOR_Eco loads RecA_Eco onto SSB_Eco-coated ssDNA ends, and RecFOR_Eco recruits RecA_Eco to the ssDNA/dsDNA boundaries of gapped DNA coated by SSB_Eco (reviewed in Refs. 14, 18, 19, 21, and 22). In B. subtilis, RecO alone or in concert with RecR loads RecA onto SsaB-coated ssDNA (11, 23). It was hypothesized that AddAB and RecFOR might facilitate RecA loading onto gapped or protein-free ssDNA, respectively, but they are poorly characterized in B. subtilis (24–26).

Unlike recombinational repair (see above), the DNA substrate during GR is linear ssDNA that can be coated by SsbA, SsbB, or any other ssDNA-binding protein upon exit from the entry channel (2, 3). Genetic data suggest that RecA, DprA, SsbB, and RecO are necessary for natural transformation (2), whereas the role of the essential SsbA was not tested. To gain insight into the mechanism of RecA loading during chromosomal transformation and in plasmid establishment, the role of the ubiquitous DprA protein was investigated. We provide biochemical evidence that DprA has two distinct activities: (i) to facilitate RecA nucleation and RecA-ssDNA filament extension onto ssDNA coated with SsbB and SsbA and (ii) to mediate ssDNA annealing of complementary strands coated preferentially by SsbB. The first activity is essential for RecA-dependent chromosomal transformation, and the second activity of DprA is required to facilitate the annealing of complementary strands with subsequent dislodging of the SSB protein (RecA-independent plasmid transformation).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—All B. subtilis strains used are listed in Table 1. E. coli BL21(DE3)[pLysS] cells bearing pCB722 ssaB, pCB777 ssbB, or pCB596 sshsPP1 under the control of a phage T7 promoter were used to overexpress SsaB, SsbB, and SsbSPP1 proteins, respectively, as described (11, 27, 28). pBT61, containing recA under the control of its own promoter, was used to overexpress RecA in BG214 cells (29). The PCR-amplified dpra gene was joined to Ncol-Xhol-cleaved pET21d to yield pCB888. The dpra construct includes an added hexahistidine affinity tag. The presence of a hexahistidine tag at the C terminus of DprA rendered a protein that complemented the null dpra mutant (Δdpra) strain for chromosomal transformation (data not shown). Plasmid pCB888, carrying the dpra gene under the control of a phage T7 promoter, was used to overexpress DprA in E. coli BL21(DE3)[pLysS] cells. CsCl-purified pGEM3 Zf(+) plasmid dsDNA and circular pGEM3 Zf(+) and pGEM3 Zf(−) ssDNA were used as recombination substrates. If not stated otherwise, DNA quantities are expressed as mol of nt.

Enzymes, Reagents, and Protein Purification—All chemicals used in this study were of analytical grade. Isopropyl β-D-galactopyranoside was from Calbiochem, and PEP, DTT, and dATP were from Sigma. DNA restriction enzymes, ligase, etc. were supplied by Roche Applied Science, New England BioLabs, or Fermentas. DEAE, Q-, and SP-Sepharose, Sephadex
G-100, and Superose 12 were from GE Healthcare, and phos- 
phocellulose was from Whatman. [$γ^{-32}P]$ATP was from 
PerkinElmer Life Sciences.

SsbA (18.7 kDa), SsbB (12.4 kDa), SsbSPP1 (17.0 kDa), and 
RecA (38.0 kDa) proteins were purified as described (11, 28, 29).

DprA (32.7 kDa) protein was purified as follows. E. coli 
BL21(DE3)[pLysS]/pCB888 cells were grown at 25 °C to mid- 
exponential phase, and expression was induced by adding 0.4 
mm isopropyl 1-thio-β-D-galactopyranoside. Rifampicin (200 
μg/ml) was added after 30 min of isopropyl 1-thio-β-D-galac- 
topyranoside induction. Cells grew for an additional 180 min 
and were harvested by centrifugation. The cell pellet was resus- 
pended in Buffer A (50 mM potassium phosphate buffer, pH 7.5, 
1 mM DTT, 10% glycerol) containing 1 mM PMSF and 1 mM NaCl 
and lysed by sonication. After centrifugation, the 32.7-kDa DprA 
protein was found in the soluble fraction and loaded onto 
Ni2+-chelating column equilibrated with Buffer A contain- 
ing 1 mM NaCl and 5 mM imidazole. DprA was eluted with a linear 
gradient from 0 to 200 mM imidazole in Buffer A with 1 mM 
NaCl. Fractions containing DprA were pooled and dialyzed 
against Buffer A containing 400 mM NaCl. Dialyzed DprA was 
diluted 1:3 and loaded onto an SP-Sepharose column equili- 
brated in Buffer A (50 mM potassium phosphate buffer, pH 7.5, 
1 mM DTT, 90 mM NaCl, 10 mM MgOAc, 50 μg/ml BSA, 5% glycerol) 
containing 5 mM dATP for 25 min at 37 °C in a 50-μl reaction 
volume. The orders of addition of 3,199-nt pGEM3 
Zf(+) ssDNA (10 μM), the purified proteins, and their concen- 
trations are indicated throughout. A dATP regeneration system 
(0.5 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase 
and a coupling system (0.25 mM NADH, 10 units/ml lactate 
dehydrogenase) were also included (11). The amount of dADP 
was calculated as described previously (11, 34).

RecA dATP Hydrolysis Assays—The ssDNA-dependent 
dATP hydrolysis activity of RecA protein was performed as 
described previously (11). Absorbance measurements were 
taken with a Shimadzu CPS-240A dual beam spectrophotome- 
ter equipped with a temperature controller and six-position cell 
chamber, and the cell path length and band pass were 1 cm and 
2 nm, respectively (11). The regeneration of dATP from dADP 
and phosphoenolpyruvate driven by the oxidation of NADH 
can be followed by a decrease in absorbance at 340 nm. Rates of 
ssDNA-dependent RecA-mediated dATP hydrolysis and the 
lag times were measured in Buffer D (50 mM Tris-HCl (pH 7.5), 
1 mM DTT, 90 mM NaCl, 10 mM MgOAc, 50 μg/ml BSA, 5% glycerol) 
containing 5 mM dATP for 25 min at 37 °C in a 50-μl 
reaction volume. For RecA, the regeneration system 
(0.5 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase) 
and a coupling system (0.25 mM NADH, 10 units/ml lactate 
dehydrogenase) were also included (11). The amount of dADP 
was calculated as described previously (11, 34).

RecA-mediated dATP-dependent DNA Strand Exchange— 
Under physiological Mg2+ ion concentrations (<2 mM), 
RecA/Eco (reviewed in Ref. 18) or RecA (28) protein is completely 
inactive for recombination activities, and the SSB proteins 
occlude 35 nt (SSB53 binding mode). However, under optimal 
RecA conditions (10 mM Mg2+), the SSB proteins occlude 65 nt, 
with the ssDNA wrapping around all four subunits of the 
tetramer (SSB(Eco) (8, 9, 14). In this study, the experiments were 
performed under optimal RecA conditions; hence, the SSB pro- 
teins were expected to be in the SSB binding mode.

The 3,199-bp KpnI-cleaved pGEM3 Zf(+) dsDNA (20 μM in 
nl) and homologous circular 3,199-nt pGEM3 Zf(+) ssDNA 
(10 μM in nt) were incubated with the indicated concentrations 
of protein or protein combinations in Buffer D containing 2 mM 
dATP for 60 min at 37 °C in a final volume of 20 μl. A dATP 
regeneration system (8 units/ml of creatine phosphokinase and 
8 mM phosphocreatine) was included as indicated. The samples 
were deproteinized as described (27, 35) and separated on a 
0.8% agarose gel with ethidium bromide. The signal was quan- 
tified using a Gel Doc (Bio-Rad) system as described (23).

DprA-mediated DNA Strand Annealing—A linear 440-bp 
[$γ^{-32}P]$dsDNA (8 μM in nt), derived from pGEM3 Zf(+), was 
heat-denatured for 10 min at 100 °C and immediately shifted to 
ice water for 2 min. Heat-denatured ssDNA was incubated with 
increasing DprA concentrations or preincubated with SsbB,
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SsbA, or Ssb_{SPP1} at a fixed concentration (0.15 μM) for 10 min at 30 °C in Buffer E (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM EDTA, 100 mM NaCl, 50 μg/ml BSA, 5% glycerol) as described (10). Variable amounts of DprA (0.05–0.8 μM) were then added, and reactions were incubated for 60 min. The complexes formed were deproteinized as described (27) and fractionated through 6% PAGE. The signal was quantified as described above.

For AFM analysis, the 3,199-nt pGEM3 Zf(+) and its complementary pGEM3 Zf(−) ssDNA were incubated in Buffer C containing 50 μM spermidine with the indicated protein(s) for 30 min at 30 °C in a 20-μl reaction mixture. A fraction of the sample was deposited on a freshly cleaved mica surface, and the sample was processed as described previously (10).

RESULTS

Analysis of SsbA and SsbB Bound to ssDNA—Previously, it was shown that SsbA, SsbB, SSB_{Eco}, and SsbA_{Spn} interact with ssDNA in the SSB_{35} binding mode in the absence of Mg^{2+} and with the SSB_{65} mode in the presence of Mg^{2+}, but SsbB_{Spn} binds homopolymeric ssDNA in an SSB_{65} binding mode in the presence or the absence of Mg^{2+} (see Refs. 8, 14, and 36). We used AFM to gain insight into the mechanism by which ssDNA interacts with SsbA and/or SsbB. SSB-ssDNA binding experiments were carried out in the absence of Mg^{2+} (SSB_{35} mode) with the aim of detecting any possible difference between SsbA and SsbB proteins when bound to ssDNA (see Ref. 2). The 3,199-nt circular ssDNA (1 nm in molecules) behaved as a disordered coil that made length measurements difficult. The ssDNA usually appears smaller than normal in AFM images, and the mean height of the collapsed pGEM3 Zf(+) ssDNA was ~0.4 nm, which deviates from the theoretical height for ssDNA (1 nm).

SsbA or SsbB specifically bound to ssDNA (Fig. 1, A and B) but failed to form a stable complex with duplex DNA (10). At low ratios (1 SsbA tetramer/320 nt), SsbA extended the collapsed state of the ssDNA and facilitated the formation of discrete beads of ssDNA-protein complexes, with an average as low as ~6 ± 2 SsbA beads/ssDNA molecule (Fig. 1A, n = 200). At a ratio of 1 SsbA/64 nt, circular beaded complexes were more densely packed, with an average of ~24 ± 4 SsbA beads/ssDNA molecule (Fig. 1A). The number of SsbA beads per ssDNA molecule did not significantly increase at higher SsbA/ssDNA ratios (1 SsbA/32 nt) (Fig. 1A).

SsbB shows a 6–8-fold lower affinity for ssDNA when compared with SsbA-ssDNA (11). In the presence of limiting SsbB (1 SsbB/320 nt) only ssDNA was observed (data not shown). At a ratio of 1 SsbB tetramer/64 nt, there were an average of ~10 ± 2 SsbB beads/ssDNA molecule (Fig. 1B, n = 150). At a ratio of 1 SsbB/32 nt, this increased to ~24 ± 4 SsbB beads/ssDNA molecule (Fig. 1B), with no further increase at higher SsbB/ssDNA ratios (1 SsbB/16 nt) (data not shown).

The morphologies of the SsbA-ssDNA and SsbB-ssDNA complexes were similar in these experiments (Fig. 1, A and B) and were consistent with observed AFM images of tetrameric SSB_{Eco} bound to different circular ssDNA molecules in the presence or absence of Mg^{2+}, respectively (37). It is likely, therefore, that an SsbA or SsbB tetramer is the ssDNA binding unit. To test this hypothesis, the volume of the SsbA or SsbB beads was estimated. The observed volumes of SsbA (~135 ± 30 nm^3) and SsbB (~109 ± 22 nm^3) were in good agreement with (i) the theoretical volume of tetrameric SsbA (~120 nm^3) and SsbB (~82 nm^3) and (ii) the volume determinations for SsbA as well as volumes that were deduced from the co-crystal structures of SsbB-ssDNA, respectively (10, 11). At low ratios (1 SsbA/320 to 64 nt), the SsbA or SsbB beads had a height of ~1.6 ± 0.20 nm, and this height slightly increased to ~1.8 ± 0.18 nm at a higher protein-ssDNA ratio (1 SsbA/32 nt) (Fig. 1C). The results, showing beaded morphology along the circular DNA, suggest that ssDNA wound around an SsbA or SsbB tetramer. At present, we cannot rule out the possibility that at low SSB/ssDNA ratios, both proteins may bind ssDNA in one binding mode and that at high SSB/ssDNA ratios, another binding mode may be favored.

To establish whether there is a coordinate interaction between the SSB proteins, SsbA or SsbB was bound to ssDNA, and then SsbB or SsbA was added to maintain the stoichiometry (1 SSB/32 nt). Regardless of which protein was added first, there were ~24 ± 3 (SsbA added first) and ~26 ± 3 (SsbB added first) SsbB beads/ssDNA molecule (data not shown). Because the numbers of SsbA or SsbB beads, alone or in concert, observed per ssDNA molecule are similar but ~3-fold lower than if the ssDNA lacked secondary structures (i.e. 3,199 nt/SSB_{35} ~90 beads), it is likely that SsbA and SsbB bind to preexisting ssDNA tracts.

DprA Preferentially Binds to ssDNA—To analyze the mechanism by which DprA interacts with ssDNA, increasing concentrations of the protein were incubated with ssDNA, and the complexes were visualized by AFM. DprA-ssDNA complex formation was detected at DprA concentrations as low as 0.1 nm (1 DprA dimer/ssDNA molecule), but formation of DprA-dsDNA complexes was not observed even at DprA concentrations as high as 20 nm (data not shown).

DprA-bound ssDNA formed discrete globular structures on ssDNA. At low ratios (1 DprA/320 nt), DprA formed protein-ssDNA complexes with an average of ~4 ± 1 DprA-ssDNA molecule (Fig. 1C, n = 100), but at higher protein-ssDNA ratios, DprA formed structures that were larger than expected for a dimer (Fig. 1C, n = 200). Because the DprA-ssDNA complex increased in size with increased protein concentrations, we termed them “blobs” to differentiate them from the SSB-ssDNA beads that were similar in shape and size. At ratios of 1 DprA/64 to 16 nt, DprA formed large blobs on ssDNA with an average of 1.2 DprA complexes/ssDNA molecule (Fig. 1C, c), implying that DprA bound to ssDNA interacted with DprA free in solution to form a discrete higher order DprA-ssDNA complex rather than nonspecific aggregates of proteins on DNA (see “Discussion”).

In our AFM images, the DprA structures were globular in shape, and their height and width increased with increasing protein concentrations (Fig. 1C, c and d). The theoretical volume of DprA was ~54 nm^3 for a monomer and ~108 nm^3 for a dimer. The volume of free DprA, which did not vary with protein concentration under these experimental conditions, correlated with DprA monomers (Fig. 1C, d, denoted by an arrow). The volume of DprA-ssDNA complexes, however, varied with
protein concentration. At low DprA concentrations, two discrete DprA subpopulations with volumes of \( \approx 80 \) and 140 nm\(^3\) that might correlate with DprA monomers and dimers were observed (Fig. 1C, a). At high protein concentrations, large DprA aggregates bound to one or more ssDNA molecules were observed with a volume increase of 10–60-fold (Fig. 1C, d).

**DprA Promotes Limited SSB Dislodging from ssDNA**—To gain insight into the DprA interaction with ssDNA precoated by SsbA or SsbB, ssDNA was preincubated with an SSB protein at ratios of 1 SSB/64 nt, and then variable amounts of DprA were added (Fig. 1, A and B). At low (1:10) DprA/SSB molar ratios, the SsbA or SsbB beaded complexes were similar to those formed in the absence of DprA (Fig. 1, A and B). When the DprA concentration was increased up to 1:1 ratios, the average number of SsbA and SsbB beads per ssDNA molecule was reduced to 15 ± 3 and 13 ± 2, respectively (Fig. 1, D (c) and E (c)). The DprA blob increased in size with increasing protein concentrations. At 2:1 DprA/SSB ratios, the clearly distinguishable DprA blobs further dislodged SSB beads from the ssDNA. In the presence of DprA, the number of SsbA or SsbB beads per ssDNA molecule was reduced from \( \sim 24 \pm 4 \) to \( \sim 13 \pm 1 \) and \( \sim 10 \pm 2 \) beads/ssDNA molecule, respectively (Fig. 1, D (d) and E (d)). Similar results were observed when stoichiometric amounts of SsbA and SsbB (1 SSB/32 nt) were co-assembled onto ssDNA and then DprA (1 DprA/\( \sim 64 \) nt) was added (data not shown).

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exchange with dATP as a cofactor, rATP-bound RecA decreases filament formation onto SsbA-coated ssDNA by about 8-fold and impairs the DNA strand exchange between SsbA-coated ssDNA and homologous duplex DNA by roughly 15-fold; thus, dATP may be the preferred cofactor for RecA activities at least in vitro (28). Therefore, RecA nucleation and filament growth were analyzed in the presence of dATP.

In the absence of homologous duplex DNA, dATP binding by RecA is essential for RecA nucleation onto ssDNA, and dATP hydrolysis makes RecA-ssDNA filaments dynamic; both steps are crucial for a successful GR reaction (reviewed in 2). Measuring the initial rate of dATP hydrolysis provides an indirect measure of RecA nucleation, and the dynamic self-assembly onto ssDNA with subsequent disassembly from this ssDNA lattice is inferred by steady state of dATP hydrolysis (RecA-ssDNA filament formation) (11, 28, 38–40). The lag time of nucleation is derived from the time intercept of the linear regression based on the steady state rate of dATP hydrolysis (34).

At a ratio of 1 RecA monomer/12 nt, the rate of RecA nucleation onto ssDNA and subsequent filament formation was biphasic, with a slow nucleation step (4 ± 0.5-min lag phase). After nucleation, the RecA filaments formed quickly, hydrolyzing dATP near the previously observed $K_{\text{cat}}$ of 17.1 ± 0.4 min$^{-1}$ (Fig. 2A) (28).

To test whether DprA facilitates nucleation and/or RecA filament extension onto ssDNA, the rate of dATP hydrolysis was determined. In control experiments in the absence of RecA, DprA (1 DprA/11 nt) did not exhibit dATP hydrolysis activity when compared with the mock reaction in the absence of both DprA and RecA (data not shown), indicating that the hydrolysis of dATP observed in the assays can be solely attributed to the RecA protein.

The addition of low DprA concentrations (1 DprA/125 to 66 nt) did not significantly facilitate RecA nucleation onto ssDNA when compared with RecA alone (Fig. 2A, inset). DprA did not significantly stimulate the rate of dATP hydrolysis when compared with RecA alone (Fig. 2A). It is likely that low DprA concentrations might not contribute to the removal of regions of secondary structure within ssDNA to facilitate RecA nucleation.

When the DprA concentration approached parity with RecA (1 DprA/16 nt and 1 RecA/12 nt), the lag time was significantly increased (8 ± 0.6-min lag phase) (Fig. 2A, inset), but the final rate of RecA dATP hydrolysis did not significantly change (16.7 ± 0.5 min$^{-1}$) (Fig. 2A). Furthermore, at equimolar concentrations with RecA (1 DprA/11 nt and 1 RecA/12 nt), DprA reduced nucleation (10.5 ± 0.3-min lag phase), and the final rate of RecA dATP hydrolysis also decreased (13.8 ± 0.2 min$^{-1}$) (Fig. 2A). It is likely that DprA, at about equimolar concentrations with RecA, antagonized its polymerization onto ssDNA.

Previously, it was shown by Förster resonance energy transfer that DprA interacts with RecA in vitro (4). To test whether DprA physically interacted with RecA in vitro, we used a Ni$^{2+}$ column and purified His-tagged DprA and native RecA (Fig. 2B). As expected, DprA was retained on the Ni$^{2+}$ column (Fig. 2B, lane 4), but RecA was present in the flow-through (Fig. 2B, lane 5, FT). In the presence of DprA, the association of RecA with the Ni$^{2+}$ column was observed (Fig. 2B, lane 10), indicating that the interaction of RecA with the resin was dependent on the presence of DprA. However, the addition of RecA partially destabilized the His-tagged DprA-matrix interaction (Fig. 2B, lane 8), suggesting that DprA makes specific protein-protein interactions that sequester RecA. Indeed, when the stoichiometry of the reaction was altered, by increasing RecA (data not shown) or the ssDNA concentration (Fig. 2C), the antagonism...
exerted by DprA on RecA nucleation and filament extension was overridden. These data suggest that the inhibition of RecA loading onto ssDNA at high DprA concentrations might be due to an interaction between DprA and RecA that forms an inactive binary complex. This seems more plausible than the possibility that traces of a putative “inhibitory factor” present in the DprA preparation inhibited dATP hydrolysis.

**DprA and SsbB Interaction Facilitates RecA Nucleation onto ssDNA**—Förster resonance energy transfer suggested that DprA physically interacts with SsbB in vivo during GR (4), although this interaction was not detected in our in vitro studies. To test whether DprA stimulated RecA nucleation onto ssDNA coated by a SSB protein, the kinetics of DprA-mediated nucleation of RecA onto SsbB- or SsbA-coated ssDNA was analyzed by measuring RecA-mediated hydrolysis of dATP (Fig. 3). At a ratio of 1 RecA monomer/12 nt, RecA nucleation onto ssDNA was slow (4 ± 0.5-min lag phase) (Fig. 3, inset), and subsequent filament extension approached the maximal rate of dATP hydrolysis (17 ± 0.5 min⁻¹). In control experiments in the absence of RecA, none of the SSB protein exhibited dATP hydrolysis activity (data not shown).

Preincubation of ssDNA with SsbB or SsbA, sufficient to saturate the binding sites (1 SSB/33 nt) delayed the nucleation of RecA and reduced the RecA filament extension phase, albeit to different extents (Fig. 3, A and C, inset) (11). Independent of the order of addition, SsbB (1 SsbB/33 nt) and DprA (1 DprA/33 to 125 nt) added prior to RecA significantly reduced the lag time to ~1 min when compared with RecA nucleation onto SsbB-coated ssDNA (~8-min lag time) (Fig. 3, A [inset] and B). Under this condition and depending on the stoichiometry of the reaction, the final rate of RecA dATP hydrolysis approached the maximum rate of 25–30 min⁻¹ (Fig. 3, A and B). These results suggest that DprA, by promoting partial SsbB dislodging (Fig. 1E), facilitates new RecA nucleation events and thereby accelerates the dynamic assembly and disassembly of RecA from SsbB-coated ssDNA with subsequent hydrolysis of dATP (see Fig. 3, A and B).

*In vivo*, the absence of SsbB resulted in only a 5–10-fold reduction in chromosomal and plasmid transformation (2), suggesting that the essential SsbA, perhaps in concert with SsbB, might play a role in RecA nucleation. Adding SsbA (1 SsbA/33 nt) and DprA (1 DprA/66–125 nt) prior to RecA reversed the SsbA-mediated antagonism of RecA nucleation (Fig. 3C). The presence of DprA (1 DprA/125 to 33 nt) did not facilitate RecA nucleation onto SsbA-coated ssDNA (lag time ~5 ± 1 min) when compared with RecA alone (Fig. 3C, inset). In the presence of low DprA concentrations (1 DprA/125 to 66 nt), the final rates of dATP hydrolysis (17.2 ± 0.3 min⁻¹) were comparable with the final rate with RecA alone (Fig. 3C). The addition of DprA (1 DprA/33 nt) to the preformed SsbA-ssDNA complex increased the final rate of dATP hydrolysis (25 ± 0.5 min⁻¹) to a level comparable with the rate in the presence of SsbB (Fig. 3A). These results reveal a difference between DprA and DprA_{Spn}. Unlike DprA (Figs. 2 and 3), it was suggested that DprA_{Spn} binds to the protein-free ssDNA and nucleates RecA_{Eco} onto ssDNA (see Ref. 41). When the DprA concentrations approached the RecA concentrations (1 DprA/16 to 11 nt), the lag in RecA-mediated dATP hydrolysis (>9 min) and the final rate (13 ± 0.5 min⁻¹) decreased to levels comparable with those of reactions lacking DprA and containing SsbA (Fig. 3C).

**DprA Facilitates RecA Nucleation onto SsbB- and SsbA-coated ssDNA**—To test whether both SSB proteins (1 SSB/33 or 16 nt) affected the ability of DprA to facilitate RecA nucleation, SsbA was preincubated with ssDNA (10 μM in nt) was preincubated with 0.3 μM SsbB (5 min) and incubated with increasing DprA concentrations. Then RecA (0.8 μM) was added, and the ssDNA-dependent dATPase activity was measured for 25 min. The measured lag times are plotted against the concentration of DprA (inset). The filled square denotes the lag time of RecA alone. B, ssDNA was preincubated (5 min) with increasing DprA concentrations and then incubated with SsbB (0.3 μM), and the ssDNA-dependent dATPase activity of RecA (0.8 μM) was measured for 25 min at 37 °C. C, ssDNA was preincubated with 0.3 μM SsbA (5 min) and incubated with increasing DprA concentrations. Then RecA (0.8 μM) was added, and the ssDNA-dependent dATPase activity was measured for 25 min. All reactions were repeated three or more times with similar results. The amount of dADP and the lag time were calculated as described in the legend to Fig. 2. The ssDNA-dependent dATP hydrolysis by RecA alone is denoted as a broken dotted line, and the hydrolysis in the presence of an SSB protein is represented by a broken line.

**FIGURE 3.** SsbB or SsbA and DprA play a role in the rate-limiting nucleation of RecA A, the 3,199-nt pGEM3 Zf (+) ssDNA (10 μM in nt) was preincubated with 0.3 μM SsbB (5 min) and incubated with increasing DprA concentrations. Then RecA (0.8 μM) was added, and the ssDNA-dependent dATPase activity was measured for 25 min. The measured lag times are plotted against the concentration of DprA (inset). The filled square denotes the lag time of RecA alone. B, ssDNA was preincubated (5 min) with increasing DprA concentrations and then incubated with SsbB (0.3 μM), and the ssDNA-dependent dATPase activity of RecA (0.8 μM) was measured for 25 min at 37 °C. C, ssDNA was preincubated with 0.3 μM SsbA (5 min) and incubated with increasing DprA concentrations. Then RecA (0.8 μM) was added, and the ssDNA-dependent dATPase activity was measured for 25 min. All reactions were repeated three or more times with similar results. The amount of dADP and the lag time were calculated as described in the legend to Fig. 2. The ssDNA-dependent dATP hydrolysis by RecA alone is denoted as a broken dotted line, and the hydrolysis in the presence of an SSB protein is represented by a broken line.
**DprA Has Two Activities**

**FIGURE 4. DprA facilitates RecA loading on SsbA-ssDNA-SsbB complexes.** A, the 3,199-nt pGEM3 Zf(+) ssDNA (10 μM in nt) was preincubated with SsbA and then with SsbB (0.3 μM) in Buffer D containing 5 mM dATP. Increasing DprA concentrations were added and incubated for 5 min. RecA (0.8 μM) was then added, and the absorption was measured for 25 min. B, the 3,199-nt ssDNA (10 μM in nt) was preincubated with 0.3 μM SsbSPP1 and then incubated with DprA. Then RecA (0.8 μM) was added, and the ssDNA-dependent dATPase activity was measured for 25 min. All reactions were repeated three or more times with similar results. The ssDNA-dependent dATP hydrolysis by RecA alone is denoted as a broken dotted line, and the hydrolysis in the presence of an SSB protein is shown by a broken line.

**DprA Overcomes the Interference of a Non-cognate SSB Protein with RecA Nucleation**—To test whether DprA bound to ssDNA constrained the diffusion of SSB along the ssDNA lattice and facilitated its spontaneous dislodging, the steady state rate of RecA-mediated dATP hydrolysis was measured in the presence of a non-cognate SSB protein (SsbSPP1) (Fig. 3B). Of interest is the fact that SsbA and SsbB share a similar degree of identity with SSB_Eco (177 residues long) and SsbSPP1 (B. subtilis SPP1 virus-encoded protein, 159 residues long) at their N-terminal domains. SsbA shares 39 and 40% identity, respectively, in the first 130 residues, and SsbB shares 40 and 39% identity with SSB_Eco and SsbSPP1 in their first 105 or 111 residues, respectively. Little homology was revealed at the C-terminal domain except for the acidic C-terminal tail. It is absent in SsbB.

Preincubation of ssDNA with sufficient SsbSPP1 to saturate the binding sites (1 SsbSPP1/33 nt) retarded the nucleation of RecA onto ssDNA, with a lag phase of 7–8 min, and also reduced the RecA filament extension (12.4 ± 0.4 min⁻¹) (Fig. 4B) (23). The presence of DprA reversed the inhibitory effect exerted by SsbSPP1 on RecA nucleation onto SsbSPP1-coated ssDNA (Fig. 4B), suggesting that DprA might facilitate partial displacement of a non-cognate SSB (SsbSPP1 protein).

**DprA Facilitates RecA-mediated DNA Strand Exchange**—To understand the role of DprA in RecA-mediated DNA strand exchange, we performed three-strand exchange assays in the presence of dATP as a nucleotide cofactor (Fig. 5). RecA initiates DNA strand exchange by pairing the free end of the lds with the homologous circular css, leading to jm intermediates. Continued strand exchange in these hybrid complexes ultimately generates nc dsDNA and lss products (Fig. 5A). In the strand exchange reaction, the displaced lss product could be further engaged in a reverse reaction with subsequent reversion of the nc product to the initial substrates or a network of interlinked intermediates if an SSB protein is omitted in the reaction mixture (reviewed in Ref. 21); hence, the strand exchange reaction rarely goes to completion, and the specific role of the RecA accessory protein is not always easy to evaluate.

In vitro, RecA promotes a set of DNA strand exchange reactions that mimics its presumed role in GR. To test the role of DprA on RecA-mediated DNA strand exchange, we first performed the three-strand exchange assays in the presence of low RecA concentrations. Limiting RecA (1 RecA/28 nt) catalyzed DNA strand exchange between the css and the lds DNA, converting <10% of the substrate into jm intermediates during a 60-min reaction (Fig. 5B, lane 2). The addition of half-saturating to saturating amounts of SsbA (1 tetramer/66, 33, or 22 nt) prior to RecA stimulated RecA strand exchange 6–9-fold, with nc products amounting to 32.7 ± 0.2 to 42.9 ± 0.5% of the total (Fig. 5B, lanes 3–5). When SsbB replaced SsbA, a significant stimulation of RecA-mediated DNA strand exchange (4–5-fold) was also observed (nc products, 21.8 ± 0.7 to 23.3 ± 0.4%) (Fig. 5B, lanes 6–8). It is likely that the SSB protein aids RecA-mediated DNA strand exchange by facilitating spontaneous melting of secondary structure on ssDNA and/or by sequestering the displaced lss DNA strand, albeit with different efficiency.
The addition of DprA (1 DprA/66, 33, or 22 nt) prior to limiting RecA stimulated RecA-mediated DNA strand exchange (4–5-fold), but this effect was insensitive to DprA concentration (with average nc products, 21.6 ± 0.8%) (Fig. 5B, lanes 9–11). It is likely that DprA facilitates RecA-mediated DNA strand exchange, but the mechanism is poorly understood. To

FIGURE 5. **DprA facilitates RecA-mediated DNA strand exchange in the presence of both SSB proteins.** A, scheme of the three-strand exchange reaction between circular ssDNA (css, in red) and the linear duplex (lds, in black and red) substrate and the expected intermediates (jm) and final products (nc and lss) by RecA-mediated DNA strand exchange. B, circular 3,199-nt pGEM3 Zf(+) ssDNA (10 μM in nt) and homologous KpnI-linearized dsDNA (20 μM in nt) were preincubated with increasing concentrations of SsbA (0.15, 0.3, and 0.45 μM; lanes 3–5), SsbB (0.15, 0.3, and 0.45 μM; lanes 6–8), and DprA (0.15, 0.3, and 0.45 μM; lanes 9–11) for 10 min at 37 °C in Buffer D containing 2 mM dATP. The circular ssDNA and homologous dsDNA were preincubated with a constant amount of SsbA (0.15 μM in lanes 12–14) or SsbB (0.15 μM in lanes 15–17). Then increasing concentrations of DprA (0.15, 0.3, and 0.45 μM) were added and incubated for 10 min at 37 °C. Then a constant amount of RecA (0.35 μM; lanes 2–17) was added, and the reaction was incubated for 60 min at 37 °C. C, circular ssDNA and homologous linear dsDNA were preincubated with decreasing concentrations of SsbB and then increasing concentrations of SsbA (lanes 3–6) or vice versa (lanes 8–11) for 5 min at 37 °C in Buffer D containing 2 mM dATP. The complex was incubated with a constant amount of DprA (0.1 μM; lanes 3–12) for 5 min at 37 °C, followed by the addition of a constant amount of RecA (0.7 μM; lanes 2–12) and incubated for 60 min at 37 °C. The products of the reactions were deproteinized, separated, and quantified as described under “Experimental Procedures.” The positions of the bands corresponding to css, cds, lds, nc, and jm are indicated. + or −, presence or absence of the indicated protein. Data of recombination products (jm, nc, or the sum of both) are indicated as percentages and are the average values obtained from more than three independent experiments (the results given are within a 5% S.E.).
test whether DprA could recruit RecA onto SsbA- or SsbB-coated ssDNA, RecA-mediated strand exchange in the presence of a constant amount of SSB protein (1 SS/66 nt) and increasing DprA concentrations was analyzed. In the presence of SsbA and low DprA concentrations (1 DprA/66 nt), the accumulation of nc product by RecA-mediated DNA strand exchange was increased and reached levels comparable with RecA plus SsbA (Fig. 5B, lanes 3–5 and 12). In the presence of SsbA and higher DprA concentrations (1 DprA/33 or 22 nt) equimolar with RecA (1 RecA/28 nt), the accumulation of nc products decreased (35.0 ± 0.1 and 33.1 ± 0.4%) (Fig. 5B, lanes 13 and 14). It is likely that in the presence of SsbA, an excess of DprA contributed to RecA dislodging, as described for RecA nucleation (see above). When Ssb replaced SsbA, DprA moderately increased RecA-mediated DNA strand exchange (Fig. 5B, lanes 15–17).

The presence of suboptimal RecA (1 RecA/14 nt) catalyzed strand exchange between the css and the lds DNA, converting 27.4 ± 0.6% of the homologous lds DNA into nc and lss products during a 60-min reaction (Fig. 5C, lane 2). To test whether limiting DprA concentrations could facilitate RecA-mediated DNA strand exchange with an ssDNA substrate coated by SsbA and SsbB, the ssDNA was preincubated with various concentrations of both SSB proteins (1 tetramer/66, 33, or 22 nt). SSB-bound css DNA was incubated with lds DNA and limiting DprA concentrations (1 DprA/100 nt), followed by the addition of suboptimal RecA concentrations (1 RecA/14 nt). In the absence of SSB proteins, the effect of DprA on RecA-mediated DNA strand exchange was not markedly increased when compared with RecA alone (Fig. 5C, lanes 2 and 12). The addition of DprA to the preformed SsbA-ssDNA, SsbB-ssDNA, or SsbB-ssDNA-SsbA complexes significantly increased RecA-mediated DNA strand exchange (Fig. 5C, lanes 3–11). The order of addition of the SSB proteins might play a minor role if any (Fig. 5C). It is likely that the substrate for RecA-mediated DNA strand exchange is SSB-coated ssDNA rather than protein-free ssDNA.

DprA is Important for Plasmid Transformation—DprA is required for chromosomal transformation in different bacterial species. In the absence of DprA, chromosomal transformation is reduced 10–100-fold (2, 3), but the requirement of DprA for plasmid transformation is less clear. Plasmid transformation is marginally reduced if at all in ΔdprA 1/sig or ΔdprA 1/hs, competent cells (3), but it is 40-fold reduced in ΔdprA cells (11). Similarly, the absence of the strand-annealing protein RecO resulted in a 30-fold reduction in plasmid transformation. Chromosomal and plasmid transformation, however, were blocked in a ΔrecO ΔdprA double mutant strain (Table 1) (11), suggesting that both DprA and RecO are crucial for efficient GR in otherwise rec− cells.

To confirm the role of DprA in plasmid transformation, we constructed a ΔdprA ΔrecA double mutant strain (Table 1). Chromosomal transformation is RecA-dependent, and plasmid transformation is a RecA-independent reaction (see Table 1) (2). As expected, chromosomal transformation was blocked in the ΔdprA ΔrecA or ΔrecO ΔrecA context (Table 1). In ΔrecO cells, defects in plasmid transformation were not due to the unavailability of ssDNA, because the absence of RecA suppressed the need for RecO during plasmid transformation (Table 1) (11). However, plasmid transformation was also impaired in ΔdprA ΔrecA cells (Table 1), suggesting that DprA is required for plasmid transformation.

DprA Facilitates Annealing of Protein-free or SSB-coated DNA Strands—Above it was shown that (i) SsbA or SsbB bound to ssDNA failed to bridge two non-complementary DNA molecules by a direct protein-protein interaction (Fig. 1, A and B), (ii) DprA on one ssDNA molecule interacted with DprA-ssDNA on a second ssDNA molecule and bridged them (Fig. 1C, denoted by an arrowhead), and (iii) DprA is necessary for effective plasmid transformation (Table 1). However, it was reported that DprA was unable to catalyze ssDNA annealing under conditions where DprA Ssp is deficient (see Fig. 3). We tested whether DprA is able to catalyze SSA, using heat-denatured 440-nt-long complementary ssDNA strands coated or not by an SSB protein as substrate.

In the presence or absence of Mg2+, spontaneous reannealing of the protein-free 440-nt DNA strands was measured to be ~16 and ~22%, respectively (Fig. 6) (10, 11). DprA (1 DprA/160 nt) facilitated the annealing of both complementary DNA strands by ~2-fold when compared with the absence of DprA (Fig. 6, A (lane 4) and E), but increasing DprA concentrations (1 DprA/80 to 10 nt) did not further enhance the SSA reaction (Fig. 6, A (lanes 5–8) and E).

As reported previously (10, 11), the presence of any of the SSB proteins (1 SSB/53 nt) prevented spontaneous reannealing of the complementary strands (Fig. 6, B–D, lane 2). At a ratio of 1 DprA/80 nt, DprA facilitated the annealing of the two complementary ssDNAs coated by SsbB (1 SSB/53 nt), and at a ratio of 1 DprA/40 nt, the SSA reaction reached its maximal level (Fig. 6, B (lane 5) and E). When SsbB was replaced by SsbA, ~30% of the complementary strands were reannealed at ratios of 1 DprA/80 to 20 nt (Fig. 6, C (lanes 4–7) and E). By contrast, DprA protein annealed Ssb Ssp-coated ssDNA complexes very poorly, and a high DprA/ssDNA ratio (1 DprA/20 nt) was needed (Fig. 6, D (lane 6) and E). Because the ssDNA was the same for the Ssb Ssp and the SsbB experiments, it is likely that
DprA mediates annealing of complementary ssDNA tracts coated with SsbB by interacting with SsbB. Moreover, DprA-DprA interactions bridging two ssDNA molecules probably facilitate the annealing of complementary ssDNA tracts coated by SsbB.

**DprA Facilitates the Annealing of SsbB-coated Plasmid DNA Strands**—To test whether DprA could mediate SSA of plasmid-sized circular ssDNA substrates (pGEM3 Zf(+)) and pGEM3 Zf(−)) coated or not by an SSB protein, the SSA reaction was performed and then monitored by AFM. When SsbA (1 SsbA/64 nt) was preincubated with the ssDNA and then increasing concentrations of DprA and the complementary ssDNA were added, DprA-mediated accumulation of duplex DNA was rarely observed (<0.8%, n = 112) (Fig. 7A, a–c). At low concentrations (1 DprA/250 nt), DprA formed discrete blobs on ssDNA, whereas at higher DprA/ssDNA ratios (1 DprA/125 nt), the accumulation of bridged molecules was observed (Fig. 7C, b, denoted by an arrowhead). DprA (1 DprA/125) facilitated intermolecular bridging that amounted to 44% of total molecules, but annealed duplex DNAs were seldom observed (<1%, n = 85) (Fig. 7C, c). When the ssDNA was precoated with SsbB and DprA (1 DprA/250 nt), bridging molecules were readily observed, and dsDNA comprised ~18% (n = 76) of total molecules (Fig. 7B, b and c). It is likely that the annealing of plasmid-sized ssDNA strands is preferentially dependent on SsbB. It should be noted that the annealed duplex is largely devoid of associated proteins, consistent with the lower affinity of SsbB and DprA for dsDNA compared with ssDNA (Fig. 7B).

**DISCUSSION**

The present study demonstrates that DprA has two activities: to recruit RecA onto SsbB-coated or SsbB plus SsbA-coated ssDNA and to mediate annealing of two complementary strands coated by SsbB and to a lesser extent by SsbA. The first activity is intrinsic to directing RecA loading onto SsbB- and SsbA-coated ssDNA (chromosomal transformation). The second activity is required for the annealing of the incoming oligomeric plasmid ssDNA to reconstitute a dsDNA circular plasmid molecule (plasmid transformation). From this and previous data, we proposed that DprA in concert with SsbB and SsbA promotes both chromosomal and plasmid transformation. These properties of DprA might not universally hold for transformable bacteria. It is proposed that SsbA/Ssb, which is not induced in competence, cannot substitute for SsbB Ssb and the SsbB Ssb, ssDNA complexes only represent a reservoir of internalized ssDNA (42). Here DprA/Ssb is likely to interact with ssDNA as soon as it exits from the entry channel, and then the DprA Ssb/ssDNA complex might recruit RecA Ssb onto ssDNA (41). This study, however, was carried out with heterologous proteins (DprA Ssb, RecA Ssb, and SSB Ssb) (41).

**DprA Promotes the Nucleation of RecA Filaments onto SSB-ssDNA Complexes**—The circular ssDNA was arranged in a compact collapsed state, and with the addition of SsbA or SsbB, circular extended beaded complexes were observed. The morphologies of the SbA-ssDNA and SsbB-ssDNA were similar (Fig. 1) (10) and were similar to those observed for tetrameric SSB Ecoli in the presence or absence of Mg2+ (37).
At low concentrations (1 DprA/320 nt), DprA formed 3–6 discrete blobs on ssDNA, whereas at higher DprA/ssDNA ratios, those blobs mainly converged in a large, single globular structure per ssDNA molecule. Similar types of complexes were observed for DprA with DprA or DprA (1 DprA or DprA/20 nt) by transmission electron microscopy (41). DprA coating the ssDNA was not observed (Fig. 1C), and, independent of the technique and experimental conditions used, long extensive regions of protein-free ssDNA could be detected (Fig. 1C); thus, the mode of interaction of DprA (this work) or DprA (41) with ssDNA was incompatible with the hypothesis that the Firmicutes DprA protein at physiological concentrations might protect the ssDNA from endo- or exonuclease degradation.

We reported that the rate-limiting step in RecA filament assembly is the nucleation process, and the presence of substoichiometric DprA concentrations does not significantly facilitate the nucleation of RecA onto ssDNA. Stoichiometric concentrations of DprA, relative to RecA, however, decreased RecA nucleation onto ssDNA (Fig. 2A). We propose that (i) DprA might not contribute to the removal of secondary structure within ssDNA and (ii) the inhibitory effect exerted by DprA on RecA nucleation could be attributed to a specific interaction of DprA with RecA free in solution (Fig. 2B), resulting in sequestration of an inactive binary (RecA-DprA) or ternary (RecA-ssDNA-DprA) complex.

We reported that when an SSB protein (SsbA or SsbB) at approximately physiological conditions is prebound to ssDNA, it creates a significant kinetic barrier to RecA nucleation, highlighting the role of DprA as a RecA mediator (Fig. 3, A and C). We proposed that SsbB, in concert with SsbA, contributes to the role of DprA as a genuine mediator. However, how DprA facilitates partial SSB dislodging remains poorly understood. In the presence of DprA, the number of SSB beads decreased (Fig. 1, D and E). A spontaneous dislodging of about half of the SSB beads in the 10-min reaction was unanticipated because the half-lives for both SsbA-ssDNA and SsbB-ssDNA complexes were longer than 25 min (data not shown), even for short ssDNA segments (e.g. dT80) (11). It is likely that the interaction of DprA with a SSB protein might reduce the diffusion rate of the latter and would indirectly facilitate a limited dislodging of cognate (SsbA and SsbB) or non-cognate (e.g. SsbSPP1) SSB proteins. A similar model was previously proposed for RecO Eco interacting with SSB Eco (14) and RecO with a SSB-ssDNA complex (11).

**FIGURE 7.** DprA-mediated strand annealing analysis by AFM in the absence or presence of SsbA and SsbB. 3,199-nt pGEM3 Zf(+) and pGEM3 Zf(−) ssDNA (0.1 nM each in ssDNA molecules) were preincubated with SsbA (5 nM) (A) or SsbB (5 nM) (B) and then incubated with increasing DprA concentrations in Buffer C containing 50 μM spermidine for 30 min at 30 °C in a 20-μl reaction mixture. C, 3,199-nt pGEM3 Zf(+) and pGEM3 Zf(−) ssDNA (0.1 nM each in ssDNA molecules) were incubated with DprA in Buffer C containing 50 μM spermidine for 30 min at 30 °C in a 20-μl reaction mixture. A fraction of the sample was deposited onto freshly cleaved mica and processed as described under “Experimental Procedures.” The white arrow denotes the annealed ssDNA, and the arrowhead shows DprA-ssDNA bridged complexes. Scale bars = 100 nm.
SSB sliding along ssDNA contributes to the spontaneous destabilization of DNA secondary structures (14). RecA_{Eco}, RecT_{Eco}, and RecO_{Taq} bind ssDNA and have been shown to produce a local increase in the distance between adjacent bases (43). Based on previous data and the data presented here, we propose that DprA binding to ssDNA might generate a local “distortion” between adjacent bases, and this hypothetical non-canonical ssDNA structure might facilitate dislodging of the SSB proteins. Similar models were proposed for RecOR_{Eco} and RecO-mediated displacement of SSB_{Eco} and SsbA, respectively (11, 34, 44).

**DprA Facilitates the Annealing of SsbB-coated or SsbB plus SsbA-coated ssDNA during Plasmid Transformation**—Different plasmid transformation models have been postulated, and all of them invoke one or more strand annealing steps prior to circularization, with RecO and DprA as the potential SSA proteins (reviewed in Ref. 2). Any SSB protein bound to complementary DNA strands inhibits spontaneous strand annealing. Here we have shown that DprA preferentially bridges, by protein–protein interactions, two discrete ssDNA strands (Fig. 1C). In the presence of SsbB, DprA promotes annealing of plasmid-size homologous ssDNA molecules coated by SsbB (Fig. 7B) or SsbB and SsbA (data not shown). DprA and SsbB (or both SSBs) then lose affinity for the duplex once the DNA becomes base-paired (Fig. 7B). When SsbB was replaced by SsbA (or Ssb_{dprA}; data not shown), the efficiency of SSA was decreased (Fig. 6, C and D).

The absence of DprA decreased chromosomal transformation 10–100-fold and plasmid transformation 40-fold, and the absence of RecO decreased plasmid transformation about 30-fold (2). In the absence of both mediators, DprA and RecO, chromosomal and plasmid transformation was markedly inhibited (>10^3-fold) (Table 1). The absence of RecA partially suppressed the need for RecO (11) but reduced the frequency of plasmid transformation 100-fold in the ΔdprA context (Table 1). We proposed that SsbA, which shows higher affinity for ssDNA than SsbB and interacts with RecO (11), might be titrated by coating the incoming ssDNA in the ΔrecA context. If SsbA is titrated, free SsbB bound to plasmid ssDNA might redirect the SSA reaction toward DprA and exert a negative effect on a RecO-mediated SSA reaction in the absence of RecA. Collectively, these data suggest that there are two alternative SSA pathways operative for plasmid transformation in rec^- cells, with RecO and DprA potentially having redundant roles (Table 1) (11).

**How the Mediator Is Selected**—For DNA repair, it is known that the selection of the RecA effector depends on the particular substrate onto which RecA is to be loaded (see Introduction). During GR, the DNA substrate exits the entry channel as linear ssDNA; hence, there must be a mechanism that selects the recruitment of the mediator. The molecular mechanism by which a specific mediator, DprA or RecO, promotes RecA loading onto ssDNA is not yet clear, and the relative interplay between both SSB proteins and both mediators (DprA and RecO) is poorly understood. The KD_{dprA} or RecO for ssDNA is of the same order of magnitude, but their relative abundance is unknown. On the other hand, SsbB is 5–20-fold more abundant than SsbA (42), but its abundance might be offset by its lower affinity (6–12-fold) for ssDNA when compared with SsbA (11).

We hypothesize that the choice of which SSB protein coats the ssDNA influences which mediator (DprA interacts with SsbB and RecO with SsbA) is favored to load RecA onto ssDNA. We assume that both SSB proteins, which are induced during competence, protect the incoming ssDNA, facilitate the spontaneous removal of DNA secondary structures when ssDNA exits the entry channel, and limit RecA nucleation to inhibit non-productive RecA binding to ssDNA. The DprA interaction with both RecA and SsbB implies that RecA can nucleate onto DprA-SsbB-ssDNA complexes more efficiently than it can onto DprA-ssDNA-SsbA complexes.

In the absence of DprA, RecO interaction with SsbA favors RecA nucleation on SsbA-coated or SsbB plus SsbA-coated ssDNA (10, 11). However, when both SSB proteins co-assemble onto ssDNA, to form a SsbA-ssDNA-SsbB complex, both effectors, DprA or RecO, can facilitate RecA loading and DNA strand annealing. In the absence of RecA, however, the relative concentrations of the SSB proteins are altered, and plasmid transformation is mainly carried out via DprA.

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