Interaction of Branch Migration Translocases with the Holliday Junction-resolving Enzyme and Their Implications in Holliday Junction Resolution*

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Background: Bacillus subtilis RuvAB and RecG translocases and RecU resolvase are crucial in homologous recombination.

Results: RecU interacts with RuvB and re-localizes it at a Holliday junction (HJ). RuvB stimulates RecU. RecG and RuvAB unwind HJs.

Conclusion: A RecU-HJ-RuvAB complex might be formed.

Significance: RecU, which interacts with RecA, may help to discriminate between RuvAB and RecG at HJs.

Double-strand break repair involves the formation of Holliday junction (HJ) structures that need to be resolved to promote correct replication and chromosomal segregation. The molecular mechanisms of HJ branch migration and/or resolution are poorly characterized in Firmicutes. Genetic evidence suggested that the absence of the RuvAB branch migration translocase and the RecU HJ resolvase is synthetically lethal in Bacillus subtilis, whereas a recU recG mutant was viable. In vitro RecU, which is restricted to bacteria of the Firmicutes phylum, binds HJs with high affinity. In this work we found that RecU does not bind simultaneously with RecG to a HJ. RuvB by interacting with RecU bound to the central region of HJ DNA, loses its nonspecific association with DNA, and re-localizes with RecU to form a ternary complex. RecU cannot stimulate the ATPase or branch migration activity of RuvB. The presence of RuvB-ATPγS greatly stimulates RecU-mediated HJ resolution, but the addition of ATP or RuvA abolishes this stimulatory effect. A RecU-HJ-RuvAB complex might be formed. RecU does not increase the RuvAB activities but slightly inhibits them.

At the postreplicative stage of homologous recombination, branch migration helicases (RuvAB and RecG) and junction-resolving enzymes (RuvC or RecU) transform the four-stranded DNA recombination intermediate or HJ1 into two duplex molecules in bacteria (2, 3). This fundamental process, which is common to cells from all domains of life, shows a different degree of complexity among organisms (4–7).

Biochemical and structural studies with proteins from Escherichia coli show that RuvABC (RuvABC_Eco) is a coordinated protein machine that acts in the late stages of recombination to migrate and resolve HJs. First two tetramers of RuvAB_Eco bind to the center of the HJ and impose a 4-fold symmetric square-planar structure on the DNA (8–10). Then two RuvB_Eco hexameric rings, recruited through contacts with RuvA_Eco, to the HJ, drive branch migration (11). Finally, binding of the junction-specific endonuclease, RuvC_Eco, leads to the formation of a RuvABC_Eco complex with the potential dislodging of one RuvA_Eco tetramer (6, 12). The RuvABC_Eco complex resolves HJs and completes the recombination process (13). The conclusion from the biochemical data that the three proteins act together forming a resolvasome is further supported by genetic data that show that (i) increased expression of RusA_Eco suppresses the DNA repair and recombination defects of ruvAB_Eco, ruvB_Eco, and ruvC_Eco mutants (14, 15), and (ii) ruvC_Eco recG_Eco double mutants have the same extreme sensitivity to UV light and recombination deficiency as ruvA_Eco recG_Eco or ruvB_Eco recG_Eco strains (16). However, the distribution of repair genes suggests that at least in some bacteria the HJ helicase RuvB might act separately of RuvA and/or the HJ-resolving enzymes (RuvC or RecU). Indeed, among free-living bacteria only <1% of the genomes examined (782) lack recA and ruvB, <4% lack ruvA, <5% lack recG, and ~7% lack a HJ-resolving enzyme (ruvC or recU) (17).

RecG_Eco is another helicase implicated in recombination that can also bind to HJs, from which it promotes ATP-dependent branch migration. RecG_Eco is able not only to migrate HJs but also to form HJs by fork regression (reversal) in vitro, a mechanism thought to aid in restarting replication at blocked replication forks (18). Such an activity was also demonstrated in vivo.

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5 The abbreviations used are: HJ, Holliday junction; ATPγS, adenosine 5′-O-(thiotriphosphate); AFM, atomic force microscopy; KD_app, apparent binding constant; HJ-J1 and HJ-J3, non-spontaneously migrating Junctions 1 and 3; HJ-Jbm6, spontaneously mobile junction 6.
for the RuvAB<sub>Eco</sub> complex (19, 20), and it was demonstrated in vitro for the Mycobacterium tuberculosis RuvAB complex (21). However, recent biochemical data suggest that both helicases might have separated roles in the cell, being RecG<sub>Eco</sub> responsible for stalled replication fork regression, whereas the preferred substrate for RuvAB<sub>Eco</sub> would be a HJ (22). Note that in the remainder of this paper, unless stated otherwise, the indicated genes and products are from *Bacillus subtilis* origin.

The bacterial Holliday junction-specific endonucleases comprise two classes of unrelated enzymes either of the integrase superfamily (e.g. RuvC<sub>Eco</sub>) or of the restriction endonuclease superfamily (e.g. RecU) (6). The RecU HJ resolvase is a dimeric enzyme that shares structural similarity with viral (T7 Endo I) and archaeal (Hje and Hjc) resolvases (23). From the results obtained with *E. coli* enzymes, it is believed that bacterial HJ resolvases work in concert with RuvAB. However, recent data from other bacteria suggest that the in vivo role of ubiquitous HJ translocases (RecG and RuvAB) might not be conserved: (i) the absence of both branch migration translocases, RecG and RuvAB, is synthetically lethal in *B. subtilis* (Firmicutes phylum) or *Neisseria gonorrhoeae* (representative of β-Proteobacteria class) (24–26); (ii) RuvAB and recG show a synergistic defect in DNA repair in *E. coli* cells (γ-Proteobacteria class); (iii) recG suppresses the recombination defect of the ruvB mutations in *Helicobacter pylori* (representative of e-Proteobacteria Class) (27); (iv) ruvAB is synthetically lethal in the recU context (24); (v) the resolvases from phages or Archaea seem to act independently of the presence of a branch migration helicase (6, 12, 28).

The N-terminal region of RecU (residues 1–31), which is essential for stable HJ–RecU complex formation, is involved in the interaction with RuvB (29). Based on the RecU structure, we proposed a model of how RecU binds to the HJ DNA. The RecU stalk region, by penetrating in the center of the HJ, distorts it so that the HJ adopts a square planar conformation with a central hole (23). This model was recently confirmed by the identification of residues located in the stalk region that are essential for HJ recognition and flexibility (30, 31). From this model we hypothesized that RecU could act as a steric barrier for RuvA binding to the center of the junction, so that the existence of a stable RecU–HJ–RuvAB complex might be hindered. Furthermore, genetic studies suggested that RecU could work independently of RuvAB and in coordination with the RecG helicase. This is consistent with the observations that: (i) the *ruvA*, *ruvB*, *recU*, or *recG* mutation renders cells very sensitive to DNA-damaging agents (24, 25); (ii) RuvA, RecU, and RecG share common suppressors (32); (iii) Δ*ruvAB* Δ*recG* and Δ*ruvAB* Δ*recU* strains are synthetically lethal, whereas a Δ*recG* Δ*recU* strain could be obtained, although with reduced viability (24, 25); (iv) the *ruvA* and *ruvB* genes are SOS inducible, whereas the *recU* gene is located in another operon under the control of σ<sup>M</sup>, and its expression is increased upon cell envelop stress (e.g. vancomycin addition or by acid, heat, ethanol, and superoxide stresses) (33, 34). These results altogether prompted us to analyze the possible coordination of RecU with the RuvAB and RecG translocases.

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**EXPERIMENTAL PROCEDURES**

**DNA Manipulations**—The NdeI-BamHI cleaved *ruvA* gene was joined to pET-3b plasmid (Novagen) to render pCB632-borne *ruvA*. The *recG* gene (obtained from digestion of plasmid pQW101 (35)) was cloned into NdeI-Clal-digested pET-3b, rendering pCB951. The accuracy of the constructions was verified by DNA sequencing. Plasmid-borne recU or its variants (recLR31A, recUE36A, and recUY80A) and ruvB gene were previously described (29, 30, 36, 37).

Plasmids were transformed onto *E. coli* BL21(DE3)[plysS] for protein overexpression. Cells were grown to an *A<sub>660</sub> = 0.8* at 37 °C, 2 mM IPTG was added, and after 120 min cells were harvested by centrifugation and stored at −20 °C.

The X-structure substrate was obtained as described previously (38, 39). The radiolabeled HJ-J1 and HJ-J3 were assembled and purified from four oligonucleotides each of 80 nucleotides as previously described (23, 38). The radiolabeled HJ-Jm6 was assembled from four 40-nucleotide oligonucleotides and gel-purified. It contains a 13-bp homologous core (36).

**Proteins**—RecU, RecUE36A, RecUR31A, RecUY80A, and RuvB were purified, and their concentrations were determined as previously described (23, 29, 30, 36, 37). Cells overexpressing the RuvA protein were harvested and resuspended in buffer A (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 10% glycerol) containing 100 mM KCl and lysed by sonication. Cell debris were removed by centrifugation (18,000 rpm in an SS-34 rotor, 40 min), and DNA was precipitated by the addition of polyethyleneimine (final concentration, 0.25% v/v with *A<sub>660</sub> = 120* and centrifugation at 12,000 rpm in a SS-34 rotor for 10 min at 4 °C. RuvA, which remains in the supernatant, was loaded onto a hydroxyapatite column (Bio-Rad). The protein was eluted with buffer A containing 100 mM KCl and 250 mM phosphate and loaded onto a Q-Sepharose column (GE Healthcare). RuvA was eluted by a step gradient from 150–300 mM KCl and dialyzed against 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 50% glycerol to store it at −20 °C. RuvA was >98% pure, and the concentration of the protein was determined by using a 11,920 M<sup>−1</sup> cm<sup>−1</sup> molar extinction coefficient.

For RecG purification, 5 g of wet weight cells were resuspended in 25 ml of buffer A containing 100 mM NaCl. Lysis was accomplished by sonication, and cell debris was removed by centrifugation. The supernatant was diluted 1:2 in buffer A, and DNA was precipitated from the supernatant by the addition of polyethyleneimine as described above. RecG, which remains in the supernatant, was loaded onto a SP-Sepharose column, and after washes with 50 and 100 mM NaCl, it was eluted at 200 mM NaCl. NaCl was adjusted to 450 mM and directly loaded onto a hydroxyapatite column (Bio-Rad). The protein was eluted with buffer A containing 450 mM NaCl and 20 mM phosphate. RecG (>98% pure) was dialyzed against 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 50% glycerol to store it at −20 °C. The concentration of the protein was determined by using a 63,260 M<sup>−1</sup> cm<sup>−1</sup> molar extinction coefficient. The concentrations of RecG, RecU, RuvB, and RuvA are expressed as moles of monomers, dimers, hexamers, and tetramers, respectively.
HJ Binding and Cleavage Assays—HJ binding was assayed by EMSA using the indicated [γ-32P]HJ DNA (0.4 nm) as follows: the binding of RecU, RecUE36A, or RecUR31A in the presence of RuvB to the synthetic HJ was performed in buffer B (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 0.05 mg/ml BSA, 5% glycerol) containing 15 mM CaCl2 or 5 mM MgCl2 and 1 mM ATP or ATPγS for 15 min at 37 °C. When indicated the reactions were additionally incubated for 10 min with 0.1% glutaraldehyde before electrophoresis. Binding of RecG and RecU or RecUE36A to the junction was performed in buffer B containing 5 mM EDTA or MgCl2 and 1 mM ATPγS, and incubation was for 15 min at 37 °C. Protein-DNA complexes were separated using 6% PAGE in Tris-acetate-EDTA buffer and visualized by autoradiography.

To measure the stability of the protein-HJ complexes, γ-32P-labeled HJ-J3 DNA was incubated with RecUE36A or RecG (4× K_D(app)) for 15 min at 37 °C in buffer B containing 1 mM ATPγS and 5 mM MgCl2 or 5 mM EDTA in a volume of 120 μl. Then a 50-fold excess of cold HJ-J3 was added, and 20-μl aliquots were taken at the indicated times and directly loaded on a 6% polyacrylamide gel run at 180 V for 2 h. The half-life of the complex is the time required to have 50% of the labeled DNA remaining in the complex.

Cleavage of the HJs (HJ-J3 and HJ-J1, labeled in arm 1) at the indicated concentrations of RecU, RecG, RuvA, and RuvB was assayed for 30 min at 37 °C in buffer B containing 10 mM MgCl2 and 1 mM ATPγS. After deproteination by the addition of 1/5 volume of stop mix solution (5% SDS, 100 mM EDTA, 5 mg/ml proteinase K) and further incubation for 10 min at 37 °C, the products of the cleavage were analyzed either by 15% denaturing PAGE or by 6% native PAGE and autoradiography.

Immunodetection of RecU, RuvB, and RuvA in Protein-DNA Complexes—Binding reactions (20 μl) contained 0.4 nm γ-32P-labeled HJ-J3 or HJ-J3 DNA and the indicated amounts and combinations of proteins in binding buffer B containing 5 mM MgCl2 and 1 mM ATPγS. After 10 min of incubation at 37 °C, 1 μl of anti-RuvA or anti-RecU polyclonal antibodies, 1 μl of Clontech His6 monoclonal antibodies, or 1 μl of a mock buffer (1× PBS) was added as indicated, and incubation was continued for 10 min at 37 °C. Complexes were fixed by the addition of glutaraldehyde (0.1% final concentration) and incubating samples for a further 10 min at 37 °C. Samples were then resolved using 6% PAGE in Tris acetate-EDTA buffer. Gels were dried and autoradiographed.

HJ Branch Migration Assays—In a standard assay γ-32P-labeled HJ-J3 DNA (0.4 nm) was incubated with RecG in buffer B containing 5 mM MgCl2 and 5 mM ATP for 30 min at 37 °C. Standard helicase assays for measurements of RuvAB activity were performed in buffer B containing 10 mM MgCl2 and 1 mM ATP. Reactions were terminated by adding 1/5 volume of stop mix solution (5% SDS, 100 mM EDTA, 5 mg/ml proteinase K) and further incubation for 10 min at 37 °C to deproteinize the sample. Unwound products were analyzed by 6% PAGE in Tris acetate-EDTA buffer and autoradiography.

ATPase Assays—ATP hydrolysis was measured as described previously (37). RuvB (20 nm) was premixed on ice with 25 nm RuvA and/or 50–150 nm RecUE36A. The reaction mixtures contained buffer B, 10 mM MgCl2, and cold synthetic HJ-J3 (100 μM in nucleotides) as effector DNA. Reactions were initiated by the addition of 1 mM ATP (mixed with [γ-32P]ATP, ratio 1:100,000) and incubated at 37 °C for 15 min. Then the reactions were stopped by the addition of 25 mM EDTA and analyzed by thin layer chromatography.

Atomic Force Microscopy—The binding of RecU and RuvB to purified χ-structures was visualized by AFM. Reaction mixtures assayed in buffer C (50 mM Tris-HCl pH 7.5, 1 mM MgCl2, 50 mM NaCl, 1 mM ATPγS) were spotted onto a freshly cleaved mica, washed several times with Milli-Q water, and dried with nitrogen. AFM observations were performed using a Nanoscope IIIa microscope (Veeco) in air using the tapping mode. The cantilever (OMCL-AC160TS-W2, Olympus) was 160 μm in length with a spring constant of 33–62 newtons/m. The scanning frequency was 2–3 Hz, and images were captured with the height mode in a 512 × 512 pixel format. The obtained images were plane-fitted and flattened by the computer program accompanying the imaging module. Image processing of the topographs and height and width measurements were performed as described (40–42). The volumes obtained (V_m) were calculated measuring the diameter at half-maximal height of the individual molecules, which compensates the tip effect (40). The theoretical molecular volume based on molecular weight and assuming that the proteins were globular was calculated using the equation V_m = (M_w/ρ) V_H, where M_w is the molecular mass, ρ is Avogadro’s number, and V_H is the partial specific volume of the particle (0.74 cm3/g), respectively (43).

RESULTS

RecU Recruits RuvB onto HJ DNA—We previously detected a specific interaction between the N-terminal region of RecU and RuvB in the presence of ATPγS-Mg2+ (denoted as RuvB-ATPγS) but in the absence of DNA (29, 37). Here, we analyzed the effect of this interaction in the presence of HJ DNA. First, the binding of RuvB to HJs in the presence of RecU was analyzed under conditions that neither allowed RecU HJ cleavage nor RuvB HJ branch migration and that were previously used (e.g. 15 mM CaCl2 and 1 mM ATP; see Ref 44). When RuvB, in the ATP-bound form (RuvB-ATP), was incubated with non-spontaneously migrating HJ DNA (HJ-J3) no retarded band was observed at the protein concentrations used (100 RuvB/HJ DNA molecule). It is likely that the protein has either low affinity for HJ DNA, forms unstable complexes with HJ DNA, or that the length of the arms are not sufficient for the formation of a stable RuvB-HJ complex (Fig. 1A, lane 2). A RuvB-HJ complex was also not detected in the presence of 5 mM MgCl2 and 1 mM ATPγS even if 0.1% glutaraldehyde was added before electrophoresis (see below). When HJ DNA was incubated with RecU and RuvB, the appearance of a band of more retarded mobility than that of the RecU-HJ complex alone was observed (Fig. 1A, lanes 6 and 7). It is likely that in the presence of RecU, RuvB, and HJ DNA, a ternary complex could be formed. To test this hypothesis RecU was replaced by the RecUR31A mutant variant, which is impaired in the interaction with RuvB (29). As shown in Fig. 1A, lanes 9–12, a band of lower mobility corresponding to a RuvB-RecUR31A-HJ complex was not observed, suggesting that formation of the ternary complex requires the physical interaction between RecU and RuvB.
RecU Discriminates between HJ Translocases

Previously it was shown that RuvBeco enhanced the association of RuvCeco with a synthetic junction X12 in the presence of 15 mM CaCl$_2$ and 1 mM ATP$_S$ (see Ref. 44). In contrast, in the presence of ATP$_S$, RuvB neither bound to HJ DNA (Fig. 1B, lane 2) nor enhanced RecU binding to HJ DNA (Fig. 1B, lanes 7–10). When HJ DNA was incubated with RuvB and the catalytically inactive RecUE36A in the presence of 5 mM MgCl$_2$ and 1 mM ATP$_S$, complexes were fixed with glutaraldehyde before separation, no enhanced binding of RecU to the HJ was observed (see below, and data not shown). RecU Brings RuvB to the Center of the Junction—AFM was used to study the nature of the RuvB-RecU-HJ ternary complex. We conducted a binding assay in the presence of both proteins with a longer HJ intermediate (the $\chi$-structure) (see Ref. 45) which facilitated the visualization of proteins bound to the center or to the arms of the structure. To properly visualize these protein-HJ complexes by AFM, 1 mM MgCl$_2$ was used. The $\chi$-structure has four arms with different lengths (R1, R2, R3, and R4 arms are 620-, 773-, 1565- and 1990-bp, respectively), with a central homologous region of 320 bp. The length of each arm was calculated assuming that the crossover site is located in the middle of the homologous region. The purified $\chi$-structures were easily distinguished by AFM because of the characteristic four-DNA arms length (Fig. 2A). The experimental values obtained for R1, R2, R3, and R4 were very close to the theoretical values (Table 1). We also measured the height of the crossover point for which we obtained a mean value of 0.55 $\pm$ 0.12 nm (mean $\pm$ S.D.) ($n = 63$, where $n$ is the total number of molecules analyzed). The variations in length and height measurements can be attributed to spontaneous branch migration between the 320 bp of homology in the $\chi$-structures (Ref. 45 and Fig. 2A).

When this HJ DNA was incubated with RecU, a globular structure only on the center of the $\chi$-structure was observed (Fig. 2B). RecU appeared as blobs of 11.87 $\pm$ 0.3 nm in length with a height of 1.09 $\pm$ 0.03 nm ($n = 138$). The volume calculated from these data was $V_m = 62.89 \pm 2.98$ nm$^3$. Given that RecU was shown to crystallize as a dimer (23), the theoretical volume for a protein of 47.9 kDa measured in dry (43) would give a $V_c = 59.7$ nm$^3$, which is in good agreement with the experimental value obtained. We can conclude that the volumes obtained reflect a population of RecU dimers bound to the $\chi$-structure. Furthermore, this binding was very specific because no binding on the arms of $\chi$-structure DNA was observed.

When the $\chi$-structure DNA was incubated with RuvB in the ATP$_S$-bound form, RuvB-HJ complexes were observed (Fig. 2C). The position of the RuvB blobs on the arms of the $\chi$-structure was nonspecific (Fig. 2C). RuvB bound to an arm of the $\chi$-structure appeared as a globular structure of 18.34 $\pm$ 0.06 nm in length and with a height of 2.68 $\pm$ 0.04 nm, which gives a calculated volume of $V_m = 286.92 \pm 12.07$ nm$^3$ ($n = 50$). Like RuvBeco (2), RuvB was shown by electron microscopy to form hexamers (224.8 kDa) at the nanomolar range in the presence of Mg$^{2+}$ and ATP or ATP$_S$. The theoretical volume for a RuvB hexamer would be $V_c = 276$ nm$^3$. It is likely, therefore, that the volumes obtained reflect a uniform population of hexamers bound to the DNA.

When both RecU and RuvB were incubated with $\chi$-structure DNA, RuvB-HJ DNA complexes with RuvB bound to the arms of the $\chi$-structure were not observed. The protein blobs observed at the center of the HJ were clearly larger than those observed in the presence of only RecU (Fig. 2D). It is likely that RecU-HJ DNA promoted the capture and tether of RuvB to the center of the junction so that the complexes observed by AFM might correspond with the ternary RuvB-RecU-HJ complex detected by EMSA (see Fig. 1A).

The analysis of the histogram obtained when both RecU and RuvB were incubated with the $\chi$-structure revealed the presence of two types of species at the center of the junction (denoted as UBI and UBII, Fig. 2D). The majority were UBI complexes (66% of total molecules; $n = 62$). The UBI complexes had an average height of 2.84 $\pm$ 0.14 nm and a width of 19.22 $\pm$ 0.12 nm, which gives a $V_m = 478.65 \pm 7.27$ nm$^3$. Taking into account the presence of two types of species at the center of the junction, it is likely that the $V_m$ for UBI complexes would be 294.38 nm$^3$.
account the calculated theoretical volumes of the individual proteins, a RecU (dimer)-RuvB (hexamer) complex, in a 1:1 stoichiometry, would have an expected $V_c$ of 335 nm$^3$. A 1:2 stoichiometry (i.e., one RecU dimer and two hexamers of RuvB bound each at one side of the junction), would give a $V_c$ of 611 nm$^3$. The analysis of the measured volumes did not allow us to discriminate between the 1:1 and the 1:2 stoichiometry. Probably UBI complexes correspond to hexamers or double hexamers of RuvB recruited by RecU to the center of the junction, respectively.

The UBII complexes showed a significantly higher height ($6.68 \pm 0.10$ nm) and width ($28.02 \pm 0.47$ nm), which gives a $V_m = 1854.41 \pm 160$ nm$^3$ ($n = 32$, Fig. 2D). From the volumes measured for the separated proteins bound to the DNA and the
Control experiments were also done to discard any nonspecific interactions. The presence of RuvB was also required to observe RuvB complexes with the inactive RecUE83A variant, but the same results were obtained when the RuvU, RuvA, and RuvB complexes were analyzed on native PAGE (Fig. 4C). This result discarded the hypothesis that RuvB may help to localize the cleavage site at the crossover by promoting limited branch migration. Because both proteins interacted and formed a complex on HJ DNA in the absence of ATP hydrolysis (Fig. 1), it is likely that RuvB in the nucleotide-bound form positioned at the center of the junction may introduce a certain flexibility in the HJ that facilitates the second cleavage and the efficiency of resolution. Indeed, it was previously shown that HJ flexibility is required for efficient RecU-mediated HJ cleavage (31). Conversely, the dynamic RuvB-ATP form could slightly migrate the junction, and RecU then fails to find its cognate cleavage sequence. This hypothesis is based on the low but detectable ATPase activity of RuvB in the presence of HJ DNA. However, this condition (i.e., RuvB-ATP) was not sufficient to detect DNA unwinding (see below). Similar results were obtained when another non-spontaneously migrating HJ was analyzed (HJ-J1; Fig. 4B). We hypothesized that (i) RecU transiently positions RuvB-ATPγS at the center of the HJ, and (ii) RuvB-ATPγS may promote the distortion of HJ DNA, and by this way it facilitates HJ resolution (23, 31). This is in agreement with the observation that ternary complexes are more stable in the presence of ATPγS. To test this hypothesis we used the RecUY80A mutant variant, which interacts with RuvB. RecUY80A binds with poor affinity to HJs, and it is not able to distort HJs, and as a consequence of this, it cleaves HJs with very low efficiency (30). In the presence of RuvB-ATPγS, RecUY80A efficiently resolved HJ-J3 DNA (Fig. 4C). It is likely, therefore, that RuvB-ATPγS partially suppressed the RecUY80A defect by facilitating the proper distortion of the HJ-3 structure.

### RecU Discriminates between HJ Translocases

RecU was analyzed on denaturing PAGE, preferentially a nick on one arm (arm 4) was observed (Fig. 4A). The sequence recognized by RecU on this arm of synthetic HJ-J3 was 5’-GG ↓ CT-3’ that is in good agreement with the previously published consensus recognition site for RecU (36). No symmetric cleavage of HJ-J3 DNA was observed in the denaturing gel (Fig. 4A), and this correlated with the poor or non-resolution of this HJ when the products of these reactions were analyzed on native PAGE (Fig. 4B).

The presence of RuvB-ATPγS greatly stimulated RecU-mediated cleavage of arms 1, 2, and 3 that were only poorly recognized by RecU alone (Fig. 4A). This correlated well with increased resolution observed in PAGE when both RecU and RuvB-ATPγS were present (Fig. 4B). However, when ATPγS was replaced by ATP, this stimulatory effect was not observed (Fig. 4B). This result discarded the hypothesis that RuvB may help to localize the cleavage site at the crossover by promoting limited branch migration. Because both proteins interacted and formed a complex on HJ DNA in the absence of ATP hydrolysis (Fig. 1), it is likely that RuvB in the nucleotide-bound form positioned at the center of the junction may introduce a certain flexibility in the HJ that facilitates the second cleavage and the efficiency of resolution. Indeed, it was previously shown that HJ flexibility is required for efficient RecU-mediated HJ cleavage (31). Conversely, the dynamic RuvB-ATP form could slightly migrate the junction, and RecU then fails to find its cognate cleavage sequence. This hypothesis is based on the low but detectable ATPase activity of RuvB in the presence of HJ DNA. However, this condition (i.e., RuvB-ATP) was not sufficient to detect DNA unwinding (see below). Similar results were obtained when another non-spontaneously migrating HJ was analyzed (HJ-J1; Fig. 4B). We hypothesized that (i) RecU transiently positions RuvB-ATPγS at the center of the HJ, and (ii) RuvB-ATPγS may promote the distortion of HJ DNA, and by this way it facilitates HJ resolution (23, 31). This is in agreement with the observation that ternary complexes are more stable in the presence of ATPγS. To test this hypothesis we used the RecUY80A mutant variant, which interacts with RuvB. RecUY80A binds with poor affinity to HJs, and it is not able to distort HJs, and as a consequence of this, it cleaves HJs with very low efficiency (30). In the presence of RuvB-ATPγS, RecUY80A efficiently resolved HJ-J3 DNA (Fig. 4C). It is likely, therefore, that RuvB-ATPγS partially suppressed the RecUY80A defect by facilitating the proper distortion of the HJ-3 structure.

### RuvA Inhibits the RecU-RuvB Resolution of HJs—To test whether the stimulatory effect exerted by RuvB was observed also in the presence of RuvA or to analyze if the presence of RuvA could further increase the activity of RecU, resolution assays with RecU in the presence of RuvB-ATPγS and RuvA were performed (Fig. 4D). The presence of RuvA, at a concentration equimolar to RecU, reversed the enhancement of RuvB-ATPγS on RecU-mediated resolution of HJs (HJ-J3 and HJ-J1, Fig. 4D, lanes 4 and 8).

### RuvA Is Essential for HJ Branch Migration—We then analyzed the effect of RecU on RuvB activities. RuvB, as other bacterial RuvBs, showed an ATPase activity stimulated by RuvA and DNA (Fig. 5A). The effector DNA used was HJ DNA to see if binding of RecU to its target facilitates RuvB-catalyzed ATP

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### TABLE 1

| Arm | Length* (nm) | Size* (bp) | Predicted size (bp) |
|-----|--------------|------------|---------------------|
| R1  | 193.4 ± 26.0 | 568.7 ± 76.4 | 620                 |
| R2  | 263.6 ± 28.0 | 775.4 ± 82.3 | 773                 |
| R3  | 523.3 ± 42.6 | 1539.0 ± 125.3 | 1565               |
| R4  | 671.4 ± 50.3 | 1974.7 ± 148.1 | 1990               |

* Observed length and size of the four arms of the χ-structure. The experimental values obtained by AFM (n = 63 molecules) are presented. Errors represent the S.D.
hydrolysis. However, the addition of RecU (data not shown) or its catalytic mutant RecUE36A (Fig. 5A) did not stimulate the RuvB ATPase activity and even inhibited the stimulatory effect exerted by RuvA.

RuvB did not show helicase activity in the absence of RuvA under the conditions tested (different Mg$^{2+}$ and Ca$^{2+}$ concentrations, ATP:ATPγS ratios and protein concentrations; Fig. 5, B and C, and data not shown). The RuvB helicase activity required the presence of RuvA (Fig. 5, B and C). In contrast, other bacterial RuvBs have a limited but detectable helicase activity in the absence of RuvA (44, 46, 47).

When RuvA was replaced by RecUE36A (Fig. 5, B and C) or RecU (data not shown) the branch migration activity of RuvB was undetectable. At high RecU protein concentrations, an inhibition of the DNA unwinding activity of the RuvAB complex was obtained (Fig. 5C, lane 10). Unlike RecUE36A-RuvB

**FIGURE 3. Detection of the RecU-RuvA-HJ, RecU-RuvB-HJ and RecU-RuvA-RuvB-HJ complexes.** A, high RecU concentrations favored the appearance of a quaternary complex. γ-$^{32}$P-labeled HJ-jbm6 DNA (0.4 nM) was incubated with different combinations of RecUE36A (from 20 to 80 nM), RuvB (40 nM), and RuvA (80 nM) as indicated for 10 min at 37 °C in buffer B containing 5 mM MgCl$_2$ and 1 mM ATPγS. Then anti-RecU (lanes 14 –19) or 1× PBS was added (lanes 1–13), and incubation was continued for 10 min at 37 °C. Before electrophoresis, complexes were fixed by the addition of glutaraldehyde to a final concentration of 0.1% and 10 min incubation at 37 °C. B, antibody supershift experiments suggest the existence of ternary (RecU-RuvA-HJ and RecU-RuvB-HJ) and quaternary (RecU-RuvA-RuvB-HJ) complexes. γ-$^{32}$P-Labeled HJ-jbm6 DNA was incubated with different combinations of RecUE36A (40 nM), RuvB (40 nM), and RuvA (80 nM) and then the indicated antibodies. In the lanes where no antibody was added, 1× PBS was added. + and − indicate the presence absence of protein, respectively. FD, free DNA; UD, RecU-DNA complexes; AD, RuvA-DNA complexes; AUD, RecU-RuvA-DNA complexes; ABD, RuvA-RuvB-DNA complexes; UABD, RecU-RuvA-RuvB-DNA complexes. The amounts of the different complexes observed are indicated as percentages with respect of total DNA and are the average values obtained from >3 independent experiments (the results given stand within a 5% S.E.). In lanes where an antibody was added, discrete complexes cannot be quantified because they were mostly shifted by the antibody.
RecG Binds and Efficiently Unwinds HJ DNA—To test whether the stimulation of RecU-mediated HJ cleavage by RuvB also takes place by the other bacterial branch migration helicase, wild type RecG was overexpressed in *E. coli* and purified by a series of chromatography steps. Peptide mass fingerprinting of the purified sample confirmed the presence of the purified protein and the absence of contaminant *recG* (*data not shown*).

In the presence of EDTA, RecG showed robust binding to HJ-J3 DNA with a $K_D$ app of 5 nM (Fig. 6A, lanes 2–8). The RecG $K_D$ app is similar to the one determined for other purified RecG proteins (48–50).

RecG-mediated branch migration was analyzed at 5 mM MgCl$_2$ and several ATP concentrations. Maximum activity was achieved at 5 mM ATP (Fig. 6B). RecG produced two dissimilar flayed duplex products, which were further unwound to render the single-stranded DNA product (Fig. 6, A and B). The flayed duplex products mimic a stalled fork with single-stranded DNA gaps on both strands. These results are slightly different to the ones obtained with a *B. subtilis* His-tagged RecG (35). His-RecG unwound DNA preferentially at ATP concentrations higher than 10 mM, and only flayed products of the unwinding of the HJ were observed, which were not further unwound (35).

RecG Fails to Stimulate HJ Cleavage—We then examined if RecG-ATP$_\gamma$S stimulated RecU-mediated resolution of HJ-J3 or HJ-J1. RecG (or RuvB as a control) was preincubated with HJ-J1 or HJ-J3 DNA in the presence of ATP$_\gamma$S. Then RecU was added, and reactions were further incubated at 37 °C for 30 min. No RecG stimulation of RecU-mediated HJ cleavage was observed (Fig. 7, lanes 3 and 4 and lanes 8 and 9). No stimulation in the cleavage was observed when ATP was used, and the reactions were analyzed on denaturing PAGE (*data not shown*). Furthermore, when disuccinimidyl suberate cross-linking assays were performed, we were unable to detect the appearance of a new band that could correspond to a putative RecU-RecG complex (*data not shown*).

A RecU-Hj-RecG Ternary Complex Was Not Observed—The effect of RecU on RecG activities was then examined. Experi-
ments were performed with the catalytic mutant of RecU (RecUE36A). In the presence of 5 mM MgCl2 and 5 mM ATP, the addition of low amounts of RecUE36A clearly inhibited RecG binding and unwinding activities with HJ-J3 DNA (Fig. 8A). The same results were observed when the mobile HJ-jbm6 DNA was used (data not shown). To test whether a putative RecU/HJ-J3/RecG complex can be formed in the absence of branch migration, EMSA experiments in the presence of 5 mM EDTA were performed. The preformed RecG/HJ DNA complexes were challenged with two different RecU concentrations (Fig. 8B). The RecG/HJ-J3 DNA complex seemed to be efficiently dislodged to form RecU/HJ-J3 DNA complexes, even at protein ratios as low as 1 RecUE36A dimer against 24 RecG monomers. The same results were observed when complexes were fixed with glutaraldehyde (0.1%) before PAGE (data not shown).
RecG Forms Unstable Complexes with HJ DNA—We hypothesized that RecG may form unstable complexes with HJ-J3 DNA so that even at low RecU:RecG ratios only RecU-HJ-J3 DNA complexes were detected. To test this hypothesis, we analyzed the dissociation of RecG and RecUE36A from HJ-J3 in the presence of 1 mM ATP and 5 mM MgCl2, and 5 mM ATP. Then the indicated amounts of RecG were added, and reactions were continued for 20 min at 37 °C. After incubation, loading buffer was added. Samples separated on 6% PAGE allowed the observation of both, the dissociation products of RecG and RecUE36A-HJ-J3 complexes. The amounts of UD and GD complexes formed and unwound products are indicated as percentages with respect of total DNA and are the average values obtained from >3 independent experiments (the results given stand within a 5% S.E.).

**DISCUSSION**

The results presented in this paper show that a RecU-RecG-HJ complex is not observed and that RecU inhibits RecG catalyzed branch migration on HJ DNA. In contrast, RecU recruits and tethers RuvB toward the HJ central region, and a quaternary RecU-HJ-RuvA-RuvB complex might be formed. However, the RuvAB complex does not stimulate RecU-mediated HJ cleavage, and branch migration mediated by RuvAB is also not stimulated by the presence of RecU.

Proper resolution of HJ DNA requires the cleavage of both DNA strands symmetrically. RecU binding and distortion of the HJ is necessary but not sufficient for symmetric cleavage of both strands (23, 36) when the junction contains the RecU recognition sequence at only one arm. The recruitment of RuvB facilitates a distortion in the junction core so that symmetric HJ cleavage is enhanced, albeit with decreased sequence specificity at the second cleavage site (Fig. 4A). In the presence of ATP, such a stimulatory effect is not observed (Fig. 4B). This result is similar to the one observed previously with RuvCEco and RuvBeco; stimulation required the presence of ATPγS, and less enhanced cleavage was observed when ATPγS was replaced by ATP (44). We show here that in the absence of ATP hydrolysis, a RuvB-RecU interaction is stable and can be detected by EMSA and be observed by AFM (Figs. 1 and 2). The complexes visualized by AFM show that similarly to RuvAeco, RecU is able to recruit RuvB to the center of the HJ. A RecU dimer recruits one or two RuvB hexamers to the center of the junction (Fig. 2). Furthermore, RecU cannot stimulate the ATPas or branch migration activity of RuvB, and for these activities RuvA is required. Conversely, the RuvBeco-RuvCEco complex can promote branch migration (44), although the physical interaction of RuvCEco with RuvBeco was neither detected by EMSA nor by electron microscopy (51).
RecU Discriminates between HJ Translocases

Previously, a RuvA<sub>Eco</sub>-RuvC<sub>Eco</sub>-HJ complex was detected by EMSA (52), and a RuvAB<sub>Eco</sub>-HJ complex was observed by co-immunoprecipitation (53). Our results show that RuvA<sub>RecU</sub>-HJ and RuvAB<sub>RecU</sub>-HJ DNA complexes may also exist in <i>B. subtilis</i>. The identification of these complexes is significant, because RecU does not share any structural nor sequence similarity with RuvC and suggest that the transition between RuvAB-mediated branch migration and resolvase-mediated cleavage might be conserved. The presence of RuvA inhibits the resolution mediated by the RecU-RuvB-ATP<sub>S</sub> complex (Fig. 4), and high RecU concentrations slightly inhibit RuvAB-mediated branch migration on HJ DNA (Fig. 5). These observations conserved among species of the same phylum? Mycoplasma pneumoniae, which belongs to the same phylum as <i>B. subtilis</i>, is unable to express a functional RecU HJ resolvase, RuvA<sub>Mpn</sub>, displayed similar affinities for both HJs and single-stranded DNA, and RuvA<sub>Mpn</sub> did not stimulate the RuvB<sub>Mpn</sub> activity (47, 54). In contrast, Mycoplasma genitalium RuvA bound to HJs, stimulated the DNA helicase and ATPase activities of RuvB, and did not compete RecU HJ binding (47). In the absence of RuvA, RuvB<sub>Mge</sub> but not RuvB<sub>Mpn</sub> showed helicase activity (55). It is likely, therefore, that although sharing similar proteins, the process of HJ resolution by the RuvAB-RecU machinery could act differently in the different bacteria and requires further analysis.

The results presented here do not explain in which circumstances RecU might work in concert with RecG and not with RuvAB as the genetic data suggest (24, 25). It is worth mentioning that RuvC<sub>Eco</sub> may be also able to perform some activity without RuvAB<sub>Eco</sub> and participate with recG<sub>Eco</sub> in some repair pathways (56), which suggests that this versatility in the use of branch migration translocases together with HJ resolving enzymes seems to occur also in other bacteria. From previous results and the ones obtained here, we hypothesize that in <i>B. subtilis</i> the RecU protein could have an important role in discriminating which recombination protein acts at the late stages of the recombination reaction. RecU acts at the synaptic and post-synaptic stages of homologous recombination. The interaction of RecU with RecA might inhibit RecA-mediated fork regression and strand exchange and might facilitate RecG- or RuvAB-mediated fork regression and/or HJ translocation (30, 37, 57). RecU bound to the formed HJ could inhibit RecG-mediated branch migration and loading (Fig. 8), whereas the interaction of RecU with RuvB could facilitate the loading of the RuvAB complex at the recombination intermediate before the RuvA protein is bound to the HJ, with at least a transient formation of a quaternary RecU-HJ-RuvA-RuvB complex. Then, the RuvAB complex could facilitate branch migration of the HJ until the RecU cognate site is positioned at the junction point. Finally, RuvB, as part of a transient RuvB-RecU complex, could facilitate RecU-mediated symmetric HJ cleavage by modulating the HJ structure by an unknown manner.

The interaction of RecU with RuvB, but not with RecG, could help in the discrimination between RuvAB and RecG at HJs. We
propose that RecG would be devoted to fork regression and also fork rewinding, as observed for RecGEco (22, 58, 59), whereas RuvAB should be mainly involved in HJ translocation. This is consistent with the observation that RuvABEco branch migration of the HJ is faster than its migration by RecAEco-mediated strand exchange (60). RuvAB should have also an unknown activity unrelated with RecU or vice versa because a ΔruvAB ΔrecU strain is synthetically lethal (24). Alternatively, in the absence of both RuvAB and RecU, RecG or another unknown protein might accumulate toxic recombination intermediates.

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