The RuvA Homologues from *Mycoplasma genitalium* and *Mycoplasma pneumoniae* Exhibit Unique Functional Characteristics

Marcel Sluijter, Silvia Estevão, Theo Hoogenboezem, Nico G. Hartwig, Annemarie M. C. van Rossum, Cornelis Vink

Laboratory of Pediatrics, Pediatric Infectious Diseases and Immunity, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

**Abstract**

The DNA recombination and repair machineries of *Mycoplasma genitalium* and *Mycoplasma pneumoniae* differ considerably from those of gram-positive and gram-negative bacteria. Most notably, *M. pneumoniae* is unable to express a functional RecU Holliday junction (HJ) resolvase. In addition, the RuvB homologues from both *M. pneumoniae* and *M. genitalium* only exhibit DNA helicase activity but not HJ branch migration activity in vitro. To identify a putative role of the RuvA homologues of these mycoplasmas in DNA recombination, both proteins (RuvA<sub>Mge</sub> and RuvA<sub>Mpn</sub> respectively) were studied for their ability to bind DNA and to interact with RuvB and RecU. In spite of a high level of sequence conservation between RuvA<sub>Mge</sub> and RuvA<sub>Mpn</sub> (68.8% identity), substantial differences were found between these proteins in their activities. First, RuvA<sub>Mge</sub> was found to preferentially bind to HJs, whereas RuvA<sub>Mpn</sub> displayed similar affinities for both HJs and single-stranded DNA. Second, while RuvA<sub>Mge</sub> is able to form two distinct complexes with HJs, RuvA<sub>Mpn</sub> only produced a single HJ complex. Third, RuvA<sub>Mge</sub> stimulated the DNA helicase and ATPase activities of RuvB<sub>Mge</sub> whereas RuvA<sub>Mpn</sub> did not augment RuvB activity. Finally, while both RuvA<sub>Mge</sub> and RecU<sub>Mge</sub> efficiently bind to HJs, they did not compete with each other for HJ binding, but formed stable complexes with HJs over a wide protein concentration range. This interaction, however, resulted in inhibition of the HJ resolution activity of RecU<sub>Mge</sub>.

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* E-mail: c.vink@erasmusmc.nl

**Introduction**

A significant proportion of the genomes of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* (approximately 8% and 4%, respectively) is composed of repeated DNA elements. These elements are referred to as RepMP elements in *M. pneumoniae* [1,2,3] and MgPa repeats (MgPar) in *M. genitalium* [4,5,6]. Although the different variants of these elements show a high level of sequence homology, they are not identical. Moreover, one or more of these variants are contained within open reading frames (ORFs) that encode antigenic surface proteins. Among these proteins are P1, P40 and P90 of *M. pneumoniae* and MgPa and P110 of *M. genitalium*. As these proteins can display amino acid sequence variation within the regions encoded by the RepMP and MgPar sequences, it has been proposed that this variation originates from recombination between different variants of RepMP or MgPar [7,8,9,10,11,12,13]. Consequently, homologous recombination between the repeated DNA elements in both *Mollicutes* species may play a crucial role in immune evasion [14].

It has previously been suggested that the mechanism of recombination between repeated DNA elements in *M. pneumoniae* and *M. genitalium* is similar to that of general homologous DNA recombination in these species [13,16]. As a consequence, these processes may utilize the same enzymatic machinery. Recent studies that were aimed at elucidation of the mechanism of recombination between repeated DNA elements therefore focused on the characterization of *Mycoplasma* proteins predicted to be involved in homologous DNA recombination, such as RecA [15], single-stranded DNA-binding protein (SSB) [16], RuvA [17], RuvB [18] and RecU [19,20]. The RecA proteins from *M. pneumoniae* and *M. genitalium* (RecA<sub>Mge</sub> and RecA<sub>Mpn</sub> respectively) were reported to possess similar activities as their counterparts from other bacterial classes. Specifically, the RecA proteins from *M. pneumoniae* and *M. genitalium* (RecA<sub>Mge</sub> and RecA<sub>Mpn</sub>, respectively) were studied for their ability to bind DNA and to interact with RecU. In spite of a high level of sequence conservation between RecA<sub>Mge</sub> and RecA<sub>Mpn</sub> (83.2% identity), substantial differences were found between these proteins in their activities. First, RecA<sub>Mge</sub> was found to preferentially bind to HJs, whereas RecA<sub>Mpn</sub> displayed similar affinities for both HJs and single-stranded DNA. Second, while RecA<sub>Mge</sub> is able to form two distinct complexes with HJs, RecA<sub>Mpn</sub> only produced a single HJ complex. Third, RecA<sub>Mge</sub> stimulated the DNA helicase and ATPase activities of RecU<sub>Mge</sub> whereas RecA<sub>Mpn</sub> did not augment RuvB activity. Finally, while both RecA<sub>Mge</sub> and RecU<sub>Mge</sub> efficiently bind to HJs, they did not compete with each other for HJ binding, but formed stable complexes with HJs over a wide protein concentration range. This interaction, however, resulted in inhibition of the HJ resolution activity of RecU<sub>Mge</sub>.
possess Mg^{2+}-dependent resolvase activity. Second, RecU_{Mge} has a unique target DNA sequence, cleaving HJ substrates at the sequence 5'-GAGCAACC-3' [28]. This cleavage site differs from the cleavage sites of RecU_{Mpn} and RecU_{Mge} (5'-A/G/C-3', 5'-A/G/TG-3', and 5'-CC-3', respectively) [21,22,23,24,25]. Third, unlike the RecU_{Mpn} protein [21], RecU_{Mge} is unable to anneal circular ssDNA to homologous, linear double-stranded DNA (dsDNA). Fourth, RecU_{Mge} does not stably bind to long ssDNA substrates, in contrast to the RecU_{Mpn} protein [21].

Another crucial finding regarding the RecU orthologues from M. pneumoniae and M. genitalium was the inability of M. pneumoniae to produce a functional RecU protein [19,20]. While a subset of M. pneumoniae strains (so-called subtype 2 strains) is able to express and produce a functional RecU protein [19,20].

The activity of RuvB_{Eco} and RuvB_{Mge} were used throughout this study. The estimated molecular masses of the purified proteins matched the theoretical molecular masses of 23.7 kDa for both RuvA_{Mge} (lane 3).

RuvA_{Mge} and RuvA_{Mpn} can bind to synthetic oligonucleotide substrates

Both RuvA_{Mge} and RuvA_{Mpn} were expressed in E. coli as poly histidine (His10)-tagged proteins and were purified to near homogeneity using similar protocols (as described in Materials and Methods). The His10-tagged proteins were found to have activities that were indistinguishable from that of their non-tagged counterparts (data not shown). Because the His10-tagged proteins were obtained at higher concentrations and at a higher purity than their ‘native’ versions (>95% versus ~90% homogeneity), they were used throughout this study. The estimated molecular masses of the purified proteins matched the theoretical molecular masses of 23.7 kDa for both RuvA_{Mge} and RuvA_{Mpn} (Fig. 1B, lane 2) and RuvA_{Mpn} (lane 3).

To test and compare the DNA-binding characteristics of RuvA_{Mge} and RuvA_{Mpn}, both proteins were incubated with HJs, double-stranded (ds) and single-stranded (ss) oligonucleotide substrates, and analyzed by electrophoretic mobility shift assay (EMSA). As described before [17], two distinct complexes (complex I and complex II) were formed between RuvA_{Mge} and HJs in a protein-concentration dependent fashion (Fig. 2A). Similar complexes were reported to be generated between RuvA_{Mpn} and HJs, and were found to consist of a single protein tetramer (complex I) or a double tetramer (complex II) bound to a HJ [30,31,32,33,34,35]. The HJ binding activity of both RuvA_{Mge} and RuvA_{Mpn} was strongly reduced in the presence of Mg^{2+} (compare Fig. 2A to Fig. 2B, and Fig. 2C to Fig. 2D). A similar inhibitory effect of Mg^{2+} on DNA-binding activity has previously also been observed for RuvA_{Mge} [31,36]. In contrast to RuvA_{Mge} and RuvA_{Mpn}, RuvA_{Mpn} produced only a single complex with HJs (Fig. 2C, lane 6), even at protein concentrations up to 4 µM (see below). This complex migrated through the gels with a mobility similar to that of RuvA_{Mge}-HJ complex I. These data indicated that: (i) the RuvA_{Mge}-HJ complex is composed of a tetramer of RuvA_{Mge} bound to a HJ, and (ii) RuvA_{Mge} may not stably bind to HJs as an octamer. These notions were supported by gel filtration chromatography data, which indicated that RuvA_{Mge} exists as a single, major protein species with a molecular mass of ~108 kDa (Fig. S1). This molecular mass corresponds to the theoretical molecular mass of a tetramer of RuvA_{Mge} (95 kDa).

In contrast to RuvA_{Mge}, RuvA_{Mpn} was previously reported to form stable complexes with linear duplex oligonucleotides [17]. As shown in Fig. 2E and 2F, both RuvA_{Mge} and RuvA_{Mpn} are able to form DNA-protein complexes in the presence of ds oligonucleotides (substrate HJ11/HJ11rv). Interestingly, at least part of these complexes consisted of RuvA molecules bound to non-annealed, ss oligonucleotide HJ11, which was present as a minor ‘contaminant’
of the ds substrate; this oligonucleotide (designated ‘Free ss’ in Fig. 2E and 2F) was completely complexed by the RuvA proteins at the highest protein concentrations tested (Fig. 2E, lane 4–6 and Fig. 2F, lane 6). In a separate EMSA, we could confirm the binding of RuvA<sub>Mpn</sub> to oligonucleotide HJ11; this binding appeared to occur with an efficiency similar to that observed with the four-stranded HJ substrate (compare Fig. 2G to Fig. 2A). Conversely, while the RuvA<sub>Mge</sub> protein also displayed binding to the ssDNA (Fig. 2H), this binding was considerably less efficient than that observed with the HJ substrate (Fig. 2C).

The preferences of RuvA<sub>Mpn</sub> and RuvA<sub>Mge</sub> for binding to either ssDNA or HJ DNA were further investigated in DNA-binding competition experiments, in which a labeled DNA substrate was kept at a constant concentration and another, unlabeled substrate was included at different concentrations. As shown in Fig. 3A, the binding of RuvA<sub>Mge</sub> to the labeled HJ substrate was not significantly influenced by inclusion of up to a 20-fold excess of unlabeled ssDNA in the reaction (lanes 3–6). In contrast, the binding of RuvA<sub>Mpn</sub> to the HJ substrate was already clearly reduced in the presence of a 2.5-fold excess of unlabeled ssDNA in the binding reactions (Fig. 3B, lane 3). Although the dsDNA substrate also competed with the HJ substrate for binding by RuvA<sub>Mpn</sub>, this competition was less efficient than that observed with ssDNA (Fig. 3C). The high affinity of RuvA<sub>Mpn</sub> for ssDNA was further demonstrated in an experiment in which the binding of RuvA<sub>Mpn</sub> to labeled ssDNA was assayed in the presence of different concentrations of unlabeled HJ substrate. As shown in Fig. 3D, the ssDNA-binding of RuvA<sub>Mpn</sub> was only marginally

**Figure 1.** Multiple alignment and purification of RuvA<sub>Mpn</sub> and RuvA<sub>Mge</sub> (A) An alignment was generated with amino acid sequences predicted to be encoded by the following ORFs (with GenBank accession numbers in parentheses), M. pneumoniae MPN535 (P75243), M. genitalium G37 MG358 (Q49424), Streptococcus pneumoniae ruvA (Q975Y4), Staphylococcus aureus ruvA (Q5HFC1) and E. coli ruvA (P0A809). Predicted secondary structural features and domains of the RuvA proteins are shown below the alignment and are based on the crystal structure of the RuvA protein from E. coli [27,28,30,33,54]. The position of the ‘acidic pin’, between β sheets 6 and 7 of RuvA<sub>Eco</sub>, two helix-hairpin-helix (HhH) motifs, and the flexible linker (between domain II and III), are also indicated. The multiple alignment was performed using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The program BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html) was used to generate white letters on black boxes (for residues that are identical in at least three out of five sequences) and white letters on grey boxes (for similar residues). (B) Purification of RuvA<sub>Mge</sub> and RuvA<sub>Mpn</sub>. Samples of purified H10-tagged RuvA<sub>Mge</sub> (lane 2) and H10-tagged RuvA<sub>Mpn</sub> (lane 3) were analyzed by SDS-PAGE (12%) and Coomassie brilliant blue (CBB)-staining. The sizes of protein markers (lane 1; PageRuler<sup>TM</sup> Prestained Protein Ladder [Fermentas]) are shown on the left-hand side of the figure in kDa.

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Figure 2. Binding of RuvA<sub>Mpn</sub> and RuvA<sub>Mge</sub> to HJs and other oligonucleotide substrates. (A) Binding of RuvA<sub>Mpn</sub> to HJ substrate HJ 1.1 in the absence of Mg<sup>2+</sup>. The DNA-binding reactions were performed as indicated in Materials and Methods. Reactions were performed in volumes of 10 µl and contained 12.3 nM DNA substrate and either 0 nM (marked ‘-', lane 1), 27 nM (lane 2), 81 nM (lane 3), 243 nM (lane 4), 729 nM (lane 5) or...
reduced in the presence of a 10-fold (lane 5) or 20-fold (lane 6) molar excess of unlabeled HJ DNA in the reactions. Thus, in contrast to RuvA

Mge

(Fig. 2H and 3A), RuvA

Mpn

is able to bind with a relatively high affinity to ssDNA.

The interaction between RuvA

Mge

and RecU

Mge

on HJs

The RecU protein from M. pneumoniae (RecU

Mpn

) was previously found to be inactive in HJ-binding and cleavage [19]. In contrast, the M. genitalium RecU protein (RecU

Mge

) was reported to be a potent HJ-resolving enzyme [19,20]. Because it is possible that RecU

Mge

functionally interacts with RuvA

Mge

in the processing of HJs, both proteins were included in HJ binding and resolution assays. The binding of RecU

Mge

to HJ substrate HJ 1.1 was previously demonstrated to result in a single DNA-protein complex [19,20]. Interestingly, at relatively high RecU

Mge

concentrations and at different binding conditions than those used previously (i.e., binding on ice instead of at room temperature and in the absence of BSA), a range of discrete RecU

Mge
-HJ DNA-protein complexes were generated, with an inverse correlation between protein concentration and mobility of the complexes through EMSA gels (Fig. 4A, lanes 2–4). At 500 nM of RecU

Mge

three major DNA-protein complexes and one minor complex can

Figure 3. DNA binding preferences of RuvA

Mge

and RuvA

Mpn

(A) Binding of RuvA

Mge

(3 μM) to HJ substrate HJ 1.1 (6-FAM-labeled on strand HJ11) in the presence of various concentrations of unlabeled ssDNA (oligonucleotide HJ11). The molar excess of unlabeled DNA over labeled DNA in the reactions was 0 × (lane 2), 2.5 × (lane 3), 5 × (lane 4), 10 × (lane 5) and 20 × (lane 6). The protein was added as final component in the reactions. Protein was omitted from the reaction shown in lane 1. The positions of the free HJ substrate (Free HJ) and RuvA

Mge
-HJ complexes (Complex) are indicated at the right-hand side of the gel. (B) Binding of RuvA

Mpn

(3 μM) to HJ substrate HJ 1.1 (6-FAM-labeled on strand HJ11) in the presence of various concentrations of unlabeled ssDNA (oligonucleotide HJ11). The experiment was performed similarly as in (A). The two major RuvA

Mpn
-HJ complexes (Complex I and II) are indicated at the right-hand side of the gel. (C) Binding of RuvA

Mpn

(3 μM) to HJ substrate HJ 1.1 (6-FAM-labeled on strand HJ11) in the presence of various concentrations of unlabeled dsDNA (oligonucleotide HJ11/HJ11rv). The experiment was performed similarly as in (A). The two major RuvA

Mpn-HJ complexes (Complex I and II) are indicated at the right-hand side of the gel. (D) Binding of RuvA

Mpn

(3 μM) to ssDNA (6-FAM-labeled oligonucleotide HJ11) in the presence of various concentrations of HJ DNA (HJ 1.1). The experiment was performed similarly as in (A). Protein was omitted from the reaction shown in lane 1. The positions of the unbound ssDNA (Free ssDNA) and RuvA

Mpn-ssDNA complex (Complex) are indicated at the right-hand side of the gel.

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be discerned (lane 4). A similar range of complexes was previously also observed after binding of *E. coli* resolvase RuvA to HJ substrates [25]. Due to the distinct nature of the RecU_Mge-HJ complexes and their relative migration in the gel, we hypothesize that they represent different multimeric forms of RecU_Mge bound to a single HJ substrate. Upon addition of RuvA_Mge to these complexes (after preincubation of RecU_Mge with the HJ substrate), novel complexes were formed with a considerably slower mobility than the RecU_Mge-HJ complexes (Fig. 4B, lanes 3–7). At the highest concentration of RuvA_Mge used (4 μM), all RecU_Mge-HJ complexes appeared to have shifted to a higher position in the gel (lane 7). Because the novel complexes had a slower mobility than the RuvA_Mge-HJ complex (Fig. 4B, lane 8), it is likely that they represent HJs bound by both RecU_Mge and RuvA_Mge. This notion was corroborated by a reciprocal experiment in which the HJ substrate was preincubated with RuvA_Mge (at 4 μM), followed by the addition of RecU_Mge at concentrations ranging from 0 nM to 500 nM (Fig. 4C, lanes 2–7). Already at a RecU_Mge concentration of 31 nM (lane 3), a ‘supershift’ of the RuvA_Mge-HJ complex was observed; this supershift was virtually complete at a RecU_Mge concentration of 250 nM (lane 6). At the latter concentration, a single major supershifted complex was observed. At 500 nM of RecU_Mge, however, four discrete supershifted complexes were formed, which corresponded in mobility with the complexes generated in the previous experiment (Fig. 4B, lane 7). Again, the supershifted complexes displayed a slower mobility than did the RecU_Mge-HJ and RuvA_Mge-HJ complexes (Fig. 4C, lanes 2 and 8), indicating that they indeed represent RecU_Mge•RuvA_Mge-HJ complexes. The interactions between RecU_Mge and RuvA_Mge on HJ substrates differ significantly from those reported between RuvA_Eco and the RuvC resolvase from *E. coli* (RuvC_Eco). Specifically, RuvA_Eco appears to have a significantly higher affinity than RuvC_Eco for HJ substrates, and a fully saturated RuvA_Eco-HJ complex (complex II) cannot be obtained detectably by RuvC_Eco [32]. As a consequence, RuvAC_Eco-HJ complexes are only observed at relatively low RuvA_Eco concentrations (1–20 nM); at higher RuvA_Eco concentrations, RuvAC_Eco-HJ and RuvC_Eco-HJ complexes are either not formed or rapidly dissociated [32]. In contrast, RecU_Mge and RuvA_Mge do not appear to compete with each other in HJ binding, but rather associate readily and stably on a HJ substrate at a wide range of concentrations of both RuvA_Mge (Fig. 4B) and RecU_Mge (Fig. 4C). As yet, the multimeric protein composition of the different RecU_Mge•RuvA_Mge-HJ complexes is unknown. Nevertheless, while a single stable complex is generated between RuvA_Mge and HJs, it is likely that each of the RecU_Mge•RuvA_Mge-HJ complexes only contains a single tetramer of RuvA_Mge.

RuvA_Mge inhibits HJ resolution by RecU_Mge

Because RuvA_Mge readily binds to RecU_Mge-HJ complexes, we investigated the influence of RuvA_Mge on the activity of RecU_Mge in HJ resolution assays. In these assays, substrate HJ 1.1 was preincubated on ice with either RecU_Mge (at 0.2 μM; Fig. 5A) or RuvA_Mge (at 0 to 4 μM; Fig. 5B), followed by the addition of the other protein. After incubation for 30 min at 37°C, the resolution products were analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 5A and 5B, RuvA_Mge inhibited the resolution activity of RecU_Mge in a RuvA_Mge concentration-dependent fashion. The inhibition of HJ resolution was most effective when RuvA_Mge was added to the HJ substrate before RecU_Mge (Fig. 5B and 5C). In that case, HJ resolution by RecU_Mge was already inhibited by ~20% at a RuvA_Mge concentration of 60 nM (Fig. 5B, lane 3 and Fig. 5C). At RuvA_Mge concentrations of 1 μM or higher, RecU_Mge activity was reduced by ~80% (Fig. 5B, lanes 7–9). When the HJ substrate was incubated with RecU_Mge before the addition of RuvA_Mge, a significant inhibition of HJ resolution activity (≥20%) was only observed at RuvA_Mge concentrations of ~250 nM (Fig. 5A and 5C). Moreover, inhibition levels of >80% were not observed at RuvA_Mge concentrations lower than 4 μM. When RecU_Mge and RuvA_Mge were added simultaneously to the HJ substrates, a similar pattern of HJ resolution was observed as that shown in Fig. 5A (in which RecU_Mge was added to the reactions before RuvA_Mge). This finding corroborates the notion that RecU_Mge-HJ complexes cannot be dissociated by RuvA_Mge. Despite the significantly higher activities in the dynamics of the RecU_Mge•RuvA_Mge-HJ complexes and RuvAC_Eco-HJ complexes, RuvA_Mge inhibits the resolution activity of RecU_Mge in a similar fashion as RuvA_Eco, inhibits RuvC_Eco activity.

The influence of the RuvA proteins on the activities of RuvB_Mge and RuvB_FH

The RuvB protein that is expressed by *M. pneumoniae* subtype 2 strains, RuvB_Mge, was recently reported to act as a DNA helicase on specific, partially double-stranded DNA substrates [18]. Interestingly, while this activity of RuvB_Mge was not influenced by RuvA_Mge, the RuvB protein from *M. genitalium*, RuvB_Mge, did show RuvA_Mge-dependent helicase activity. The latter activity, however, was only detected on a single helicase substrate, i.e. Substrate IV from Fig. 6A [18]. To further delineate the functional interactions between the RuvA and RuvB proteins from *M. pneumoniae* and *M. genitalium*, the proteins were combined at various concentrations (including considerably higher RuvA concentrations than used previously) in DNA helicase or branch migration assays, using the DNA helicase substrates shown in Fig. 6A. While the helicase activity of RuvB_Mge was not influenced by RuvA_Mge (data not shown), the helicase activity of RuvB_Mge on Substrate II (Fig. 6B and 6C) and Substrate I (Fig. 6D) was stimulated in the presence of high concentrations of RuvA_Mge. As expected, RuvA_Mge alone did not display any DNA helicase activity (lane 7 in Fig. 6C and 6D). This stimulatory effect of RuvA_Mge was observed at various concentrations of RuvB_Mge from 0.9 μM (Fig. 6B) to 2.7 μM (Fig. 6C). These results indicated that the activation of RuvB_Mge by RuvA_Mge is a general phenomenon that is not restricted to a specific DNA substrate. Nevertheless, irrespective of the presence of high concentrations of the RuvA proteins, both RuvB_Mge and RuvB_Mge were unable to unwind small, double-stranded oligonucleotide substrates (data not shown).

The ATPase activity of RuvB_Mge is stimulated by RuvA_Mge

While RuvB_Mge and RuvB_FH were previously found to possess intrinsic ATPase activity, this activity was significantly higher for RuvB_FH than for RuvB_Mge [18]. To investigate whether the ATPase activities of the RuvB proteins can be modulated by their corresponding RuvA proteins, ATPase assays were carried out in which the RuvA and RuvB proteins were tested together. In accordance with previous findings [18], RuvB_FH was found to possess a significantly higher ATPase activity than RuvB_Mge (Fig. 7). However, while the activity of RuvB_Mge was not significantly influenced by RuvA_Mge, the activity of RuvB_Mge was strongly stimulated by RuvA_Mge. Thus, the ATPase activities of RuvB_Mge and RuvB_Mge directly reflect the DNA helicase activities of these proteins in two important aspects. First, the intrinsic enzymatic activity of RuvB_Mge is higher than that of RuvB_Mge. Second, RuvB_Mge activity can be stimulated by RuvA_Mge whereas RuvB_FH activity is not influenced by RuvA_Mge. As expected, both RuvA_Mge and RuvA_Mge did not show any ATPase activity on their own (Fig. 7).
above the lanes). The nature of the various protein-DNA complexes is indicated at the right-hand side of the gel; RuvA of RecU a similar fashion as described in Fig. 3. Reactions were performed in volumes of 10 μl and contained 12.3 nM HJ 1.1 and the indicated concentrations of RecU<sub>Mge</sub>. The positions of unbound HJs (HJ) and RecU<sub>Mge</sub>-HJ complexes are depicted at the right-hand side of the gel. (B) The binding of RuvA<sub>Mge</sub> to RecU<sub>Mge</sub>-HJ complexes. RuvA<sub>Mge</sub> (0.5 μM) was incubated with HJ 1.1, followed by the addition of RuvA<sub>Mge</sub> (at different concentrations, as indicated above the lanes). The nature of the various protein-DNA complexes is indicated at the right-hand side of the gel; RuvA<sub>Mge</sub>-HJ complexes are indicated with a dot (●). (C) The binding of RecU<sub>Mge</sub> to RuvA<sub>Mge</sub>-HJ complexes. RuvA<sub>Mge</sub> (4 μM) was incubated with HJ 1.1, followed by the addition of RecU<sub>Mge</sub> (at various concentrations, as indicated above the lanes). The labeling of the figure is similar to that shown in (B).

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Discussion

The DNA recombination and repair machineries of mycoplasmas differ considerably from those of gram-positive and gram-negative bacteria. Most importantly, in contrast to the latter micro-organisms, mycoplasmas do not possess homologues of LexA, RecBCD, AddAB, RecQ, RecF and RecF [37,38]. In addition, some components of the putative DNA recombination machineries of M. pneumoniae and M. genitalium were found to have characteristics that diverge from those of their homologues from other bacterial classes. These components include the RecU and RuvB proteins [18,19,20]. In Table 1, the characteristics of these as well as the other (putative) components of the DNA recombination machineries of M. pneumoniae and M. genitalium are listed and compared.

We here report that the RuvA proteins from both Mycoplasma spp., RuvA<sub>Mge</sub> and RuvA<sub>Mge</sub> also possess exceptional properties as opposed to their well-characterized counterpart from E. coli, RuvA<sub>Eco</sub>. While both RuvA<sub>Mge</sub> and RuvA<sub>Eco</sub> [31,39] preferentially bind to HJs, RuvA<sub>Mge</sub> displayed a high affinity for both HJ and ssDNA. In addition, while RuvA<sub>Mge</sub> and RuvA<sub>Eco</sub> are both able to form two distinct complexes with HJ substrates, RuvA<sub>Mge</sub> only formed a single complex with HJs. As this RuvA<sub>Mge</sub>-HJ complex had a similar mobility through polyacrylamide gels as RuvA<sub>Eco</sub>-HJ complex I and RuvA<sub>Eco</sub>-HJ complex I [17,30,31,32,33,34,35], and because RuvA<sub>Mge</sub> is a tetramer in solution, it is highly likely that this complex is composed of a tetramer of RuvA<sub>Mge</sub> bound to a single HJ. This implies that RuvA<sub>Mge</sub> may only stably bind to HJs as a tetramer. This notion can have important consequences for the interaction of the RuvA<sub>Mge</sub>-HJ complex with other proteins that are potentially targeted to HJs, such as RuvB<sub>Mge</sub> and RecU<sub>Mge</sub>. It was previously reported that the ability of RuvA<sub>Eco</sub> to form stable octamers on HJs was vital for full activity of the protein. This notion was inferred from the activities of four different octamerization-deficient RuvA<sub>Eco</sub> mutants [34,35,40]. Three of these mutants carried amino acid substitutions in a protein region known to be involved in tetramer-tetramer interactions [34,35,40]. This region was identified within the crystal structure of HJ-bound of the Mycobacterium leprae RuvA protein (RuvA<sub>Mle</sub>) [33]. Within this structure, the two RuvA tetramers make direct protein-protein contacts through specific amino acid side chain interactions at four equivalent points, which are localized to the 96 helix of domain II (Fig. 1A). The interacting 96 helices from two RuvA monomers are in an antiparallel configuration, such that ion pair interactions are formed between three pairs of amino acid residues. On the basis of sequence alignments, we predict that only two of such pairs may be formed between two antiparallel 96 helices of both RuvA<sub>Mge</sub> and RuvA<sub>Mge</sub>. In RuvA<sub>Mge</sub> these pairs would consist of Lys121-Asp133 and Arg124-Glu130, whereas in RuvA<sub>Mge</sub> they would consist of Lys121-Glu133 and Arg124-Glu130. While this prediction emphasizes the sequence similarity between RuvA<sub>Mge</sub> and RuvA<sub>Mge</sub> it does not provide an explanation why RuvA<sub>Mge</sub> is able to form stable octameric complexes with HJs, and RuvA<sub>Mge</sub> is not. It should be considered,
however, that the octamerization signals of RuvA
Mpn (which are absent from RuvA
Mge) may differ considerably from those of RuvA
Mle, and are not (solely) determined by contacts between
amino acid residues located in the a6 helix. In this regard, it is
relevant to note that one of the reported RuvA
Eco mutants that is unable to form stable octamers on HJs, RuvAz87, does not carry
mutations in helix a6, but in two other regions of the protein, i.e. in
the region between helices a2 and a3 and in helix a4 [40].

Despite its inability to octamerize on HJs in a stable fashion, RuvA
Mge was found to stimulate the DNA helicase and ATPase
activities of RuvB
Mge. The octamerization-competent RuvA
Mpn protein, however, did not augment RuvB FH activity. It is possible
that the relatively high intrinsic DNA helicase activity of RuvB FH
obscured the observation of any additional stimulatory effect on
this protein by RuvA
Mpn. An alternative explanation for the
inability of RuvA
Mpn to boost RuvBFH activity is that these proteins
are unable to physically interact. In agreement with this notion, we
have not yet been able to detect direct or indirect interactions
between these proteins in DNA-binding studies.

Another unique feature of RuvA
Mge is the mode in which this
protein forms tripartite complexes with HJ resolvase RecU
Mge and HJs. This is the first report to demonstrate an interaction between a member of the RecU protein family and a RuvA protein. RuvA
Mge and RecU
Mge were found to associate readily and stably
on HJ substrates at a broad protein concentration range. In contrast, tripartite complexes of RuvA
Eco, RuvC
Eco and HJs were only observed at relatively low concentrations of RuvA
Eco because the latter protein has a higher affinity than RuvC
Eco for HJ DNA [32]. At relatively high RuvA
Eco concentrations, the HJ DNA will be saturated with protein, such that two RuvA
Eco tetramers are bound to opposite faces of the junction. Thus, the binding of RuvC
Eco to the junction is excluded [32,33,41]. At low RuvA
Eco concentrations, however, the main protein-HJ complex that is formed is complex I, which consists of a single tetramer of RuvA
Eco bound to a single face of the junction. This structure may allow the binding of RuvC
Eco to the other face of the DNA substrate, thereby generating a tripartite RuvAC
Eco-HJ complex [32]. In analogy with this model, a tetramer of RuvA
Mge bound to one side of a HJ may permit the binding of (multimers of) RecU
Mge at the opposite side of the junction. Because RuvA
Mge is unable to
form stable octameric-HJ complexes, as discussed above, the
tetrameric RuvA
Mge-HJ complex may always be accessible, at one face of the junction, for binding by RecU
Mge. This may explain why RecU
Mge and RuvA
Mge do not compete with each other for
binding to HJs, but rather interact readily by forming a stable tripartite complex. This interaction does, however, lead to inhibition of the HJ resolution activity of RecU
Mge, a phenomenon that parallels the inhibition of RuvC
Eco-catalyzed HJ resolution by

Figure 5. RuvA
Mge inhibits HJ resolution by RecU
Mge. (A, B) HJ resolution assays [19] were performed in volumes of 10 μl and contained
12.3 nM HJ substrate HJ 1.1 (6-FAM-labeled), RecU
Mge (0.2 μM) and various concentrations of RuvA
Mge, as indicated above the lanes. Reactions were
preincubated for 2 min with either RecU
Mge (A) or RuvA
Mge (B), followed by addition of the other protein. After incubation for 30 min at 37°C, the
reaction products were separated on 12% polyacrylamide gels, and analyzed by fluorometry. The locations of the HJ substrate and resolution
products are indicated schematically at the right-hand side of the gels. (C) Quantification of the influence of RuvA
Mge on RecU
Mge activity. The relative
RecU
Mge (resolution) activity was measured from the gels shown in (A) and (B) and expressed as percentage of the protein’s activity in the absence of
RuvA
Mge. The data from (A) and (B) are represented by the closed squares (■) and the open squares (□), respectively.

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It remains to be determined whether the RecUMge-RuvA
Mge-HJ complexes are stabilized exclusively by protein-DNA
interactions or also by RecUMge-RuvA
Mge interactions; experiments
aimed at the detection of such protein-protein interactions have
hitherto not produced conclusive results. In addition, it is clear
that the physiological role will have to be established of the
RecUMge-RuvA
Mge interaction and the RuvA
Mge-mediated inhibi-
tion of the HJ resolution activity of RecUMge. Nevertheless, it is
likely that a functional coupling exists between these proteins and
that the combined activities of a complex of RuvB
Mge and RuvA
Mge may be linked to the resolvase activity of RecUMge. Such a situation
could be similar to that in E. coli, in which the RuvAB DNA
branch migration complex is coupled to the RuvC resolvase in a
RuvABC
Eco resolvasome complex. In this regard, it is also
interesting to note that a close association between RecUMge and
RuvA
Mge (plus RuvB
Mge) is also reflected in the genome of M.
genitalium, in which the ORF encoding RecUMge (MG352) is
localized in the vicinity of the ORFs encoding RuvA
Mge (MG358)
and RuvB
Mge (MG359).

Another issue that remains to be addressed is the nature of the
four different RecUMge-HJ complexes that were formed at
relatively high concentrations of RecUMge. In previous studies on
this protein, only a single RecUMge-HJ complex was observed due
to the use of different DNA binding conditions [19,20]. It was
shown by protein crystallography and structure determination that
the RecU homologues from Bacillus subtilis [42] and Bacillus
stearothermophilus [43] exist as dimers. Based on this information, we
speculate that the four RecUMge-HJ complexes that were observed
in this study consist of HJs bound by dimers, tetramers, hexamers
and octamers, respectively, of RecUMge. How the larger multimers
would be accommodated on a single HJ, and how these would also
leave room for binding of a RuvA
Mge tetramer, which was
observed for each of the four RecUMge-HJ complexes, are
challenging questions. The formation of large assemblies of
proteins bound to a junction, however, is not unprecedented, as
RuvA
Eco mutant RuvA3m was reported to generate HJ-protein
complexes consisting of six protein tetramers [35].

In conclusion, the studies of the RuvA, RuvB and RecU
homologues from mycoplasmas have revealed that these proteins
each have distinctive properties as opposed to their counterparts
from other bacterial classes. It is possible that these unique features
have emerged as a consequence of the evolutionary reduction that
the genomes of the mycoplasmas are believed to have undergone.
Specically, the loss of a significant portion of an ancestral set of

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**Figure 6. The influence of RuvA
Mge on the DNA helicase activity of RuvB
Mge** (A) Schematic illustrations of the DNA substrates used in the DNA helicase assays. The substrates are composed of a combination of oligonucleotides (oligonucleotide 1, oligonucleotide 2 or oligonucleotide 2/1) and single-stranded, circular 5,386-bp φX174 DNA, as described previously [18]. (B, C) RuvA
Mge stimulates the DNA helicase activity of RuvB
Mge on Substrate II, 6-FAM-labeled at the 5′ end of oligonucleotide 1, was incubated with either 0 μM, 0.9 μM (B) or 2.7 μM (C) of RuvB
Mge in the presence of various concentrations of RuvA
Mge as indicated above the lanes. (D) RuvA
Mge stimulates the DNA helicase activity of RuvB
Mge on Substrate I, 6-FAM-labeled at the 5′ end of oligonucleotide 1, was incubated with either 0 μM (lanes 1 and 7) or 1.6 μM of RuvB
Mge in the presence of various concentrations of RuvA
Mge as indicated above the lanes. After the reaction (5 min at 37°C), the samples were deproteinized, electrophoresed through native 12% polyacrylamide gels, and analyzed by fluorometry. The positions of the substrates, which are too large to enter the gels, as well as the positions of the oligonucleotide reaction products, are indicated at the right-hand side of the gels by schematic illustrations. In these illustrations, the position of the 6-FAM label is indicated by a black dot.
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DNA recombination and repair enzymes may have required an accompanying modification of the function of the RuvA, RuvB and RecU proteins in order to preserve certain functionalities of the recombination and repair system. Nevertheless, the complete set of functions of this system in mycoplasmas is yet to be determined. In this regard, it is particularly interesting to learn how DNA recombination processes are achieved in M. pneumoniae in the absence of a functional RecU resolvase [19]. Although HJ resolvasome activities may be exerted by other proteins, such proteins have not yet been identified in M. pneumoniae. Moreover, the lack of a functional RecU was proposed as a possible cause of the relatively low frequency of homologous DNA recombination events in M. pneumoniae [19]. Also, the HJ resolvasome deficiency of M. pneumoniae may be associated with the difference between M. pneumoniae and M. genitalium in the specific mechanism by which homologous DNA recombination events occur in these species. In M. genitalium, the repeated DNA elements appear to recombine predominantly in a reciprocal fashion [7,8,12], whereas in M. pneumoniae such elements seem to recombine via a gene conversion-like mechanism, in which donor sequences are copied to the acceptor site and the original acceptor sequence is lost [9,10,11,13,14]. To address these and other issues related to the mechanism of homologous recombination in M. pneumoniae and M. genitalium, it is crucial that the entire set of putative DNA recombination and repair enzymes of these species be delineated. This will therefore be the goal of future studies.

Materials and Methods

Cloning of the M. pneumoniae MPN535 gene and M. genitalium MG358 gene

Bacterial DNA was purified from cultures of M. pneumoniae strain M129 (ATCC® no. 29342™) and M. genitalium strain G37 (ATCC® no. 33530™), as described previously [16,44]. The MPN535 ORF of M. pneumoniae strain M129, which encodes a RuvA homologue, was amplified by PCR. The PCR reaction was performed using the following primers: RuvAmpn_fw (5’-GGTCGTCGATATGATGCTTCATTTGATTGGAGCCATTTTGGAA-3’), which overlaps with the translation initiation codon [underlined] of MPN535 and primer RuvAmpn_rev (5’-GCAGCCGGATTCCTTAGGCGGTTTTATTTG-3’), which overlaps with the antisense sequence of the translation termination codon [underlined] of the gene. The resulting 0.6-kilobase pairs (kb) PCR fragment was digested with NdeI and BamHI (the recognition sites for these enzymes are indicated in italics in the sequences of primers RuvAmpn_fw and RuvAmpn_rev, respectively), and cloned into NdeI- and BamHI-digested Escherichia coli protein expression vectors, i.e. pET-11c and pET-16b (Novagen), generating plasmids pET-11c-RuvAmpn and pET-16b-RuvAmpn, respectively. Plasmid pET-11c-RuvAmpn was used for expression of native RuvAmpn, while plasmid pET-16b-RuvAmpn was employed for expression of RuvAmpn as an N-terminally poly histidine (H10)-tagged protein in E. coli.

Before cloning of the MG358 ORF of M. genitalium into E. coli protein expression vectors, a TGA codon within the ORF (encoding the Trp residue at position 27 of RuvA) was changed to a TAG codon using a PCR-based mutagenesis procedure [19]. Following mutagenesis, MG358 was amplified by PCR using the primers RuvAmg_pETfw (5’-CGTCGTCGATATGATGCTTCATTTTGGAGCCATTTTGGAA-3’), which overlaps with the translation initiation codon [underlined] of the gene. The 0.6-kb PCR product was digested with NdeI and BamHI, and ligated into NdeI- and BamHI-digested vectors pET-11c and pET-16b, resulting in plasmids pET-11c-RuvAmg and pET-16b-RuvAmg, respectively. These plasmids were used for expression of native and H10-tagged RuvAmpn, respectively, in E. coli. The integrity of all DNA constructs used in this study was checked by dideoxy sequencing, as described before [15].

Figure 7. The influence of RuvAmpn and RuvAmpn on the ATPase activities of RuvBFH and RuvBFH respectively. ATP hydrolysis by RuvBFH and RuvBFH was measured at a protein concentration of 0.5 μM, either in the absence of any protein. (+), RuvAmpn alone; (○), RuvAmpn alone; (■), RuvAmpn plus RuvAmpn (△), RuvBFH alone; (▲), RuvBFH plus RuvAmpn. The graph shows a representative experiment. doi:10.1371/journal.pone.0038301.g007
Table 1. Compilation of the activities of the RecA, SSB, RuvA, RuvB and RecU proteins from *M. pneumoniae*, *M. genitalium* and reference bacteria.

| Protein | Species | ORF     | Activities (in vitro)                                                                 | Divalent cations, nucleotide and protein cofactors | Interacting proteins (physical and/or functional) | Reference |
|---------|---------|---------|-------------------------------------------------------------------------------------|--------------------------------------------------|--------------------------------------------------|-----------|
| RecA    | *E. coli* | *recA*  | Exchange of homologous DNA strands                                                  | Mg\(^{2+}\), ATP, SSB\(_{Eco}\)                  | SSB\(_{Eco}\)                                      | [47]      |
| RecA\(_{Mpn}\) | *M. pneumoniae* | MPN490 | Exchange of homologous DNA strands                                                  | Mg\(^{2+}\), ATP, SSB\(_{Mpn}\)                 | SSB\(_{Mpn}\)                                      | [15]      |
| RecA\(_{Mge}\) | *M. genitalium* | MG339  | Exchange of homologous DNA strands                                                  | Mg\(^{2+}\), ATP, SSB\(_{1}\)                  | SSB\(_{1}\)                                       | [15]      |
| SSB     | *E. coli* | *ssb*   | ssDNA-binding, stimulation of RecA\(_{Eco}\), various other roles in DNA replication, repair, and recombination | None                                             | RecA\(_{Eco}\), other proteins                   | [48]      |
| SSB\(_{Mpn}\) | *M. pneumoniae* | MPN229 | ssDNA-binding, stimulation of RecA\(_{Mpn}\)                                       | None                                             | RecA\(_{Mpn}\)                                      | [16]      |
| SSB\(_{Mge}\) | *M. genitalium* | MG091  | Unknown                                                                              | Unknown                                          | Unknown                                          | n.a.      |
| RuvA    | *E. coli* | *ruvA*  | HJ-binding, stimulation of RuvB\(_{Eco}\), inhibition of RuvC\(_{Eco}\)           | None                                             | RuvB\(_{Eco}\), RuvC\(_{Eco}\)                   | [31,32]   |
| RuvA\(_{Mpn}\) | *M. pneumoniae* | MPN35  | HJ- and ssDNA-binding                                                                | None                                             | Unknown                                          | This study; [17] |
| RuvA\(_{Mge}\) | *M. genitalium* | MG358  | HJ-binding, stimulation of RuvB\(_{Mge}\), inhibition of RecU\(_{Mge}\)            | None                                             | RuvB\(_{Mge}\), RecU\(_{Mge}\)                   | This study |
| RuvB    | *E. coli* | *ruvB*  | HJ branch migration, DNA unwinding                                                 | Mg\(^{2+}\), ATP, RuvA\(_{Eco}\)               | RuvA\(_{Eco}\), RuvC\(_{Eco}\)                   | [49,50,51]|
| RuvB\(_{Mpn}\) | *M. pneumoniae* | MPN36  | DNA unwinding                                                                       | Mg\(^{2+}\), ATP                                | Unknown                                          | This study; [18] |
| RuvB\(_{Mge}\) | *M. genitalium* | MG359  | DNA unwinding                                                                       | Mg\(^{2+}\), ATP, RuvA\(_{Mge}\)               | RuvA\(_{Mge}\)                                      | This study; [18] |
| RecU    | *Bacillus subtilis* | *recU*  | HJ resolution, annealing of homologous DNA substrates, modulation of RecA function | Mg\(^{2+}\)                                      | RuvB\(_{Mge}\), RecA\(_{Mge}\)                   | [21,52,53]|
| RecU\(_{Mpn}\) | *M. pneumoniae* | MPN528a | None                                                                                 | n.a.                                             | Unknown                                          | [19]      |
| RecU\(_{Mge}\) | *M. genitalium* | MG352  | HJ resolution                                                                       | Mn\(^{2+}\)                                      | RuvA\(_{Mge}\)                                      | This study; [19,20] |

1. The RuvBFH protein is exclusively expressed by subtype 2 strains of *M. pneumoniae*. Subtype 1 strains express a RuvB protein (RuvBM129) that differs in a single amino acid residue from RuvB\(_{Mge}\). RuvBM129 has significantly lower ATPase and DNA helicase activities than RuvB\(_{Mge}\) [18].
2. RecU\(_{Mpn}\) is only expressed by subtype 2 strains of *M. pneumoniae*. Subtype 1 strains are unable to express a full-length RecU protein due to the presence of a nonsense mutation in the RecU gene (MPN528a) [19].
3. The SSB-dependence of the RecA\(_{Mge}\) protein was determined using SSB\(_{Mge}\) and SSB\(_{Eco}\) [15]. The SSB\(_{Mge}\) protein has not yet been characterized.

n.a., not applicable.

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Protein expression and purification

The various pET-11c- and pET-16b-derived vectors were introduced into E. coli BL21 (DE3) and the resulting strains were grown overnight at 37°C in LB medium containing 100 µg/ml ampicillin. The cultures were diluted 1:100 in 300 ml LB medium with ampicillin and grown at 37°C to an optical density at 600 nm of 0.6. Protein expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.6 mM. After incubation for 2 hr at 30°C, the bacteria were harvested by centrifugation and stored at −20°C.

The H$_{14}$-tagged RuvA$_{Mge}$ and RuvA$_{M. pneumoniae}$ proteins were both purified using the following protocol. Bacterial pellets were resuspended in 10 ml of buffer A (20 mM Tris-HCl pH 8.0, 1 M NaCl) containing 0.5 mg/ml of lysozyme. The suspension was sonicated on ice and clarified by centrifugation for 20 min at 12,000 × g (at 4°C). The supernatant, imidazol was added to a final concentration of 5 M. Then, the supernatant was loaded onto a column containing 1 ml of Ni$^{2+}$-nitroacetic acid (Ni-NTA)-agarose (Qiagen), which was equilibrated previously in buffer A containing 5 mM imidazol. The column was washed with 5 ml of buffer A plus 5 mM imidazol and with 5 ml of buffer A plus 20 mM imidazol. The specifically bound proteins were eluted from the column with 8 ml of buffer A containing 250 mM NaCl plus 20 mM imidazol. The specifically bound proteins were eluted from the column with 5 ml of buffer A plus 5 mM imidazol and with 5 ml of buffer A plus 20 mM imidazol. The specifically bound proteins were eluted from the column with 8 ml of buffer A containing 250 mM imidazol. Fractions of 0.5 ml were collected, analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, and dialyzed against a solution of 20 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 0.1 mM EDTA, 1 mM DTT and 50% glycerol (buffer B). Aliquots of purified protein, which had an estimated homogeneity of >95%, were stored at −20°C.

The native RuvA proteins were purified by solubilization of the bacterial pellets in a buffer containing 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT and 0.5 mg/ml of lysozyme. After sonication and centrifugation (using similar procedures as described above), the RuvA proteins were precipitated with ammonium sulphate and resuspended in 20 mM Tris-HCl pH 7.4, 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT. The proteins were then subjected to affinity chromatography using Heparin Sepharose 6 Fast Flow (GE Healthcare). Proteins were eluted from the column material with a linear gradient from 0 M to 1 M NaCl in 20 mM Tris-HCl pH 7.4, 0.1 mM EDTA and 1 mM DTT. The RuvA-containing fractions were pooled, dialyzed against buffer B, and stored at −20°C.

The purifications of RecU$_{Mge}$, RuvB$_{HH}$ and RuvB$_{M. pneumoniae}$ have been described before [18,19,20].

SDS-PAGE

Proteins were separated by SDS-PAGE, as described by Laemmli [45]. Gels were stained with Coomassie brilliant blue (CBB), destained in 40% methanol/10% acetic acid, and recorded using a GelDoc XR system (Bio-Rad). Digital images were processed using Quantity One® 1-D Analysis Software (Bio-Rad).

DNA substrates

The small DNA substrates that were used in the DNA binding experiments consisted of synthetic oligonucleotide substrates that were 5’-6-FAM-labelled on a single strand. Holliday junction (HJ) substrate HJ 1.1, single-stranded oligonucleotide HJ11 and double-stranded substrate HJ11/HJ11v have been described by Sluijter et al. [19]. Substrate HJ 1.1 is composed of the following four oligonucleotides: HJ11 (5’-GGGACGCTGATCCAGCAGAT- GTAATTGCT-GAGATCTGTTTTTCCCGAAGAAGC-3’), HJ12 (5’-GGCCCTCTGTTGCGAGAAAGCATGCTTGA- GAAATCGTCTGACGTGAAAGC-3’), HJ13 (5’-GGTTCCATGCGACTGAGATT-GCTAGGCT-CAAGGCAGACTGCTACGG-3’) and HJ14 (5’-AC- CGTTAGCATCG-CCTTAGGCT-CATC/TGGTGATACGCACGG-3’). The sequence of oligonucleotide HJ11v is 5’-GGCCTCTCTTGCGAGAAAGCATT-GCCAGCAATCCTGTTGTGACGCTGCG-3’. The DNA helicase substrates (Fig. 6A) have been described in detail by Estevão and coworkers [18].

DNA-binding assays

Binding of the RuvA proteins to various DNA substrates was carried out in 10-µl volumes and included 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 12.3 mM oligonucleotide substrate and various concentrations of RuvA proteins. After incubation on ice for 10 min, 1 µl was added of a solution containing 40% glycerol and 0.25% bromophenol blue. Then, the reaction mixtures were electrophoresed through 8% polyacrylamide gels in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Following electrophoresis, the polyacrylamide gels were analyzed by fluorometry, using a Typhoon Trio™ 9200 Variable Mode Imager (GE Healthcare) in combination with the Typhoon Scanner Control v4.0 software (Amersham Bioscience). Images were processed using Quantity One® 1-D Analysis Software.

Holliday junction (HJ) resolution assays

HJ resolution assays were carried out as described by Sluijter et al. [19]. Reactions were analyzed by electrophoresis through 12% polyacrylamide/1× TBE mini-gels. The relative RecU$_{Mge}$ (resolution) activity (Fig. 5C) was expressed as percentage of the protein’s activity in the absence of RuvA$_{Mge}$.

DNA helicase and ATPase assays

DNA helicase assays were performed similarly as described before [18]. After deproteinization, the reactions mixtures were analyzed by electrophoresis through 12% polyacrylamide/1× TBE mini-gel and fluorometry. The ATPase activities of RuvB$_{HH}$ and RuvB$_{M. pneumoniae}$ were determined by using a β-nicotinamide adenine dinucleotide reduced form (NADH)-coupled assay on a VersaMax Tunable Microplate Reader (Molecular Devices) [15,46].

Supporting Information

Figure S1 RuvA$_{Mge}$ is a tetramer in solution. (A) Gel filtration analysis of RuvA$_{Mge}$. Gel filtration chromatography was performed in a similar fashion as described previously [16], using a Sephadex G-150 column (length, 1.0 m; inner diameter, 1.0 cm). The column was run at 4 ml/h in 30 mM Tris-HCl (pH 7.5)/135 mM NaCl, and calibrated with blue dextran (2,000 kDa), bovine serum albumin (BSA, 66.4 kDa), ovalbumin (42.9 kDa), and cytochrome C (12.3 kDa). Fractions of 1.0 ml were collected and monitored by measuring the optical density at 280 nm (OD280, Y-axis at the left-hand side of the graph). The fractions eluted from a subsequent run, containing 15 ng of RuvA$_{Mge}$ were precipitated with trichloroacetic acid, and separated on 12% SDS-PAGE gels. Gels were silver-stained and recorded using the GelDoc XR system. RuvA$_{Mge}$ was quantified by densitometry using Quantity One® 1-D Analysis Software (Bio-Rad). The relative concentration of RuvA$_{Mge}$ (Y-axis on the right-hand side, in arbitrary units) is shown for column fractions 23 to 39. In all other fractions, RuvA$_{Mge}$ was not detected. (B) Calibration curve range was obtained from the gel filtration experiment shown in (A). The molecular weight of protein size standards (•) is plotted against the elution volume (Ve) divided by the void volume.
(V_0) of the column (V/V_0). V_0 was determined with blue dextran. The V/V_0 of RuvA_Mg is marked on the calibration curve (×).

Author Contributions
Conceived and designed the experiments: MS SE TH CV. Performed the experiments: MS SE TH CV. Analyzed the data: MS SE TH NG AM CV R. Contributed reagents/materials/analysis tools: MS SE TH NG AM CV R. Wrote the paper: MS SE TH NG AM CV R.

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