Identification and characterization of *Thermus thermophilus* HB8 RuvA protein, the subunit of the RuvAB protein complex that promotes branch migration of Holliday junctions

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The *Escherichia coli* *ruvA* and *ruvB* genes constitute an SOS-regulated operon. The products of these genes form a protein complex that promotes branch migration of the Holliday junction, an intermediate of homologous recombination. RuvA protein binds specifically to the Holliday junction and recruits RuvB protein to the junction. RuvB is an ATP-driven motor protein involved in branch migration. We previously cloned the *ruvB* gene of the thermophilic bacterium *Thermus thermophilus* HB8 (*Tth*) and found that, in contrast to the operon structure in most mesothermic bacteria, the *ruvA* gene is absent from the vicinity of *ruvB*. In this work, we cloned the *ruvA* gene from *T. thermophilus* HB8 and analyzed its nucleotide sequence. *Tth* RuvA is a protein of 20,414 Da consisting of 191 amino acid residues, and is 37% identical in amino acid sequence to *E. coli* RuvA. *Tth* *ruvA* complemented the DNA repair defect of *E. coli* Δ *ruvA* mutants. The purified *Tth* RuvA protein stimulated *Tth* RuvB activities, such as hydrolysis of ATP and promotion of branch migration of the Holliday junction, in a manner similar to the RuvA-RuvB interactions observed in *E. coli*. In addition, *Tth* RuvA stimulated the *E. coli* RuvB activities *in vitro*, which was well consistent with the results of *in vivo* hetero-complementation experiments.

INTRODUCTION

Homologous recombination is an essential biological process that generates genetic diversity and is necessary to repair damaged DNA. The pathways of homologous recombination have been extensively studied in *Escherichia coli*. The Holliday structure, which is a crucial recombination intermediate, is formed through homologous pairing and strand exchange reactions mediated by RecA protein and its accessory proteins (Kowalczykowski

et al., 1994). The RuvABC proteins are involved in processing Holliday junctions during the late stage of homologous recombination in *E. coli* (Shinagawa and Iwasaki, 1996; West, 1997). The RuvAB protein complex promotes branch migration of Holliday junctions (Iwasaki et al., 1992; Tsaneva et al., 1992), and RuvC resolves the junction when it encounters favorable Holliday junction sequences (Iwasaki et al., 1991; Shah et al., 1994). The 22-kDa RuvA protein forms a stable tetramer, binds specifically to the junction, and facilitates the loading of RuvB to the junction (Iwasaki et al., 1992; Parsons et al., 1992). The 37-kDa RuvB protein forms a hexameric ring and is a motor protein involved in branch migration that requires ATP hydrolysis (Shiba et al., 1993; Stasiak et al., 1994).

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RuvB has intrinsic ATPase activity, which is enhanced by duplex DNA and RuvA in a synergistic manner (Iwasaki et al., 1989; Shiba et al., 1991). The 19-kDa RuvC protein forms a dimeric endonuclease, specifically binds to the Holliday junction, and resolves it by introducing two nicks at symmetrical positions across the junction (Dunderdale et al., 1991; Iwasaki et al., 1991; Shida et al., 1996).

RuvA and RuvB orthologs are highly conserved among bacteria. In *E. coli* the *ruvA* and *ruvB* genes constitute a single operon regulated by the SOS system (Benson et al., 1988; Shinagawa et al., 1988). In contrast, *ruvC* belongs to a separate non-SOS operon that is located immediately upstream of *ruvAB* (Sharples and Lloyd, 1991; Takahagi et al., 1991). In many bacteria, such as *Pseudomonas aeruginosa*, *Haemophilus influenzae* Rd, and *Mycobacterium leprae*, *ruvC*, *ruvA*, and *ruvB* constitute a single operon with the genes in this order (Hishida et al., 1996; Sharples et al., 1999). However, the *ruvA* gene of *Thermus thermophilus* HB8 (*Tth*) and two other thermophilic bacteria in which *ruvB* genes were previously cloned and analyzed was not found in the vicinity of the *ruvB* gene (Tong and Wetmur, 1996; Yamada et al., 1999). Although the *Tth ruvB* gene does not complement the UV sensitivity of the *E. coli ruvB* deletion strain, *Tth* RuvB-mediated branch migration of Holliday junctions is stimulated by *E. coli* RuvA *in vitro* (Yamada et al., 1999). This suggested the existence of a RuvA ortholog in *T. thermophilus* HB8.

In the present study we cloned the *ruvA* gene of *T. thermophilus* HB8 and analyzed its sequence. We constructed an overproduction system for *Tth* RuvA in *E. coli*,

purified the *Tth* RuvA protein, and analyzed its biochemical properties *in vitro*. *Tth* RuvA had similar properties to *E. coli* RuvA and interacted functionally with *E. coli* RuvB both *in vivo* and *in vitro*.

MATERIALS AND METHODS

***E. coli* strains and plasmids.** HRS2300 (Δ *ruvA*100::cm^r) (Hishida et al., 1996) and HRS3403 (Δ *ruvAB*200::km^r) (Ishioka et al., 1998), both derived from AB1157 (Bachmann, 1972), were used as host strains for UV sensitivity tests. HRS4000 (Δ *ruvABC*200::km^r) (Yamada et al., 1999), derived from BL21(DE3) (Studier et al., 1990), was used for overexpression of the *Tth* RuvA protein. Plasmids used in this study are listed in Table 1.

Cloning and sequencing the *Tth ruvA* gene. Since a portion of a coding region of a RuvA-like protein was found from the *Tth* genome sequencing project, two primers based on the sequence of that region (F1: 5'-AACCTCGCCCTCTTTGAACTC-3' and R1: 5'-CTTTAGGGCC-TCCTTGATGAG-3') were synthesized. These primers were subsequently used to amplify the *Tth ruvA* gene segment. The amplified DNA fragment (~400bp) was used as a hybridization probe for the cloning of *ruvA*. The *Tth* genomic DNA was digested with *Bam*HI and fractionated by agarose gel electrophoresis. A 5.5-kb DNA band that hybridized with the probe was isolated and ligated to *Bam*HI-digested pUC19. Positive clones were identified by hybridization. One of these clones was named

Table 1. Plasmid list

Plasmid	Characteristics	Source / reference
pUC19	A cloning vector	Laboratory stock
pAF101	A T7 expression vector with a pUC19 backbone	(Yamada et al., 1999)
pAF104	A T7 expression vector derived from pAF101 carrying <i>lacI</i> ^r	This study
pAF134	pAF104 derivative carrying the <i>E. coli ruvA</i> ⁺ gene	(Nishino et al., 1998)
pBAD24	An arabinose-inducible expression vector	(Guzman et al., 1995)
pBluescript II SK (+)	A cloning vector	Laboratory stock
pLysS	Used for T7 expression system to reduce the basal level expression	(Studier et al., 1990)
pSTV28	pACYC184 derivative cloning vector compatible with pUC-type plasmids	Takara-Shuzo (Kyoto)
pTF100	pBAD24 derivative plasmid in which unique <i>Nde</i> I and <i>Bgl</i> II sites were created downstream of the <i>ParaC</i> promoter	This study
pTRA104	pUC19 derivative carrying 5.5-kb <i>Bam</i> HI fragment containing the <i>Tth ruvA</i> ⁺ gene	This study
pTRA126	pBluescript II SK(+) derivative carrying 2.2-kb <i>Bam</i> HI- <i>Hind</i> III fragment containing the <i>Tth ruvA</i> ⁺ gene	This study
pTRA127	<i>Tth</i> RuvA overexpression vector derivative of pAF104	This study
pYWH500	pSTV28 derivative cloning vector carrying the same multicloning site as pTF100	This study
pYWH501	pYWH500 derivative carrying the <i>E. coli ruvB</i> ⁺ gene	This study
pYWH800	pYWH500 derivative carrying the <i>Tth ruvB</i> ⁺ gene	This study

pTRA104. The 5.5-kb DNA fragment was digested with several restriction enzymes. The pTRA126 clone was created by ligating the resulting 2.2-kb *Bam*HI-*Hind*III fragment containing the *Tth ruvA* gene to a *Bam*HI-*Hind*III-digested pBluescript II SK(+) plasmid. The nucleotide sequence of both strands of pTRA126 was determined using an automated DNA sequencer (Applied Biosystems, model 373XL).

Construction of pTRA127, a *Tth* RuvA-overproducing plasmid. A pair of DNA primers (F2; 5'-CCAGGGCATATGATCCGCTACCTCCGGG-3' and R2; 5'-GGAGGGGATCCTAGCGGAGGCGCTTTAGGG-3') were used to create an *Nde*I site at the first ATG codon of the *Tth ruvA* gene and to create a *Bam*HI site downstream from the stop codon. The underlined portion of each primer indicates the respective *Nde*I and *Bam*HI sites. The *Tth ruvA* gene, which was amplified by PCR using these primers and pTRA126 DNA as a template, was ligated to the *Nde*I-*Bam*HI site of pAF104, resulting in pTRA127.

Purification of *Tth* RuvA protein. *E. coli* HRS4000 harboring pLysS was transformed with pTRA127. The transformants were subsequently grown in 1.5 liters of LB medium containing ampicillin at 37°C until an A_{600} of about 0.6 was achieved. Isopropyl- β -D-thiogalactopyranoside was then added to a final concentration of 1 mM, and the culture was incubated for an additional 4 hours. The cells were collected by centrifugation, resuspended in R-buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 7 mM β -mercaptoethanol, and 10% glycerol) containing 500 mM NaCl, and disrupted by sonication. To denature most of the endogenous *E. coli* proteins, this crude cell extract was incubated at 75°C for 15 min. The cell extract was then centrifuged at $10,000 \times g$ for 15 min. Ammonium sulfate was gradually added to the supernatant to achieve 40% saturation. The mixture was stirred for 30 min and then centrifuged at $27,000 \times g$ for 15 min. The pellet was resuspended in R-buffer containing 100 mM NaCl and 2 M urea, and dialyzed against the same buffer overnight. Urea was added to prevent aggregation of *Tth* RuvA at high concentrations. After the precipitate was removed by centrifugation ($27,000 \times g$ for 15 min), the supernatant was applied to an SP-Sepharose column (Amersham Pharmacia Biotech) equilibrated with R-buffer containing 100 mM NaCl and 2 M urea. RuvA was eluted with a 300-ml linear gradient of R-buffer containing 2 M urea plus 100 mM to 1000 mM NaCl. The RuvA peak fractions were pooled and dialyzed overnight against R-buffer containing 100 mM NaCl and 2 M urea. The dialyzed sample was applied to a Heparin column (Amersham Pharmacia Biotech) equilibrated with R-buffer containing 100 mM NaCl and 2 M urea. RuvA was eluted with a 300-ml linear gradient of R-buffer containing 2 M urea plus 100 mM to 1000 mM NaCl. The

RuvA peak fractions were pooled and then applied to a Hiroad 26/60 Sephacryl S-300 HR gel filtration column (Amersham Pharmacia Biotech) equilibrated with R-buffer containing 500 mM NaCl and 2 M urea. The resulting RuvA peak fractions were pooled and dialyzed against R-buffer containing 100 mM NaCl and 2 M urea. The dialyzed sample was applied to another Heparin column equilibrated with R-buffer containing 100 mM NaCl and 2 M urea. The RuvA was eluted with a 300-ml linear gradient of R-buffer containing 2 M urea plus 100 mM to 1000 mM NaCl. The RuvA peak fractions were pooled and dialyzed against R-buffer containing 500 mM NaCl. The protein concentration was determined using an extinction coefficient of $\epsilon_{280} = 2600 \text{ M}^{-1} \text{ cm}^{-1}$.

***Tth* RuvB and *E. coli* RuvA and RuvB proteins.** *Tth* RuvB and *E. coli* RuvA and RuvB proteins were purified as described previously (Hishida et al., 1999; Nishino et al., 1998; Yamada et al., 1999).

Computer analysis. A multiple sequence alignment of RuvA orthologs was constructed with the CLUSTAL W program (Thompson et al., 1994) and was modified by visual inspection to increase the similarity. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987).

Construction of synthetic Holliday junctions. A synthetic Holliday junction was constructed by annealing four 72mer oligonucleotides, JY-11, JY-12, JY-13 and JY-14, as described previously (Hishida et al., 1999). Duplex DNA used for a control was constructed by annealing two complementary 72mer oligonucleotides, JY-11 and JY-15 (5'-CAGCGCTTGGTAAACACATAGAATTCTGCTCGG-TCTGAGCCGATTGAAGCTTCTCGAGGTTCTCTGTCGCTCG-3').

DNA binding assay. Specific binding of RuvA protein to a synthetic Holliday junction labeled with ^{32}P was assessed with a gel mobility retardation assay. A standard reaction mixture (20 μl) containing 10 nM synthetic ^{32}P -Holliday junction DNA and *Tth* RuvA protein in a buffer of 20 mM HEPES-NaOH (pH 7.6), 1 mM DTT and 0.01% bovine serum albumin was incubated for 30 min on ice. The concentrations of RuvA in the reaction mixture were systematically varied between 0 and 200 nM. After incubation, the reaction mixtures were analyzed by polyacrylamide gel electrophoresis (PAGE) on 6% polyacrylamide gels. The protein-DNA complexes were visualized with a Fuji BAS1500 image analyzer. The same procedure was carried out for the control in which 10 nM ^{32}P -duplex DNA was used in the reaction mixture in place of the Holliday junction DNA.

Branch migration assay. The branch migration activ-

ity of RuvB was assayed using the synthetic Holliday junction essentially as described previously (Yamada et al., 1999). Reaction mixtures containing one of the following pairings: 1) *E. coli* RuvA and *E. coli* RuvB, 2) *Tth* RuvA and *E. coli* RuvB, 3) *E. coli* RuvA and *Tth* RuvB, or 4) *Tth* RuvA and *Tth* RuvB, were prepared. The standard reaction mixture (20 µl) contained 5 nM synthetic ³²P-Holliday junction DNA, 150 nM RuvB and varying concentrations of RuvA (0 to 200 nM) in a buffer of 20 mM HEPES-NaOH (pH 7.6), 1 mM DTT, 5 mM MgCl₂, and 20% glycerol. After incubation for 10 min or 30 min at either 37°C or 55°C, the reaction products were analyzed by 9% PAGE.

ATPase assay. The ATP hydrolysis reaction catalyzed by *Tth* RuvB protein was measured essentially as described previously (Yamada et al., 1999). The standard reaction mixture (20 µl) contained 0.67 mM supercoiled pUC19 DNA, 0.2 µM RuvB, varying concentrations of RuvA (0 to 2000 nM), and 2 mM [γ-³²P] ATP in a buffer of 20 mM HEPES-NaOH (pH 7.6), 10 mM MgCl₂, 10% glycerol, and 1 mM DTT. DNA concentration is expressed in terms of the nucleotide concentration only in this assay. The hydrolyzed Pi was measured using a Fuji BAS1500 image analyzer.

RESULTS AND DISCUSSION

Cloning and sequencing of the *T. thermophilus* HB8 *ruvA* gene. The DNA fragment containing the *ruvA* gene was amplified from genomic DNA using primers that corresponded to sequences flanking the *Tth ruvA* coding region. This fragment was used as a probe to identify restriction fragments carrying the *Tth ruvA* gene. A 5.5-kb *Bam*HI genomic fragment was first identified by Southern blot hybridization and was subsequently confirmed to contain the full length of the *Tth ruvA* gene. This fragment was cloned into the *Bam*HI site of pUC19, thereby producing pTRA104. The restriction enzyme cleavage map of this fragment is shown in Fig. 1.

The 2.2-kb *Bam*HI-*Hind*III fragment that was expected to contain the entire *ruvA* gene was subcloned into the pBluescript II SK(+) vector, thereby forming pTRA126. Analysis of the nucleotide sequence of the 2.2-kb insert (DDBJ Accession No. AB048605) revealed three open reading frames corresponding to the putative enoyl-CoA hydratase α subunit gene, the *Tth ruvA* gene and a putative *ysaA* homolog of *Bacillus subtilis*, whose function is unknown (Fig. 1). *Tth ruvA* encodes a protein of 20,414 Da consisting of 191 amino acid residues. The GC content of *Tth ruvA* is 70.8%, which is similar to that of *Tth ruvB* (70.1%) (Yamada et al., 1999). In contrast to most prokaryotes, in which *ruvA* and *ruvB* constitute a single operon (Hishida et al., 1996; Sharples et al., 1999), neither *ruvB* nor *ruvC* were found in the vicinity of *ruvA* in

Fig. 1. Physical map of cloned and subcloned DNA fragments containing the *ruvA* gene from *T. thermophilus* HB8. The arrows indicate open reading frames. The bold line indicates the probe region used for cloning. Restriction enzyme recognition sites: B, *Bam*HI; H, *Hind*III; K, *Kpn*I; N, *Nco*I; X, *Xho*I.

T. thermophilus HB8.

Multiple sequence alignment and phylogenetic analysis.

The amino acid sequence of *Tth* RuvA was aligned with the sequences of other bacterial RuvA proteins (Fig. 2). X-ray crystallography of *E. coli* RuvA revealed that it consists of three domains (Nishino et al., 1998; Rafferty et al., 1996) and the secondary structure of *E. coli* RuvA is shown above the alignment (Fig. 2). The deduced sequence of *Tth* RuvA is 37% identical and 75.4% similar to the sequence of *E. coli* RuvA. The deduced sequence of *Tth* RuvB is 56% identical to that of *E. coli* RuvB (Yamada et al., 1999). This is in accord with the observation that the sequence conservation among RuvA orthologs is generally much lower than that among RuvB orthologs (Hishida et al., 1996; Sharples et al., 1999). Notably, *Tth* RuvA, as well as RuvAs from *Mycoplasma pneumoniae* (*Mpn*), *Mycoplasma genitalium* (*Mge*), *Helicobacter pylori* (*Hpy*) and *Borrelia burgdorferi* (*Bbu*), have a much shorter linker region between the α6 and α7 helices than *E. coli* RuvA.

Using the alanine scanning mutagenesis technique, we previously identified six functionally important amino acid residues in *E. coli* RuvA. That study revealed that mutations of Ile-2, Leu-110, Leu-167, Leu-170, Tyr-172 and Leu-199 resulted in loss of RuvA function *in vivo* (Nishino et al., 1998 and T. Fujita, H. I. and H. S. unpublished data). Four of these amino acids (Leu-167, -170, and -199 and Tyr-172) are hydrophobic residues located in domain III of *E. coli* RuvA and are likely to form an interface with RuvB. X-ray crystallography of the *E. coli* RuvA-Holliday junction complex revealed that two helix-hairpin-helix (HhH) motifs in domain II are involved in Holliday junction binding (Ariyoshi et al., 2000). The nitrogens of the main chain amides of Gly-80 and Gly-82 in the hairpin in HhH I interact with phosphate oxygens of the junction DNA through hydrogen bonds. A similar interaction occurs for the amides of Gly-115 and Gly-117 in the hairpin in HhH II. The side chains of Lys-84 in the second

Fig. 2. Sequence alignment of *Tth* RuvA and other bacterial orthologs. Amino acid sequences of various RuvAs from *Escherichia coli* (Eco), *Thermus thermophilus* HB8 (Tth), *Deinococcus radiodurans* (Dra), *Mycobacterium tuberculosis* (Mtu), *Mycobacterium leprae* (Mle), *Haemophilus influenzae* (Hin), *Pseudomonas aeruginosa* (Pae), *Chlamydia pneumoniae* (Cpn), *Chlamydia trachomatis* (Ctr), *Helicobacter pylori* (Hpy), *Clostridium histolyticum* (Chi), *Bacillus subtilis* (Bsu), *Rickettsia prowazekii* (Rpr), *Treponema pallidum* (Tpa), *Borrelia burgdorferi* (Bbu), *Mycoplasma pneumoniae* (Mpn), *Mycoplasma genitalium* (Mge), and *Synechocystis sp* (Syn) were aligned using the CLUSTAL W multiple sequence alignment program (Thompson et al., 1994). Amino acid positions that contained more than 12/18 similar amino acids and identical amino acids are shaded in gray and black, respectively. Similar amino acids were defined as follows: LIMV, SPTAG, YWF, DEQN, and KRH. Particularly important amino acid residues that are mentioned in the text are marked by asterisks above the *E. coli* RuvA sequence. The secondary structure of *E. coli* RuvA (α helices and β strands) is shown above the alignment and is indicated by arrows (β strands) and bold lines (α helices). Domains 1, 2, and 3 include β 1 to β 6, α 2 to α 6, and α 7 to α 9, respectively (Ariyoshi et al., 2000; Nishino et al., 1998; Rafferty et al., 1996). The striped bars between domains 2 and 3 indicate disordered structure. The entire linker region (residues 141–157) adopted a disordered structure in the DNA-free crystal, while the region contained a short α helix (α^*) (residues 144–149) in the complex with the synthetic Holliday junction (Ariyoshi et al., 2000).

Fig. 3. The unrooted phylogenetic tree of 18 bacterial RuvA orthologs. Based on the multiple alignment shown in Fig. 2, a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). Abbreviations of the species are the same as those in Fig. 2.

Fig. 4. *In vivo* complementation assay. Serial dilutions of cells were spotted on LB plates, followed by UV irradiation. The plates were incubated at 37°C for about 15 hr. (A) The UV sensitivity of an *E. coli ruvA* mutant strain transformed with either pAF104 (vector), pAF134 (*E. coli ruvA*), or pTRA127 (*Tth ruvA*). (B) The sensitivity of an *E. coli ruvA ruvB* mutant strain carrying both homologous and heterologous combinations of *ruvA* and *ruvB* genes. The plasmids carrying *ruvA* were pAF134 (*E. coli ruvA*) and pTRA127 (*Tth ruvA*), both of which are derivatives of vector plasmid pAF104. The plasmids carrying *ruvB* were pYWH501 (*E. coli ruvB*) and pYWH800 (*Tth ruvB*), both of which are derivatives of vector plasmid pYWH500. The plasmids carrying the *ruvA* or *ruvB* genes, respectively, were compatible in the strain. Bars indicate the empty vector.

helix in HhH I and those of Lys-119 and Arg-123 in the second helix in HhH II also interact with the junction DNA. The observation that the mutant protein (Leu-110 to Ala) does not bind junction DNA *in vitro* (Nishino et al., 1998), suggests that this mutation may cause structural disorder of the functional HhH II motif. The side chains of Arg-54, Glu-55 and Asp-56, the latter two of which are collectively called the “acidic pin” (Rafferty et al., 1996), are located in the vicinity of the junction center. They are involved in the disruption of base-pairing at the junction center, which facilitates RuvB-mediated branch migration (Ariyoshi et al., 2000). The amino acid residues corresponding to the important ones revealed by these *E. coli* RuvA studies are also highly conserved in *Tth* RuvA (Fig. 2).

On the basis of this alignment, we constructed an unrooted molecular phylogenetic tree of RuvA orthologs using the neighbor-joining method (Fig. 3). *Tth* RuvA is evolutionarily most related to *Deinococcus radiodurans* RuvA. The phylogenetic relationships among RuvA orthologs are similar to those among RuvB orthologs and among 16S rRNA sequences (Maidak et al., 1999). This observation may suggest that RuvA, as well as RuvB, evolved from the earliest times as an important protein with a fundamental cellular function, like DNA and RNA polymerases and many enzymes involved in translation. Genetic studies show that RuvA, RuvB, and RuvC proteins work together in sexual conjugation and recombinational DNA repair in *E. coli* (Mandal et al., 1993; Takahagi et

Fig. 5. SDS-PAGE analysis of *Tth* RuvA. Proteins were separated by 15% SDS-PAGE and stained with Coomassie brilliant blue. Lanes: a, molecular weight markers; b, purified *E. coli* RuvA; c, purified *Tth* RuvA.

al., 1991). The fact that RuvA and RuvB are widespread throughout bacterial species whereas RuvC is less common (Sharples et al., 1999), suggests that an alternative Holliday junction resolvase or resolution mechanism may exist in certain bacteria that lack RuvC. The possibility has been suggested that RuvAB, independently of RuvC, is involved in the restoration of stalled replication forks (Seigneur et al., 1998), and this may also account for the higher conservation of RuvAB than RuvC.

The *Tth ruvA* gene complements the DNA repair defect of *E. coli ruvA* mutants. To examine whether the *Tth ruvA* gene could substitute for the recombination repair function of the *E. coli ruvA* gene, a plasmid carrying the *Tth ruvA* gene (pTRA127) was introduced into the *E. coli ΔruvA* strain (HRS2300) by transformation, and the UV sensitivity of the transformant was compared with that of the control strain (Fig. 4A). The results indicated that the *Tth ruvA* gene was indeed able to complement

(A)

(B)

Fig. 6. (A) DNA-binding activity of *Tth* RuvA was analyzed by a gel retardation assay. Reactions were carried out with either 10 nM junction DNA (synthetic Holliday junction, lanes a to h) or duplex DNA (lanes i and j) and the indicated amount of *Tth* RuvA protein. The protein-DNA complexes were analyzed by 6% PAGE. (B) RuvA-induced stimulation of the branch migration activity of *Tth* RuvB for the synthetic Holliday junctions. Five nanomolar ³²P-labeled junction was incubated with 1 μM *Tth* RuvB at 55°C either in the absence of *Tth* RuvA (lane c) or in the presence of different concentrations of *Tth* RuvA (lanes d to j). Following deproteinization, the products were analyzed by 9% PAGE. ATP was added to the reaction mixtures loaded into lanes a to g. No ATP was present in the sample in lane h. ADP (lane i) and ATPγS (lane j) were substituted for ATP in the respective reaction mixtures.

the repair defect of the *E. coli* *ruvA* mutant. The repair capability of the *Tth* *ruvA* gene, however, was less efficient than that of the *E. coli* *ruvA* gene.

To examine the functional interactions between heterologous combinations of RuvA and RuvB *in vivo*, an *E. coli* Δ *ruvAB* strain was transformed with different combinations of the *ruvAB* genes, and the UV sensitivities of the transformants were compared (Fig. 4B). The combination of the *Tth* *ruvA* (pTRA127) and *E. coli* *ruvB* (pYWH501) genes complemented the *E. coli* Δ *ruvAB* strain (HRS3403) as efficiently as the intrinsic combination of the *E. coli* *ruvA* (pAF134) and *E. coli* *ruvB* (pYWH501) genes. Inefficient restoration of DNA repair by *Tth* *ruvA* in the Δ *ruvA* strain (HRS2300) is probably due to lower expression of *E. coli* RuvB from the chromosome than from the multicopy plasmid. Although *E. coli* *ruvA* complemented the UV sensitivity of the Δ *ruvA* strain, the expression level of *ruvB* was reduced compared to that in the wild-type strain after the cells were irradiated with UV at $60 \text{ J} / \text{m}^2$, due to a polar effect of the insertion mutation (data not shown). A higher amount of *E. coli* RuvB may be required to form a fully functional complex with *Tth* RuvA than with *E. coli* RuvA.

On the other hand, consistent with our previous findings that *Tth* *ruvB* does not complement the repair deficiency of an *E. coli* Δ *ruvB* strain (Yamada et al., 1999), neither combinations of *E. coli* *ruvA* (pAF134) and *Tth* *ruvB* (pYWH800) nor *Tth* *ruvA* (pTRA127) and *Tth* *ruvB* (pYWH800) restored the UV sensitivity of the *E. coli* Δ *ruvAB* strain. Possible explanations for these results are: 1) *Tth* RuvB may not function well at the optimum growing temperature for *E. coli* (37°C), a temperature which is much lower than the optimum growing temperatures of the thermophilic bacteria, and/or 2) *Tth* RuvB may not functionally interact with *E. coli* RuvC *in vivo*.

Biochemical properties of *Tth* RuvA protein. *Tth* RuvA was purified from the T7-overexpression system by successive column chromatography as described in MATERIALS AND METHODS. *Tth* RuvA preparations showed a single band in SDS-PAGE (Fig. 5). From 1.5 liters of culture, about 10 mg of pure *Tth* RuvA was obtained. Using this purified protein, we examined the biochemical properties of *Tth* RuvA *in vitro*.

First, we examined the DNA-binding activity and specificity of *Tth* RuvA for four-way junctions (synthetic Holliday junctions). As shown in Fig. 6A, *Tth* RuvA formed two kinds of complexes with the junction DNA: one that migrated relatively quickly (complex I) and the other that migrated more slowly (complex II) through the gel, as observed in the case of the *E. coli* RuvA-Holliday junction complex (Parsons et al., 1992). Complex I corresponds to the junctions bound by one RuvA tetramer, and Complex II corresponds to junctions bound by two RuvA tetramers. *Tth* RuvA did not bind to duplex DNA, even when RuvA

was present at a concentration 200 times higher than that of duplex DNA. This observation clearly demonstrates that *Tth* RuvA has a highly specific affinity for junction DNA, as shown previously for its *E. coli* ortholog (Iwasaki et al., 1992; Parsons et al., 1992).

We next examined the ability of *Tth* RuvA to promote RuvB-mediated branch migration of Holliday junctions. To avoid spontaneous branch migration of the synthetic Holliday junction, all reactions were carried out at 55°C, a temperature slightly lower than the optimal growing temperature (~70°C) of *T. thermophilus* HB8. As shown in Fig. 6B, the *Tth* RuvA-RuvB complex promoted branch migration in a manner that was dependent on the concentration of RuvA present in the reaction mixture. The reaction was also dependent on ATP hydrolysis because neither ADP nor non-hydrolyzable ATP γ S was able to promote *Tth* RuvA-RuvB-associated branch migration.

E. coli RuvB has an intrinsic ATPase activity, which is greatly stimulated by RuvA and duplex DNA, and particularly by circular duplex DNA (Marrione and Cox, 1996; Shiba et al., 1991). The ATPase activity of *Tth* RuvB in the presence of *Tth* RuvA displayed essentially the same characteristics as that of *E. coli* RuvB (Fig. 7).

The branch migration activities of heterologous combinations of *Tth* and *E. coli* proteins were examined (Fig. 8). *Tth* RuvA stimulated the branch migration activities of

Fig. 7. Stimulation of the ATPase activity of *Tth* RuvB by *Tth* RuvA. The ATPase activity of *Tth* RuvB (200 nM) in the presence of *Tth* RuvA at the indicated concentration were measured in the presence (squares) and absence (circles) of dsDNA under the standard conditions at 70°C for 10 min. Each point represents the average of four independent experiments.

Fig. 8. Effects of RuvA on branch migration activity of RuvB in combination with *E. coli* and *T. thermophilus* HB8 proteins. (A) Effects of *E. coli* and *Tth* RuvA proteins on the branch migration activity of *E. coli* RuvB at 37°C. *E. coli* RuvB (150 nM) and various concentrations of *E. coli* RuvA (lanes b to i) or *Tth* RuvA (lanes j to p) were added to reaction mixtures containing 5 nM ³²P-labeled junction and incubated for 10 min at 37°C. (B) Effects of *E. coli* and *Tth* RuvA on the branch migration activity of *Tth* RuvB at 37°C. *Tth* RuvB (150 nM) and various concentrations of *E. coli* RuvA (lanes b to i) or *Tth* RuvA (lanes j to p) were added to reaction mixtures containing 5 nM ³²P-labeled junction and incubated for 10 min at 37°C. (C) Effects of *E. coli* and *Tth* RuvA proteins on the branch migration activity of *Tth* RuvB at 55°C. The reactions were carried out under the same conditions as in (B) except that the reaction mixtures were incubated at 55°C. Protein concentrations: Lane a, no RuvA or RuvB; lane b, no RuvA; lanes c and j, 1 nM RuvA; lanes d and k, 5 nM RuvA; lanes e and l, 10 nM RuvA; lanes f and m, 25 nM RuvA; lanes g and n, 50 nM RuvA; lanes h and o, 100 nM RuvA; and lanes i and p, 200 nM RuvA. Lanes b to p also contained 150 nM RuvB.

RuvB proteins from both *T. thermophilus* HB8 and *E. coli* nearly equally (Figs. 8A and 8B). At 37°C, *E. coli* RuvA stimulated the activity of *Tth* RuvB less efficiently (Fig. 8B). These results are consistent with data from our *in vivo* complementation experiments: *Tth ruvA* complemented the UV repair defect of the *E. coli ΔruvA* mutant, whereas *Tth ruvB* did not complement this defect of the *E. coli ΔruvB* mutant (Fig. 4). At 55°C, which was closer to the optimal physiological temperature for *T. thermophilus* HB8 than 37°C, *Tth* RuvA promoted the branch migration of *Tth* RuvB, while *E. coli* RuvA did not (Fig. 8C). However, the reaction efficiency of branch migration promoted by the *Tth* RuvAB complex was almost the same at both 37° and 55°C (compare Fig. 8C lanes j - p with Fig. 8B lanes j - p) although a larger amount of products would be expected at 55°C. Essentially the same results were reproducibly obtained from several independent experiments. We cannot explain this discrepancy at present.

To evaluate the heat stability of *Tth* RuvA, we performed two assays using *E. coli* RuvA as a control. After RuvA proteins were incubated at 90°C for 1 hr, we examined whether they retained the specific ability of Holliday junction binding by the gel shift assay. Both *E. coli* RuvA and *Tth* RuvA retained full junction-binding activity (data not shown). Similarly, after pre-incubation at 90°C for 1 hr, the ability of RuvA to promote branch migration in association with *E. coli* RuvB was examined at 37°C. The results showed that neither protein exhibited any detectable loss of activity (data not shown). Thus, we conclude that *Tth* RuvA, and surprisingly *E. coli* RuvA as well, are highly heat stable, while *Tth* RuvB is more stable than *E. coli* RuvB in terms of its ATPase and branch migration activities (Yamada et al., 1999). Therefore, the fact that the hetero-complex between *E. coli* RuvA and *Tth* RuvB did not promote branch migration at 55°C (Fig. 8C) cannot be explained simply by the heat lability of *E. coli* RuvA. It is possible that the complex formation is labile because of non-optimal interfaces between the RuvA and RuvB from different bacterial sources.

Conclusions and perspectives. In this study, we cloned the *ruvA* gene from *T. thermophilus* HB8 and analyzed its sequence. Its deduced primary amino acid sequence is 37% identical to that of *E. coli* RuvA. Although this homology is not remarkably high, nearly all the amino acid residues important for RuvA functions, which have been revealed so far by mutational and structural analyses of *E. coli* RuvA, are well conserved in *Tth* RuvA. *Tth ruvA* complements the UV sensitivity of *E. coli ΔruvA* strains. In contrast, *Tth ruvB* does not complement the UV sensitivity of *E. coli ΔruvB* strains. *Tth* RuvA protein exhibits biochemical activities very similar to those exhibited by *E. coli* RuvA. In addition, *Tth* RuvA stimulates the promotion of branch migration by *E.*

coli RuvB *in vitro*, which is well consistent with the results of *in vivo* hetero-complementation experiments.

It is widely believed that proteins from thermophilic organisms are more stable and give rise to superior crystals for X-ray diffraction analysis than the corresponding proteins from mesothermic organisms. Indeed, we have been able to produce *Tth* RuvB crystals suitable for analysis, while we have failed to produce any *E. coli* RuvB crystals (K. Yamada, H. I., T. O., H. S., and K. Morikawa, unpublished results). *Tth* RuvA, in combination with the *Tth* RuvB protein isolated previously (Yamada et al., 1999), now should be excellent material for obtaining crystals of RuvAB proteins in complexes with Holliday junction DNA for X-ray crystallography. This will be critically important for understanding the branch migration mechanism at the atomic level.

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