Cloning, Sequencing, and Expression of *ruvB* and Characterization of RuvB Proteins from Two Distantly Related Thermophilic Eubacteria

JIE TONG AND JAMES G. WETMUR*

Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029

Received 6 November 1995/Accepted 21 February 1996

The *ruvB* genes of the highly divergent thermophilic eubacteria *Thermus thermophilus* and *Thermotoga maritima* were cloned, sequenced, and expressed in *Escherichia coli*. Both thermostable RuvB proteins were purified to homogeneity. Like *E. coli* RuvB protein, both purified thermostable RuvB proteins showed strong double-stranded DNA-dependent ATPase activity at their temperature optima ($\geq 70^\circ\text{C}$). In the absence of ATP, *T. thermophilus* RuvB protein bound to linear double-stranded DNA with a preference for the ends. Addition of ATP or γ -S-ATP destabilized the *T. thermophilus* RuvB-DNA complexes. Both thermostable RuvB proteins displayed helicase activity on supercoiled DNA. Expression of thermostable *T. thermophilus* RuvB protein in the *E. coli* *ruvB* *recG* mutant strain N3395 partially complemented the UV-sensitive phenotype, suggesting that *T. thermophilus* RuvB protein has a function similar to that of *E. coli* RuvB in vivo.

Formation and resolution of homologous recombination intermediates in bacteria depend on RecA and RuvA, -B, and -C proteins. *Escherichia coli* RuvA and RuvB proteins function together to catalyze the branch migration of Holliday junctions (reviewed in references 14, 21, 30, and 31). Although RecA can catalyze branch migration through mismatched sequences, RuvA and RuvB are required both to accelerate branch migration and to bypass insertions greater than 100 bp (11). Individual roles have been assigned to RuvA and RuvB proteins. RuvB is a DNA-dependent ATPase (10, 17) which forms hexameric rings on duplex DNA (18, 27). At high concentrations, RuvB alone can promote branch migration in the presence of ATP and high concentrations of Mg^{2+} (≥ 15 mM) (20, 28), indicating that RuvB is the engine which drives branch migration of Holliday junctions. RuvA is a DNA-binding protein with specificity for Holliday junctions (23). RuvA will form a stable complex with RuvB in solution (25). At low concentrations of Mg^{2+} and RuvB, RuvA is required for branch migration (19, 20). RuvA directs the RuvB helicase to its Holliday junction substrate (9, 22).

E. coli RuvA and RuvB not only function together but are expressed from the same operon. There are two operons in the *E. coli* *ruv* locus. The *ruvA* and *ruvB* genes form an operon that is regulated by LexA (4, 26). The *ruvC* gene is expressed from a separate operon which is located within 2 kb upstream of the *ruvAB* operon.

To date, only two sequences similar to that of *E. coli* *ruvAB* have been identified, one from *Mycobacterium leprae* (GenBank accession number U00011; K. Robison, Harvard University), an organism phylogenetically related to gram-positive bacteria, and the other from *Haemophilus influenzae* (GenBank accession number U32716) (3, 8), a gram-negative bacterium. In both cases, the *ruvA* and *ruvB* genes are contiguous and apparently part of one operon. The presence of *ruvAB* in both gram-negative and gram-positive bacteria suggests conservation of RuvAB function in highly divergent prokaryotes.

RecA proteins have been cloned and expressed from several thermophilic bacteria, including *Thermus thermophilus*, *Ther-*

mus aquaticus, *Thermotoga maritima*, and *Aquifex pyrophilus* (2, 12, 32). In this paper, we report the cloning, sequencing, expression, and purification of RuvB proteins from two distantly related thermophilic eubacteria, *T. thermophilus* and *T. maritima*. Surprisingly, both *T. thermophilus* and *T. maritima* *ruvB* genes were not part of an operon with *ruvA* and did not follow *ruvC*. Nevertheless, these two thermostable RuvB proteins have high amino acid identity with *E. coli* RuvB (55% for both *T. thermophilus* and *T. maritima*). In this work, we demonstrate that the thermostable RuvB proteins have functions similar to those of *E. coli* RuvB in vivo and in vitro. Expression of *T. thermophilus* RuvB partially complemented a mutation in *E. coli* *ruvB*. Both *T. thermophilus* and *T. maritima* RuvB proteins formed stable complexes with duplex DNA and demonstrated strong double-stranded DNA (dsDNA)-dependent ATPase activity. Both *T. thermophilus* and *T. maritima* RuvB proteins unwound supercoiled DNA, with *T. maritima* RuvB showing a greater degree of unwinding within the limits of the assay employed.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* DH5 α , with plasmid pUC19, and NovaBlue(DE3)pLysS, with plasmid pET11c (Novagen), were used for plasmid manipulation and RuvB expression, respectively. *E. coli* N3395 (*recG* *ruvB*) (16) and XAO (*recG*⁺ *ruvB*⁺; *E. coli* Genetic Stock Center) were used for the complementation assay. Supercoiled (form I) pUC19 DNA was purified to homogeneity by equilibrium centrifugation in CsCl-ethidium bromide.

Genomic DNA, nucleotides, and enzymes. All DNA manipulations used standard techniques and procedures (24). Genomic DNAs of *T. thermophilus* and *T. maritima* were kindly provided by David H. Gelfand, Roche Molecular Systems, Inc. All absorbance spectra were determined with a Hewlett-Packard diode array spectrophotometer. Protein concentrations were measured with a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. Deoxynucleoside triphosphates and ATP were purchased from Boehringer-Mannheim. γ -S-ATP was purchased from Sigma. α -³⁵S-dATP and [γ -³²P]ATP were purchased from NEN/DuPont. Amplitaq DNA Polymerase, purchased from Perkin-Elmer, and native *Taq* polymerase, purchased from several suppliers, were used in the buffers supplied by the manufacturers. Calf thymus topoisomerase I (topo I) was purchased from Gibco BRL, Inc. Restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs and used as recommended by the manufacturer.

Oligodeoxynucleotides. All synthetic oligodeoxynucleotide primers for PCR and sequencing were ordered from GENSET. The degenerate sense primer 5'-GCGGAATTCCTCC(G/C)GG(G/C)CT(G/C)GG(G/C)AA(G/A)AC-3' and antisense primer 5'-GCGAGATCTTGGATCTC(G/A)TC(G/A/T)AT(G/A)AA-3', corresponding to amino acids 64 to 69 (PGLGKT) and 111 to 116 (FIDEIH) in *E. coli* RuvB, were used for cloning a fragment of the *T. thermophilus* *ruvB* gene (*EcoRI* [GAATTC] and *BglII* [AGATCT] recognition sequences are underlined). The de-

* Corresponding author. Mailing address: Department of Microbiology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029-6574. Phone: (212) 241-7685. Fax: (212) 534-1684. Electronic mail address: wetmur@msvax.mssm.edu.

FIG. 1. Unrooted evolutionary tree for RuvB proteins. Abbreviations: Tma, *T. maritima*; Tth, *T. thermophilus*; Eco, *E. coli*; Hin, *H. influenzae*; Mle, *M. leprae*.

generate sense primer 5'-GCGGAATTCAG(A/G)GA(T/C)AG(A/G)TT(T/C)G G-3' and antisense primer 5'-GCG-AGATCTAAGTC(T/C)CT(A/C)AC(T/C)CT (T/C)CT-3', corresponding to amino acids 172 to 176 (RDRFG) and 228 to 233 (RRVRDF) in *E. coli* RuvB, were used for cloning a fragment of the *T. maritima* *ruvB* gene. Additional inverse PCR and sequencing primers were synthesized as required. PCR primers for cloning *T. thermophilus* and *T. maritima* *ruvB* genes into pET11c were 5'-GCGCATATGGAAGACCTCGCCCTTAGG-3' plus 5'-GCGGG ATCCAGGTCCAGCTCGGAAAGCGTG-3' and 5'-GCGCATATGAGTGAAT TTCTCACACCT-3' plus 5'-GATGGATCCCTGTTGCTCG-3', respectively, where the *Nde*I and *Bam*HI sites are underlined and the initiation codon in the forward primer is shown in bold italics. Forward *T. thermophilus* and *E. coli* *ruvB* primers for cloning into pUC19 were 5'-GCGAAGCTTGAGGAATTTTAACG CATG followed by additional *ruvB* sequences; reverse primers were 5'-GCGGAA TTC followed by antisense sequences following the translation terminator (the *Hind*III and *Eco*RI sites are underlined).

DNA amplification, cloning, and sequence analysis. PCR amplifications with degenerate and unique primers and inverse PCR on circularized templates were carried out in a Ericomp Powerblock thermocycler and ligated into pUC19 or into the *Nde*I and *Bam*HI sites of pET11c under conditions described previously (32) and transformed in the appropriate strains. Inserts in pET11c were sequenced in both orientations by use of insert-specific and vector-specific oligodeoxynucleotide primers with the Sequenase DNA sequencing kit (U.S. Biochemicals Inc.). Cycle sequencing of PCR products was carried out with *Taq* DNA polymerase by use of ³²P-labeled primers. Nucleic acid and protein sequence analyses were carried out as described previously (32) with GenBank sequences (6) and the Genetics Computer Group (3) and PHYLIP (7) programs.

Expression. NovaBlue(DE3)pLysS cells expressing *T. thermophilus* or *T. maritima* RuvB from a pET11c construct were propagated at 37°C in Luria broth (LB) containing 50 µg of ampicillin per ml and 25 µg of chloramphenicol per ml. Overnight cultures were diluted 1/100 into the same medium, grown to an *A*₆₀₀ of >0.5, induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and grown for an additional 4 to 5 h. Cells were collected, frozen, disrupted, and clarified as described previously (32). The resulting solutions were heated at 70°C for 15 min to denature thermolabile proteins, placed on ice for 30 min to aggregate the denatured proteins, and cleared of denatured proteins by microcentrifugation for 15 min at 4°C.

Purification. Crude RuvB protein, approximately 1 ml/250-ml culture, was loaded onto a 1-ml HiTrap Q anion-exchange column (Pharmacia), repeatedly washed with 20 mM Tris-HCl (pH 8.0 or 9.0 for *T. thermophilus* or *T. maritima* RuvB, respectively), and eluted with 0.2 M NaCl in the same buffer. The eluate was loaded onto a 1-ml HiTrap blue affinity column (Pharmacia). Columns containing *T. thermophilus* or *T. maritima* RuvB were washed extensively with 2 M NaCl–10 mM Tris-HCl (pH 7.8)–1 mM EDTA or with 0.1 M Na acetate in the same buffer, respectively, and eluted in 2.0 M guanidine HCl in that same buffer. After dialysis against 0.2 M KCl–0.1 M Tris-HCl (pH 8.0)–1 mM EDTA and concentration by use of Centricon-30 (Amicon), protein concentrations were determined and compared with complete absorbance spectra to determine an extinction coefficient and to verify removal of nucleic acids. Purification from other proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in which overloaded gels were stained with Coomassie brilliant blue R.

RuvB ATPase assay. The ATPase activity of *T. thermophilus* and *T. maritima* RuvB proteins was measured by colorimetric detection of released P_i as described previously (15). Reaction mixtures (0.5 ml) contained 10 µg of *T. thermophilus* RuvB or 20 µg of *T. maritima* RuvB, 20 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, and 1 mM ATP. Some reactions contained 6 µM (nucleotides) M13 single-stranded DNA, pUC19 linearized by *Eco*RI, or supercoiled pUC19.

RuvB protein-DNA complex formation. PCR-derived linear 337-bp dsDNA at 20 µg/ml was incubated with various concentrations of RuvB protein in binding buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 50 mM NaCl) at 60°C for 1 h prior to electrophoretic separation on a 1% agarose gel, staining with ethidium bromide, and fluorography. Some reactions contained 1 mM ATP or γ-S-ATP or 20 µg of competing PCR-derived linear 1,079-bp dsDNA per ml.

Topological assay for DNA unwinding by *T. thermophilus* and *T. maritima* RuvB. A topological assay for DNA unwinding was performed as described previously (1). Supercoiled pUC19 (30 µM in nucleotides) was incubated with 4.0 µM *T. thermophilus* or *T. maritima* RuvB in a 100-µl reaction mixture containing 30 mM Tris-HCl (pH 8.0), 30 mM MgCl₂, 0.1 mM EDTA, 30 mM NaCl, 0.5 mM dithiothreitol, and 30 µg of bovine serum albumin per ml at 60°C for 15 min. Some samples contained 0.5 mM ATP and/or 0.5 mM γ-S-ATP. After 5 min of temperature equilibration at 37°C, each incubation was continued for 1 h with 6 U of calf thymus topo I. The reaction was stopped by adding SDS, EDTA, and proteinase K to 0.4% (wt/vol), 40 mM, and 20 µg/ml, respectively. DNA was extracted with phenol-chloroform, ethanol precipitated, resuspended, and analyzed by electrophoresis in 1.5% agarose in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]) at ~2 V/cm for 14 h. Two-dimensional gel analyses were performed as described previously (5, 13). The first-dimension analysis was the same as described above. The gel was soaked in the same buffer plus 1 µM chloroquine for 1 h prior to electrophoresis. The second dimension analysis was carried out for the same time with the chloroquine-containing TAE buffer. Gels were stained with ethidium bromide and analyzed by fluorography.

RuvB complementation assay. Polyclonally expressed pUC19 plasmids pTth and pEc, expressing *T. thermophilus* and *E. coli* *ruvB* genes, respectively, were constructed such that a ribosome binding site and a stop codon were introduced into the pUC19 *lac* gene at the *Hind*III site and followed immediately by *ruvB* initiation codons. Single colonies of wild-type *E. coli* XAO or *ruvB* *recG* strain N3395 containing pUC19 or one of the expression plasmids were grown to the late log phase in LB-ampicillin. Cells were diluted to ~10⁴ CFU/0.1 ml, plated in the same medium, and exposed to a calibrated 254-nm UV source for various times. After overnight incubation at 37°C, the surviving fraction was determined as the CFU on exposed plates divided by the CFU on unexposed plates.

Nucleotide sequence accession numbers. The nucleotide sequences of *T. maritima* and *T. thermophilus* RuvB proteins have been deposited in GenBank under accession numbers U22817 and U38840, respectively.

RESULTS

Cloning and sequencing of *T. thermophilus* and *T. maritima* *ruvB* genes. Degenerate primers corresponding to conserved amino acid sequences in *E. coli* and *M. leprae* were used to amplify, clone, and sequence fragments of the *T. thermophilus* and *T. maritima* *ruvB* genes. The complete sequences were determined by inverse PCR, and amplified full-length genes were cloned into the expression plasmid pET11c. The *T. thermophilus* and *T. maritima* *ruvB* sequences were confirmed by sequence identity of at least two independently derived clones expressing a thermostable RuvB protein. The guanine-plus-cytosine content of *T. thermophilus* and *T. maritima* RuvB proteins were 70 and 50%, respectively.

The *T. thermophilus* and *T. maritima* translated amino acids sequences were 54% identical to each other, and both were 55% identical to *E. coli* RuvB. Figure 1 shows an unrooted evolutionary tree of all RuvB sequences known to date. *T. thermophilus* and *T. maritima* RuvB proteins are as divergent from one another and from mesophilic gram-negative organisms as are mesophilic gram-negative organisms from mesophilic gram-positive organisms.

Expression and purification of *T. thermophilus* and *T. maritima* RuvB proteins. The pET11c plasmids expressing *T. thermophilus* or *T. maritima* RuvB were transformed into Nova Blue(DE3)pLys which is an *endA* mutant rather than BL21(DE3)pLys, because BL21 expresses endonuclease I, which was determined to be not only thermostable but thermoactive. *T. thermophilus* and *T. maritima* RuvB proteins were expressed at 10 to 20% of total cellular protein (Fig. 2, lanes 2). The extensively purified crude fractions subsequent to the heat treatment step are visible in lanes 3 of Fig. 2. In the absence of expression of thermostable RuvB (vector without insert), no protein band the size of RuvB was observed (data not shown). Only one protein band was visible following anion and affinity chroma-

FIG. 2. SDS-PAGE analysis of *T. thermophilus* (Tth) (A) and *T. maritima* (Tma) (B) RuvB proteins at each step of purification. Lanes: 1, molecular weight marker (k, thousand); 2, induced cell lysate; 3, fraction after heating at 70°C; 4, fraction eluted from HiTrap Q column; 5, fraction eluted from HiTrap blue column. The SDS-polyacrylamide gel was 12.5% polyacrylamide and was stained with Coomassie brilliant blue R.

tography (Fig. 2, lanes 4 and 5). The HiTrap blue affinity chromatography was necessary for removing residual nucleic acids. Both *T. thermophilus* and *T. maritima* RuvB proteins were purified to homogeneity as determined both by SDS-PAGE (Fig. 2, lanes 5) and on absorbance spectra (data not shown). All subsequent experiments were carried out with these purified RuvB proteins.

ATPase activity of thermostable *T. thermophilus* and *T. maritima* RuvB. Figure 3 depicts the temperature dependence of ATPase activity of *T. thermophilus* RuvB (A) and *T. maritima* RuvB (B) proteins and their dependence on various DNA substrates. *T. thermophilus* and *T. maritima* RuvB proteins have activity optima at 70 and 80°C, respectively. At 70°C, *T. thermophilus* RuvB rates of ATP hydrolysis were 1.2 (no DNA), 1.2 (single-stranded DNA), 17 (linear pUC19 dsDNA), and 20 (supercoiled pUC19 DNA) mol of ATP hydrolyzed per min per mol of RuvB. At 80°C, *T. maritima* RuvB rates of ATP hydrolysis were 1.2 (no DNA), 1.9 (single-stranded DNA), 13 (linear dsDNA), and 11 (supercoiled DNA) mol of ATP hydrolyzed per min per mol of RuvB. The equivalent stimulation of *T. thermophilus* and *T. maritima* RuvB ATPase activity by linear or supercoiled dsDNA sharply contrasts with the greater stimulation of *E. coli* RuvB by supercoiled DNA (17, 18).

Binding of *T. thermophilus* and *T. maritima* RuvB proteins to linear dsDNA. Figure 4 depicts a gel retardation assay for *T. thermophilus* RuvB binding to 337-bp linear DNA at 60°C. Increasing *T. thermophilus* RuvB concentration led to increasing numbers of well-spaced bands (Fig. 4, lanes b to f), indicating complexes with increasing numbers of *T. thermophilus* RuvB molecules. The binding was apparently noncooperative. In contrast, complexes of *T. maritima* RuvB and DNA did not produce well-spaced bands (data not shown), indicating either heterogeneity of structure or instability of the complexes. Addition of ATP (1 mM) to *T. thermophilus* RuvB-DNA complexes led to release of bound *T. thermophilus* RuvB (Fig. 4, lane g). γ -S-ATP (1 mM) had the same effect (data not shown).

Figure 5 depicts a competition assay for *T. thermophilus* RuvB binding to dsDNA of equal masses of two different

FIG. 3. Temperature dependence of ATPase activity of *T. thermophilus* RuvB (A) and *T. maritima* RuvB (B) proteins in the presence of circular M13 ssDNA (closed squares), linear pUC19 (closed triangles), supercoiled pUC19 (open circles), or no DNA (open diamonds) in a 30-min reaction.

FIG. 4. Binding of *T. thermophilus* (Tth) RuvB to linear duplex DNA. Lanes: a, DNA marker; b to g, 0.2 μ g of 337-bp PCR product incubated with the indicated concentrations of RuvB protein in binding buffer for 1 h at 60°C in the absence (lanes b to f) or presence (lane g) of ATP (1 mM). Reaction products were analyzed by electrophoresis on a 1% agarose gel.

lengths (337 and 1,079 bp) under the same conditions as those described above for Fig. 4. The extent of formation of complexes with one or more RuvB molecules was independent of the length of the dsDNA molecules, indicating a preference for binding at the ends of the molecules (see especially Fig. 5, lanes e to h). The preference for ends is not exclusive. *T. thermophilus* RuvB will also bind to supercoiled DNA (see Fig. 6). The strong binding of *T. thermophilus* RuvB to the ends of linear dsDNA is consistent with the strong stimulation of *T. thermophilus* (and *T. maritima*) RuvB ATPase activity by linear dsDNA.

Unwinding supercoiled DNA by thermostable *T. thermophilus* and *T. maritima* RuvB proteins. Figure 6 demonstrates the effect of thermostable RuvB proteins on topoisomer distribution in relaxed supercoiled dsDNA as determined by use of one-dimensional (Fig. 6A and B) and two-dimensional (Fig. 6C to E) gels. One- and two-dimensional gels of supercoiled pUC19 DNA after treatment with topo I only are depicted in Fig. 6A and B, lanes a, and Fig. 6C, respectively. One positive and one negative topoisomer with less than integral writhing number contributed to the top band (relaxed) on the one-dimensional gels. The additional bands were positively supercoiled, with increasing mobility corresponding to increasing writhing number. Addition of *T. thermophilus* RuvB or *T. maritima* RuvB without ATP or γ -S-ATP resulted in little change in the topoisomer distribution (Fig. 6A and B, lanes e).

Addition of *T. thermophilus* RuvB and both ATP and γ -S-ATP resulted in a small shift toward negative superhelicity (Fig. 6D) and the appearance of numerous additional faint bands representing topoisomers of increasing negative superhelicity (Fig. 6A, lane b). Addition of ATP without γ -S-ATP eliminated the small shift toward negative superhelicity but retained the additional faint negative topoisomers (Fig. 6A, lane c). Addition of γ -S-ATP without ATP resulted in the same small shift toward negative superhelicity but increased the fraction of highly negatively supercoiled species to the extent that they represented a major fraction (supercoiled) of topoisomers (Fig. 6A, lane d).

Addition of *T. maritima* RuvB and both ATP and γ -S-ATP resulted in a large shift toward negative superhelicity (Fig. 6E) and the appearance of numerous additional very faint bands representing topoisomers of increasing negative superhelicity

(Fig. 6B, lane b). In contrast to *T. thermophilus* RuvB, addition of *T. maritima* RuvB and ATP without γ -S-ATP resulted in little change compared with *T. maritima* RuvB alone (lane c). Addition of γ -S-ATP without ATP resulted in the same large shift toward negative superhelicity and an increase in the fraction of more negatively supercoiled species (lane d). Unlike differences in the effects of cofactors, the apparent differences in the extent of unwinding of superhelical DNA by *T. thermophilus* and *T. maritima* RuvB may be a function of the assay, where the helicase and topo I have different temperature optima.

T. thermophilus RuvB can complement *E. coli* RuvB in vivo.

To determine whether *T. thermophilus* RuvB could substitute for *E. coli* RuvB helicase in vivo, the *E. coli* *ruvB revG* double mutant strain N3395 was used as a host for control pUC19 and for pUC19-based plasmids pTth or pEc expressing *T. thermophilus* RuvB and *E. coli* RuvB, respectively. Both expression plasmids produced RuvB proteins when induced, although pEc was more efficient (data not shown). Figure 7 depicts the UV sensitivity of these strains. The UV resistance was 5- to 10-fold greater for the pTth strain than for the control pUC19 strain for the UV doses tested, although the strain was still UV sensitive compared with the pEc strain or with wild-type (*ruvB*⁺) *E. coli*.

DISCUSSION

In this study, the *ruvB* genes of the highly divergent thermophilic eubacteria *T. thermophilus* and *T. maritima* were cloned, sequenced, and expressed in *E. coli*. *T. thermophilus*, *T. maritima*, mesophilic gram-negative bacteria, and mesophilic gram-positive bacteria are equally divergent. RuvA and RuvB are expressed from a single operon in both gram-negative (*E. coli* and *H. influenzae*) and gram-positive (*M. leprae*) mesophilic bacteria. Surprisingly, the sequence 1,000 or 470 bp immediately upstream from *T. thermophilus* and *T. maritima* *ruvB*, respectively, contained no sequences similar to those of *ruvA*. In fact, a search of GenBank revealed that the translation of the *T. thermophilus* upstream sequence encoded, in the opposite orientation, a protein similar to *E. coli* PanB. The sequence of the *T. maritima* upstream open reading frame did not show significant identity to any bacterial sequences in GenBank. The absence of linkage of *ruvA* to *ruvB* in the two thermophilic genera (i) may be fortuitous or (ii) may suggest

FIG. 5. Competitive binding of two different sizes of linear dsDNA with *T. thermophilus* (Tth) RuvB protein. A 0.2- μ g amount of 337-bp and 0.2 μ g of 1,079-bp PCR product (lanes b to j), 0.2 μ g of 337-bp PCR product alone (lane k), or 0.2 μ g of 1,079-bp PCR product alone (lane l) was incubated with the indicated concentrations of *T. thermophilus* RuvB protein in binding buffer for 1 h at 60°C. Lane a, molecular weight markers. Reaction products were analyzed by electrophoresis on a 1% agarose gel.

FIG. 6. Detection of topoisomers of supercoiled pUC19 DNA following *T. thermophilus* (Tth) and *T. maritima* (Tma) RuvB-mediated unwinding and topo I treatment on one- (A and B) and two- (C to E) dimensional agarose gels. (A and B) Relaxed band, writhing number is 0; supercoiled band, highly negatively supercoiled. (C to E) Two-dimensional gels followed incubation with ATP (0.5 mM) and γ -S-ATP (0.5 mM) at 60°C for 15 min before topo I treatment. (C) No RuvB; (D) *T. thermophilus* RuvB; (E) *T. maritima* RuvB. First dimension, top to bottom; R, relaxed; sign (+, -), direction of supercoiling. Second dimension, left to right.

that thermophilic RuvB binding to Holliday junctions is independent of RuvA or depends on another protein. Given the partial complementation of a *ruvB recG* *E. coli* by *T. thermophilus* RuvB, suggesting that *T. thermophilus* RuvB and *E. coli* RuvB have a similar function in vivo, the fortuitous option is the most likely possibility. Incomplete complementation was to be expected because of the low helicase activity of *T. thermophilus* RuvB at 37°C. Initial attempts to clone genomic sequences encoding a protein similar to RuvA from several thermophilic bacterial species have been unsuccessful but may reflect the poorer conservation of RuvA amino acid sequences between highly divergent genera compared with that of RuvB amino acid sequences. Additional future efforts will be directed toward cloning genes which express thermophilic RuvA proteins.

T. thermophilus, *T. maritima*, and mesophilic RuvB proteins contain conserved regions including GxGKT and DExH motifs, which are shared by several ATP-dependent helicases in *E. coli* (29), consistent with the conservation of the function of these thermostable RuvB proteins. Although *T. thermophilus* and *T. maritima* RuvB showed functional similarity to *E. coli* RuvB in their dsDNA affinity, dsDNA-dependent ATPase activity, and ability to unwind supercoiled DNA, *T. thermophilus* and *T. maritima* RuvB proteins showed some unique properties. (i) *E. coli* RuvB ATPase activity is preferentially stimulated by supercoiled DNA (17, 18). However, *T. thermophilus* and *T. maritima* RuvB ATPase activities were stimulated as

FIG. 7. UV survival of *E. coli* *recG ruvB* mutant strain N3395 containing pUC19 (open circles), pTth expressing *T. thermophilus* RuvB protein (closed triangles), pEc expressing *E. coli* RuvB protein (closed squares), and wild-type strain XAO (open diamonds). Each datum point is the average of four experiments. The error bars indicate the average $\pm$ standard deviation.

well by linear dsDNA. This result is consistent with our additional observation that *T. thermophilus* RuvB protein has a preference for binding to the ends of linear dsDNA. Furthermore, increasing the assay temperature increases the pitch of the DNA helix, decreasing the negative superhelicity of the supercoiled DNA and making it more like relaxed circular and linear dsDNA. (ii) *E. coli* RuvB requires ATP or γ -S-ATP for the binding of DNA (19). However, *T. thermophilus* and *T. maritima* RuvB proteins form complexes with linear DNA which are stable to electrophoresis in the absence of nucleotide cofactors. In fact, adding ATP or γ -S-ATP destabilized *T. thermophilus* RuvB-dsDNA complexes, suggesting that the hydrolysis of ATP might be involved in the dissociation of *T. thermophilus* RuvB from DNA. At 70°C, γ -S-ATP was slowly hydrolyzed by *T. thermophilus* RuvB (data not shown). (iii) With *E. coli* RuvB, transiently unwound forms of supercoiled DNA could be detected only after incubation and topo I treatment in the presence of both ATP and γ -S-ATP (1). However, with *T. thermophilus* and *T. maritima* RuvB, the greatest unwinding of supercoiled DNA was detected in the presence of γ -S-ATP alone, followed by the unwinding observed in the presence of a mixture of ATP and γ -S-ATP. Studies with additional substrates may help to elucidate the significance of these differences.

ACKNOWLEDGMENTS

We are grateful to Barry Rosenstein of this institution for calibration of the UV source, to David Gelfand of Roche Molecular Systems for genomic DNA from *T. aquaticus* and *T. maritima*, to Jen-i Mao of Genome Therapeutics Corp. for providing the *M. leprae* cosmid containing the *ruv* genes, to Garry J. Sharples of the University of Nottingham (Nottingham, United Kingdom) for providing the *ruvB recG* mutant strain *E. coli* N3395, and to John A. Blaho of this institution for helpful advice on the topological assay and two-dimensional gel electrophoresis and for critical reading of the manuscript.

This research was supported by a grant from the National Institutes of Health (HG00446).

REFERENCES

- Adams, D. E., and S. C. West. 1995. Unwinding of closed circular DNA by the *Escherichia coli* RuvA and RuvB recombination/repair proteins. *J. Mol. Biol.* **247**:404–417.
- Angov, E., and R. D. Camerini-Otero. 1994. The *recA* gene from the thermophile *Thermus aquaticus* YT-1: cloning, expression, and characterization. *J. Bacteriol.* **176**:1405–1412.
- Benson, D., D. J. Lipman, and J. Ostell. 1993. GenBank. *Nucleic Acids Res.* **21**:2963–2965.
- Benson, F. E., G. T. Illing, G. J. Sharples, and R. G. Lloyd. 1988. Nucleotide sequencing of the *ruv* region of *Escherichia coli* K-12 reveals a LexA regulated operon encoding two genes. *Nucleic Acids Res.* **16**:1541–1549.
- Blaho, J. A., J. E. Larson, M. J. McLean, and R. D. Wells. 1988. Multiple DNA secondary structures in perfect inverted repeat inserts in plasmids. *J. Biol. Chem.* **263**:14446–14455.
- Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387–395.
- Felsenstein, J. 1989. PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5**:164–166.
- Fleischman, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness et al. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**:496–512.
- Hion, K., and S. C. West. 1995. Branch migration during homologous recombination: assembly of a RuvAB-Holliday junction complex in vitro. *Cell* **80**:787–793.
- Iwasaki, H., T. Shiba, K. Makino, A. Nakata, and H. Shinagawa. 1989. Overproduction, purification, and ATPase activity of the *Escherichia coli* RuvB protein involved in DNA repair. *J. Bacteriol.* **171**:5276–5280.
- Iype, L. E., E. A. Wood, R. B. Inman, and M. M. Cox. 1994. RuvA and RuvB proteins facilitate the bypass of heterologous DNA insertions during RecA protein-mediated DNA strand exchange. *J. Biol. Chem.* **269**:24967–24978.
- Kato, R., and S. Kuramitsu. 1993. RecA protein from the extremely thermophilic bacterium, *Thermus thermophilus* HB8. *J. Biochem. (Tokyo)* **114**:926–929.
- Kilpatrick, M. W., J. Klysik, C. K. Singleton, D. A. Zarling, T. M. Jovin, L. H. Hanau, B. F. Erlanger, and R. D. Wells. 1984. Intervening sequences in human fetal globin genes adopt left-handed Z helices. *J. Biol. Chem.* **259**:7268–7274.
- Kowalczykowski, S. C., D. A. Dixon, A. K. Eggleston, S. D. Lauder, and W. M. Rehrauer. 1994. Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.* **58**:401–465.
- LeBel, D., G. G. Poirier, and A. R. Beaudoin. 1978. A convenient method for the ATPase assay. *Anal. Biochem.* **85**:86–89.
- Lloyd, R. G. 1991. Conjugational recombination in resolvase-deficient *ruvC* mutants of *Escherichia coli* K-12 depends on *recG*. *J. Bacteriol.* **173**:5414–5418.
- Marriane, P. E., and M. M. Cox. 1995. RuvB protein-mediated ATP hydrolysis: functional asymmetry in the RuvB hexamer. *Biochemistry* **34**:9809–9818.
- Mitchell, A. H., and S. C. West. 1994. Hexameric rings of *Escherichia coli* RuvB protein. Cooperative assembly, processivity and ATPase activity. *J. Mol. Biol.* **243**:208–215.
- Müller, B., I. R. Tsaneva, and S. C. West. 1993. Branch migration of Holliday junctions promoted by the *Escherichia coli* RuvA and RuvB proteins. II. Interaction of RuvB with DNA. *J. Biol. Chem.* **268**:17185–17189.
- Müller, B., I. R. Tsaneva, and S. C. West. 1993. Branch migration of Holliday junctions promoted by the *Escherichia coli* RuvA and RuvB proteins. I. Comparison of RuvAB- and RuvB-mediated reactions. *J. Biol. Chem.* **268**:17179–17184.
- Müller, B., and S. C. West. 1994. Processing of Holliday junctions by the *Escherichia coli* RuvA, RuvB, RuvC, and RecG proteins. *Experientia* **50**:216–222.
- Parsons, C. A., A. Stasiak, R. J. Bennett, and S. C. West. 1995. Structure of a multisubunit complex that promotes DNA branch migration. *Nature (London)* **374**:375–378.
- Parsons, C. A., I. Tsaneva, R. G. Lloyd, and S. C. West. 1992. Interaction of *Escherichia coli* RuvA and RuvB proteins with synthetic Holliday junctions. *Proc. Natl. Acad. Sci. USA* **89**:5452–5456.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
- Shiba, T., H. Iwasaki, A. Nakata, and H. Shinagawa. 1993. *Escherichia coli* RuvA and RuvB proteins involved in recombination repair: physical properties and interactions with DNA. *Mol. Gen. Genet.* **237**:395–399.
- Shinagawa, H., K. Makino, M. Amemura, S. Kimura, H. Iwasaki, and A. Nakata. 1988. Structure and regulation of the *Escherichia coli* *ruv* operon involved in DNA repair and recombination. *J. Bacteriol.* **170**:4322–4329.
- Stasiak, A., I. R. Tsaneva, S. C. West, C. J. B. Benson, X. Yu, and E. H. Egelman. 1994. The *Escherichia coli* RuvB branch migration protein forms double hexameric rings around DNA. *Proc. Natl. Acad. Sci. USA* **91**:7618–7622.
- Tsaneva, I. R., B. Müller, and S. C. West. 1992. ATP-dependent branch migration of Holliday junctions promoted by the RuvA and RuvB proteins of *E. coli*. *Cell* **69**:1171–1180.
- Tsaneva, I. R., B. Müller, and S. C. West. 1993. RuvA and RuvB proteins of *Escherichia coli* exhibit DNA helicase activity *in vitro*. *Proc. Natl. Acad. Sci. USA* **90**:1315–1319.
- West, S. C. 1994. The processing of recombination intermediates: mechanistic insights from studies of bacterial proteins. *Cell* **76**:9–15.
- West, S. C. 1995. Formation, translocation and resolution of Holliday junctions during homologous genetic recombination. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **347**:21–25.
- Wetmur, J. G., D. M. Wong, B. Ortiz, J. Tong, F. Reichert, and D. H. Gelfand. 1994. Cloning, sequencing, and expression of RecA proteins from three distantly related thermophilic eubacteria. *J. Biol. Chem.* **269**:25928–25935.