JOURNAL OF BACTERIOLOGY, Feb. 1977, p. 1110-1120

Vol. 129, No. 2
Printed in U.S.A.

Isolation and Properties of a Thermostable Restriction Endonuclease (Endo R·*Bst*1503)

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Received for publication 2 August 1976

A restriction endonuclease was isolated from *Bacillus stearothermophilus* 1503-4R (*Bst*1503) and purified to homogeneity. The enzyme required Mg^{2+} ion as a cofactor. *Bst*1503 exhibited maximal activity between pH 7.5 and 8.0, between 60 and 65°C, and with about 0.2 mM Mg^{2+}. *Bst*1503 was not inactivated after exposure at 55 or 65°C for up to 10 h. After 2 h of incubation at 70°C, *Bst*1503 was inactivated by 65%. *Bst*1503 was rapidly inactivated at 75°C. A single protein-staining band having a molecular weight of 46,000 was observed when *Bst*1503 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was found to exist in two active forms, the predominating form with an *S* value of 8.3 (180,000) and the second form with an *S* value of 5.4 (96,000). No conversion between the 8.3*S* and 5.4*S* forms was observed after storage. *Bst*1503 recognized six sites in TP-1C deoxyribonucleic acid (DNA), one site in pSC101 and simian virus 40 DNAs, and three sites in λ*vir* DNA. *Bst*1503 and *Bam*HI were determined to be isoschizomers. The effect of temperatures on the activity and stability of *Bam*HI was determined.

Studies on the structure and function of deoxyribonucleic acid (DNA) have been greatly aided by the use of bacterial restriction endonucleases. Many of these specific endonucleases recognize and cleave a specific sequence of base pairs in DNA.

Studies on the structure of these specific endonucleases are also of interest because of the specificity of the protein-DNA interaction. Reports dealing with the isolation of restriction endonucleases and the determination of their sequence specificities, however, are far in excess of those reports dealing with their structure and catalytic properties.

Restriction endonucleases have traditionally been placed into two groups based on their cofactor requirements, catalytic properties, and subunit structure. The nomenclature used for restriction and modification enzymes is that suggested by Smith and Nathans (35). Type I restriction endonucleases *Eco*B (9, 20, 31), *Eco*K (25), and *Hin*dI (12) require adenosine 5'-triphosphate (ATP), *S*-adenosylmethionine (SAM), and Mg^{2+} for activity. Endo R·*Eco*P1 (13) requires ATP and Mg^{2+} and is stimulated by SAM. With the exception of the small circular DNA genomes that contain a single site, type I enzymes do not appear to produce limit digests with the various DNA substrates (15, 16).

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*Eco*K, *Eco*B, and *Eco*P1 have molecular weights of about 400,000 (26), 450,000 (9), and 200,000 (13), respectively. Endo R·*Eco*B can exist in several forms (9), each containing in some ratio the subunits α (135,000), β (60,000), and γ (55,000). Endo R·*Eco*K does not appear to exist in several forms and has subunits identical to those of *Eco*B (α, 135,000; β, 62,000; and γ, 52,000; 26). Endo R·*Eco*P1 is composed of three subunits having molecular weights of 90,000, 62,000, and 49,000, respectively (1).

*Eco*K and *Eco*B exhibit adenosine 5'-triphosphatase activity after restriction cleavage (10, 26).

Endo R·*Eco*K (14), Endo R·*Eco*B (39, 40), and Endo R·*Eco*P1 (14) exhibit modification methylase activity. Meth M·*Eco*B (17) and Meth M·*Eco*P1 (5) do not exhibit restriction endonuclease activity, and no modification methylase having K-type specificity has been isolated.

Modification methylase *Eco*B has also been shown to exist in several forms, depending on the environment of the enzyme during storage (molecular weight of 115,000 to 235,000; 17). Each form contains in some ratio two different subunits with molecular weights of 60,000 and 55,000. These polypeptides are indistinguishable by polyacrylamide gel electrophoresis from the β and γ subunits of the *Eco*B restriction endonuclease (17). These results suggest that the *Eco*B restriction endonuclease and modifi-

cation methylase share these two subunits.

Meth M·*Eco*P1 has only one form, with a molecular weight of 115,000, which contains two nonidentical subunits (4).

In the *Eco*B, *Eco*K, and *Eco*P1 restriction and modification (R+M) systems, about half of the single-step mutants have the phenotype R^-M^- and half have the phenotype R^-M^+ (44). The letters R and M refer to restriction and modification ability, respectively.

Complementation studies (3) using a single-step mutant (R^-M^-) and a two-step mutant (R^-M^-) led to the proposal of the "three-gene model" for restriction and modification in *Escherichia coli* strain K, B, and P1 lysogens (1). This model postulated that a gene (*hsdS*) codes for a polypeptide that is responsible for the site recognition for both restriction and modification. The two other genes code for polypeptides responsible for restriction or DNA cleavage (gene *hsdR*) and modification or DNA methylation (gene *hsdM*).

The genetic studies are consistent with the subunit composition of the type I R+M systems in that the restriction enzyme contains all three of the gene products (*hsdR* = α; *hsdM* = β; *hsdS* = γ), and the modification enzyme contains the *hsdM* and *hsdS* gene products. Since the *hsdM* polypeptide (β subunit) is also required for restriction activity, a mutation in the *hsdM* gene would also affect restriction activity (producing the phenotype R^-M^-).

A majority of the restriction endonucleases isolated, however, are of the type II variety. These enzymes require Mg^{2+} as a cofactor and produce characteristic limit digests with DNA substrates.

The type II restriction endonucleases have smaller molecular weights than the type I enzymes and generally contain two subunits of identical size. *Hin*dII has been assigned a molecular weight ranging from 67,000 to 92,000 (1, 36). Restriction endonucleases *Hpa*I and *Hpa*II have molecular weights of about 65,000 and 13,370, respectively (33). Endo R·*Eco*RII has a molecular weight of about 86,000 (32) and is composed of two identical subunits with a molecular weight of about 40,000 (32).

Restriction endonuclease *Eco*RI has a molecular weight of about 59,000 and a subunit molecular weight of about 29,500 (11). The *Eco*RI methylase is a monomeric protein with a molecular weight of about 40,000 (32).

From functional and structural considerations, type II enzymes are less complex than type I enzymes. Genetic evidence for distinctions between the two types, however, is less convincing. Bannister and Glover (2) isolated single-step mutants having the phenotypes R^-M^- and R^-M^+ from the *Eco*RI and *Eco*RII R+M systems. In contrast, Yoshimori et al. (46), using the same R + M systems, were only able to isolate the R^-M^+ phenotype.

A "two-gene model" was proposed for type II restriction and modification systems (46). In this model, a mutation in the *hsdM* gene would lead to the R^-M^- phenotype. This would imply that the restriction endonuclease has two types of subunits and that one of them alone is sufficient for modification. Neither the "three-gene" nor the "two-gene" model is consistent with the subunit composition reported for the *Eco*RI endonuclease and methylase.

There are no experimental data demonstrating that type II restriction endonucleases and modification methylases share a common subunit. This conclusion is difficult to reconcile with the observation that a single-step mutation can result in equal numbers of the phenotypes R^-M^- and R^-M^+.

A restriction and modification system has been detected in each of five strains of *B. stearothermophilus*: strains 4S (6, 19), 1503-4R (6), and 2184, 10, and 9 (unpublished data). Preliminary studies on the isolation and characterization of Endo R·*Bst*4 have been reported by Catterall et al. (6). *Bst*4 requires Mg^{2+}, is stimulated by SAM, and does not produce limit digests with DNA substrates. On the basis of cofactor requirements and catalytic properties, Endo R·*Bst*4 is a type I restriction endonuclease.

This paper describes the purification and characterization of a type II restriction endonuclease from *B. stearothermophilus* 1503-4R (*Bst* 1503).

MATERIALS AND METHODS

Strains and growth conditions. *B. stearothermophilus* 1503-4R was grown in 2% Trypticase (BBL)-0.5% yeast extract (Difco)-0.5% glucose (TYG) medium containing $FeCl \cdot 6H_2O$ (7 mg/liter), $MnCl_2 \cdot 4H_2O$ (1 mg/liter), and $MgSO_4 \cdot 7H_2O$ (15 mg/liter).

A 14-liter fermentor vessel containing 11 liters of TYG medium was inoculated with 1.1 liters of culture prepared as follows. A 2,800-ml Fernbach flask containing 1.1 liters of TYG medium was inoculated with 12- to 15-h cells from a 2% Trypticase-2% agar (Difco) plate and shaken on a New Brunswick model G-25 gyratory incubator shaker for 3 h at 55°C. The fermentor culture was agitated and aerated at 55°C in a New Brunswick fermentor (model FS 114). Growth was measured in a Bausch & Lomb Spectronic-20 colorimeter at 525 nm. *Bacillus amyloliquefaciens* H (RUB 500) was obtained from G. Wilson.

DNA. Lysates of thermophilic phage TP-1C were prepared and quantitated by the methods described by Welker and Campbell (42). Phage were isolated

by a modification of the procedure described by Yamamoto et al. (45). Fresh phage lysates were placed at 5°C overnight so that the cell debris could settle to the bottom of the container. The supernatant fluids were decanted, and the debris was removed by centrifugation. The supernatant fluids were heated to 37°C and treated with 1 μg of deoxyribonuclease (Sigma) and ribonuclease (Sigma) per ml for 1 h. Solid sodium chloride was added to a concentration of 0.5 M, and the solution was equilibrated at 5°C. Solid polyethylene glycol (PEG; average molecular weight, 6,000 to 7,500; Matheson, Coleman and Bell) was added with stirring to a concentration of 10% (wt/vol). After the PEG was dissolved, the mixture was allowed to stand at 5°C for at least 20 h or until a precipitate appeared at the bottom of the container. The supernatant fluids were decanted to a level above the precipitate. The precipitate was collected by centrifugation at 8,000 × *g* for 20 min. The phage pellets were combined and suspended in 12 to 15 ml of 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5) containing 10 mM NaCl and 1 mM $CaCl_2$ (TNC buffer). The recovery of phage was between 80 and 90%.

Phage TP-1C DNA was extracted as previously described by Streips and Welker (38) except that the phenol was saturated with TNC buffer. The DNA was purified by the procedure of Marmur (23). Phage TP-1C DNA was stored in 0.15 M NaCl-0.015 M sodium citrate buffer, pH 7.0 (SSC).

Phage λ*vir* DNA and plasmid pSC101 DNA were provided by T. T. Wu and J. E. Donelson, respectively. Samples of simian virus 40 (SV40) DNA were provided by T. Vogel, R. Kotesware, and L. Overby. Phage φX174 and f1 DNAs were provided by L. Dumas.

All DNA samples were stored in SSC at 5°C.

Restriction endonucleases. Restriction endonuclease *Bam*HI was prepared using the procedures described by Wilson and Young (43; personal communication). Endo R·*Eco*RI was purchased from Miles Laboratories.

Restriction endonuclease assay. Endo R·*Bst*1503 assay mixture contained 10 mM Tris-hydrochloride, 0.2 mM $MgCl_2$, and 6.6 mM 2-mercaptoethanol (assay buffer), pH 7.8, and 1 μg of phage TP-1C DNA in a final volume of 50 μl. Reactions were run in 0.5-ml capped polyethylene sample tubes (Bel-Art Products). After equilibration at 55°C, the reaction was initiated by the addition of 5 μl of an enzyme preparation. The reaction was stopped by the addition of 5 μl of 100 mM ethylenediaminetetraacetic acid (EDTA) containing 0.125% bromophenol blue and 40% sucrose. Reaction products were analyzed by agarose-ethidium bromide gel electrophoresis (33). Samples (60 μl) were loaded on 1.0% agarose gels (6.8 cm long) containing 0.5 μg of ethidium bromide per ml. Electrophoresis was carried out at 5 mA/gel for about 100 min at 12°C.

Undiluted column fractions were assayed for 30 min at 55°C. Fractions containing low concentrations of enzyme were assayed for 1 h at 62°C.

A unit of Endo R·*Bst*1503 activity is defined as that amount of enzyme required to completely cleave 1 μg of native phage TP-1C DNA into DNA fragments (limit digest) in 30 min at 55°C. Units of enzyme activity were qualitatively determined by noting the time required to convert native DNA into fragments.

The *Bst*1503 assay was valid for amounts of enzyme that completely digested TP-1C DNA within 30 to 60 min. Digestion proceeded in a linear manner during this period, and the estimation of the time required for complete cleavage was accurate to within ±5 min.

Endo R·*Bam*HI assay mixture contained 6 mM Tris-hydrochloride (pH 7.4), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol (43), and 1 μg of DNA in a final volume of 50 μl. Endo R·*Eco*RI assay mixture contained 100 mM Tris-hydrochloride (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$ (11), and 1 μg of DNA in a final volume of 50 μl. After equilibration at 37°C, the reaction was initiated by the addition of 5 μl of *Bam*HI or *Eco*RI (0.1 to 0.2 units). The reaction was terminated by the addition of EDTA, and the products were analyzed as described for *Bst*1503.

Protein was determined by the absorbance (280 and 260 nm) method of Warburg and Christian as described by Layne (18).

Concentration of Endo R·*Bst*1503. *Bst*1503 was concentrated by ultrafiltration in an Amicon model 12 cell equipped with a PM-30 membrane filter. The enzyme was stirred under 25 lb/in² of nitrogen. Concentration of small volumes (less than 2 ml) was accomplished by surrounding a dialysis tubing (8-mm width) containing *Bst*1503 with PEG. A four- to fivefold increase in concentration with no loss of enzyme activity was obtained by both methods.

Isoelectric focusing in polyacrylamide gels. The procedure used was that described by Eder (8). Twenty microliters of *Bst*1503 (3 to 5 μg of protein) was mixed with 5 μl of an Ampholyte (30%, pH 3 to 10; LKB Produkter)-sucrose (50%) solution and loaded onto a gel. Electrophoresis was carried out at 1 mA/gel (12°C) until a maximum of 400 V was attained (approximately 4 h). After electrophoresis for an additional 5 h, the gels were stained with Coomassie brilliant blue. The pH gradient was estimated by slicing a blank gel into 1.5-mm segments. The segments were put into tubes containing 1 ml of distilled water and placed at 5°C for 12 to 15 h. The pH of each sample was determined.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out using the procedure described by Weber and Osborn (41), except that gels contained 15.6 g of acrylamide monomer (enzyme grade; Ortec) and 0.42 g of methylenebisacrylamide (Ortec) per 100 ml of water and sodium dodecyl sulfate (SDS) was not added. Twenty microliters of *Bst*1503 (3 to 5 μg) was mixed with 2 μl of a bromophenol blue (0.125%)-sucrose (40%) solution and layered onto a gel. Electrophoresis was carried out at 8 mA/gel in 200 mM sodium phosphate buffer, pH 7.2, for 4.5 h. Gels were stained with 0.025% Coomassie brilliant blue for 4 h. Destaining and storage of the gels were accomplished in an acetic acid (7.5%)-methanol (5%) solution.

When it was necessary to elute *Bst*1503 from a gel, a duplicate gel was sliced into 1.5-mm segments and placed into tubes containing 0.1 ml of 20 mM potassium phosphate (pH 6.8) containing 0.2 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol (P

buffer). The gel segments were crushed and placed at 5°C for at least 6 h.

SDS-polyacrylamide gel electrophoresis was run according to the procedure described by Weber and Osborn (41). SDS was added to *Bst*1503 and the marker proteins in P buffer to a final concentration of 1%. Twenty microliters of *Bst*1503 (3 to 5 μg) or marker protein (10 μg) was mixed with 2 μl of a bromophenol blue (0.125%)-sucrose (40%) solution and layered onto a gel. Electrophoresis was carried out at 8 mA for 5 h (12°C). Gels were stained with 0.025% Coomassie brilliant blue for 2 h. Destaining and storage were as described above.

Glycerol gradient sedimentation. Gradients (3.8 ml) of 20 to 40% glycerol (vol/vol) in P buffer (minus 10% glycerol) were prepared. Endo R·*Bst*1503 in P buffer (0.1 ml) was layered on top of the gradient, and centrifugation was carried out at 56,000 rpm in a Beckman SW56 rotor for 20 h at 5°C. Fractions (0.2 ml) were collected from the bottom of each tube, and the absorbance at 280 nm was monitored with the Gilford model 2400 spectrophotometer equipped with a turbulence-free flow cell (Molecular Instruments, Co., Evanston, Ill.). *Bst*1503 activity was measured in each fraction. Sedimentation coefficients and molecular-weight estimates were determined by the method of Martin and Ames (24) from the positions of human hemoglobin ($s_{20,w}$ = 4.56, molecular weight of 64,500; 7) and gamma globulin ($s_{20,w}$ = 7.18, molecular weight of 163,750; 37) when sedimented under the same conditions.

High-speed equilibrium ultracentrifugation. High-speed sedimentation equilibrium experiments were done with a Spinco model E ultracentrifuge fitted with interference optics, using the method described by Yphantis (47). Data were analyzed by the computer program of Roark and Yphantis (30).

*Bst*1503 was dialyzed against 20 mM potassium phosphate buffer, pH 6.8, and placed (0.13 ml) into a 12-mm, three-channel, aluminum-filled Epon centerpiece equipped with sapphire windows and interference window holders. The concentrations of protein in the three channels were 0.88, 0.52, and 0.28 mg/ml (centripetal to centrifugal), respectively. Solvent chambers contained 20 mM potassium phosphate buffer. Centrifugation was carried out at 16,000 rpm at 20°C in a Beckman An-H rotor. At the end of the run, the protein in each channel was redistributed in the cell and centrifugation was carried out at 2,000 rpm for 1 h.

Purification of Endo R·*Bst*1503. Cultures were grown to the late logarithmic phase of growth (4×10^8 to 5×10^8 viable cells/ml) and collected by centrifugation. The yield from 12 liters of culture was approximately 40 g (wet weight) of cells. The cells were washed once in TNC buffer and stored at −20°C.

Although the enzyme was prepared from 20- to 40-g batches of cells, the procedure can be scaled up by combining the 72% ammonium sulfate precipitate from up to 120 g of cells. All steps were carried out at 5°C.

Step 1: sonication. Cells were suspended in sonication buffer containing 20 mM Tris-hydrochloride, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA, 50 mM NaCl, and 10% (vol/vol) glycerol, pH 7.4 (1 g/ml), placed in an ice bath, and disrupted by sonic irradiation (Bronwill Biosonik III). Cell disruption was followed with a phase-contrast microscope.

Step 2: removal of nucleic acids. Cell debris was removed by centrifugation at 105,000 × *g* for 1 h. After the addition of 10% streptomycin sulfate (1 ml per 1,500 units of optical density at 260 nm), the nucleic acid precipitate was removed by centrifugation at 35,000 × *g* for 15 min.

Step 3: ammonium sulfate fractionation. Solid ammonium sulfate (enzyme grade) was added with stirring to a 50% saturation. After the ammonium sulfate was dissolved, the mixture was stirred for 1 h. The precipitate was collected by centrifugation at 35,000 × *g* for 20 min and discarded. Solid ammonium sulfate was added to the supernatant fluids to a 72% saturation and stirred for 1 h. The precipitate was collected by centrifugation at 35,000 × *g* for 20 min.

Step 4: Sephadex G-25 fractionation. The precipitate was suspended in 10 ml of 10 mM sodium phosphate buffer (pH 7.4) and applied to a column (2.5 by 30 cm) of Sephadex G-25 (course grade; Pharmacia, Inc.). Enzyme activity was eluted from the column using the same buffer.

Step 5: DEAE-cellulose chromatography. Active fractions were pooled and dialyzed twice (2 and 12 h) against 40 volumes of sonication buffer, pH 8.2. The preparation was passed through a column (2.5 by 20 cm) of diethylaminoethyl (DEAE)-cellulose (standard capacity; Schwarz/Mann) equilibrated with sonication buffer, pH 8.2.

Step 6: phosphocellulose chromatography. The eluate containing the enzyme was dialyzed twice (2 and 12 h) against 30 volumes of P buffer and applied to a column (1.2 by 15 cm) of phosphocellulose (Whatman P11). Phosphocellulose was washed with 0.5 N NaOH and then with 0.5 N HCl and equilibrated with P buffer. The enzyme was eluted with a 300-ml linear gradient (0 to 1.0 M KCl) in P buffer. Only one peak having endonuclease activity was detected in the eluate. *Bst*1503 eluted between 0.27 and 0.33 M KCl. Phosphocellulose peak fractions containing 200 units or higher of *Bst*1503 activity per ml were used in these studies (not pooled).

Enzyme preparations contained no detectable nuclease activity. Approximately 200,000 units (7 to 10 mg of protein) were obtained from 10 g (wet weight) of cells.

Endo R·*Bst*1503 activity could not be detected until after DEAE-cellulose chromatography because of the presence of contaminating nucleases. For this reason the purity and recovery of enzyme could not be calculated.

The enzyme can be stored at 5°C for at least 1 year in phosphocellulose elution buffer (P buffer containing approximately 0.3 M KCl) without any detectable loss of activity. The enzyme was dialyzed into P buffer as required.

RESULTS

Homogeneity of Endo R·*Bst*1503. Purity of the *Bst*1503 enzyme preparation was determined by polyacrylamide gel electrophoresis. A

single protein-staining band was observed, indicating a homogeneous preparation (Fig. 1). A duplicate gel cut into 1.5-mm segments showed that *Bst*1503 activity coincided with this band. A faint protein-staining band (accounting for less than 1% of the total protein), migrating ahead of the major band, was observed in this enzyme preparation. *Bst*1503 (3 to 5 μg) was also analyzed by isoelectric focusing on polyacrylamide gels. A single protein-staining band was observed, with an isoelectric point (pI) of about 6.6 to 6.8.

The minor protein-staining band observed after polyacrylamide gel electrophoresis (Fig. 1A) could not be detected in the isoelectric focusing gels. This component is probably not an impurity but another form (dimer) of the enzyme. Evidence in support of this will be presented later.

Characterization of Endo R·*Bst*1503. *Bst*1503 was active over a pH range of 7.0 to 8.5, with maximum activity between pH 7.5 and 8.0 (Fig. 2). No detectable activity was observed at pH 6.5 or 9.0. The enzyme required Mg^{2+} ion as a cofactor. The optimum concentration was about 0.2 mM (Fig. 3). No activity was observed if Mg^{2+} was omitted from or EDTA was added to the reaction mixture. Manganous ion could partially substitute for Mg^{2+} in the reaction. Although the optimal concentration for Mn^{2+} was the same as for Mg^{2+}, the formation of a limit digest was 10 times slower. Calcium, Fe^{2+}, Hg^{2+}, and Co^{2+} ions were not effective.

The enzyme was not stimulated by SAM or ATP or sensitive to increasing ionic strength (up to 200 mM KCl or NaCl). In this study we did not investigate the effect of the components of P buffer on the storage of the enzyme.

*Bst*1503 was active over a temperature range of 25 to 75°C, with maximal activity between 55 and 65°C (Fig. 4). The stability of *Bst*1503 with respect to temperature was determined by incubating samples of enzyme (50 μg/ml) in P buffer containing approximately 0.3 M KCl at various temperatures. Samples were removed after 1 and 2 h and assayed at 55°C. *Bst*1503 was not inactivated after a 2-h exposure at 55 or 65°C (Fig. 5). Prolonged incubation (10 h at 55 or 65°C) did not inactivate the enzyme. After 2 h of incubation at 70°C, *Bst*1503 was inactivated by 65%. *Bst*1503 was rapidly inactivated at 75°C.

Molecular weight and subunit composition of Endo R·*Bst*1503. Since high-speed sedimentation equilibrium experiments revealed that most preparations contained two forms of the enzyme, the sedimentation of enzyme activity was determined by use of glycerol gradient centrifugation with protein markers of known sedimentation coefficient. Figure 6 shows the profiles of *Bst*1503 activity as a function of fraction number (equivalent to distance of migration). A major peak of *Bst*1503 activity was observed with a sedimentation coefficient of 8.3. Assuming a spherical shape for the protein, this value is equivalent to a molecular weight of about 180,000. Some enzyme preparations contained a second peak (or shoulder) exhibiting *Bst*1503 enzyme activity with a sedimentation coefficient of 5.4 (see arrow). The 5.4*S* peak would correspond to a molecular weight of about 96,000. The proportion of the 5.4*S* component in the various enzyme preparations (phosphocellulose fractions containing at least 200 units/ml) varied from less than 1 to 10% of the total activity (or protein). The relative amounts of the 8.3*S* and 5.4*S* peaks in each fraction did not change after storage for 3 months.

The various Endo R·*Bst*1503 preparations were examined for methylase activity by the procedure of Catterall et al. (6). The *Bst*1503 preparations did not contain any detectable methylase activity.

The leading phosphocellulose fractions (less than 200 units/ml) contained a larger proportion (20 to 60% of the total *Bst*1503 activity) of the 5.4*S* peak as determined by glycerol gradient sedimentation. The presence of the 5.4*S* component in these fractions was also visualized by polyacrylamide gel electrophoresis. The catalytic properties of the two forms were indistinguishable. No other active forms of the enzyme were detected.

A single protein-staining band was observed when *Bst*1503 preparations were analyzed by SDS-polyacrylamide gel electrophoresis. The molecular weight was found to be 46,000 (see arrow) by comparison of its mobility with those of proteins of known molecular weight (Fig. 7). Endo R·*Bst*1503 could exist in two forms, each containing a subunit of molecular weight of 46,000. Peak phosphocellulose fractions were enriched with the 180,000-dalton form (tetramer), and the leading phosphocellulose fractions contained, in addition to the tetrameric form, the 96,000-dalton form (dimer). The dimer appeared to elute from phosphocellulose at a lower KCl concentration. No detectable association-dissociation between the dimer and tetramer occurred during storage.

Dissociation of the tetramer to the dimer was observed when a *Bst*1503 preparation containing only the tetramer was subjected to high-speed sedimentation equilibrium. The experiments gave weight-average molecular weights

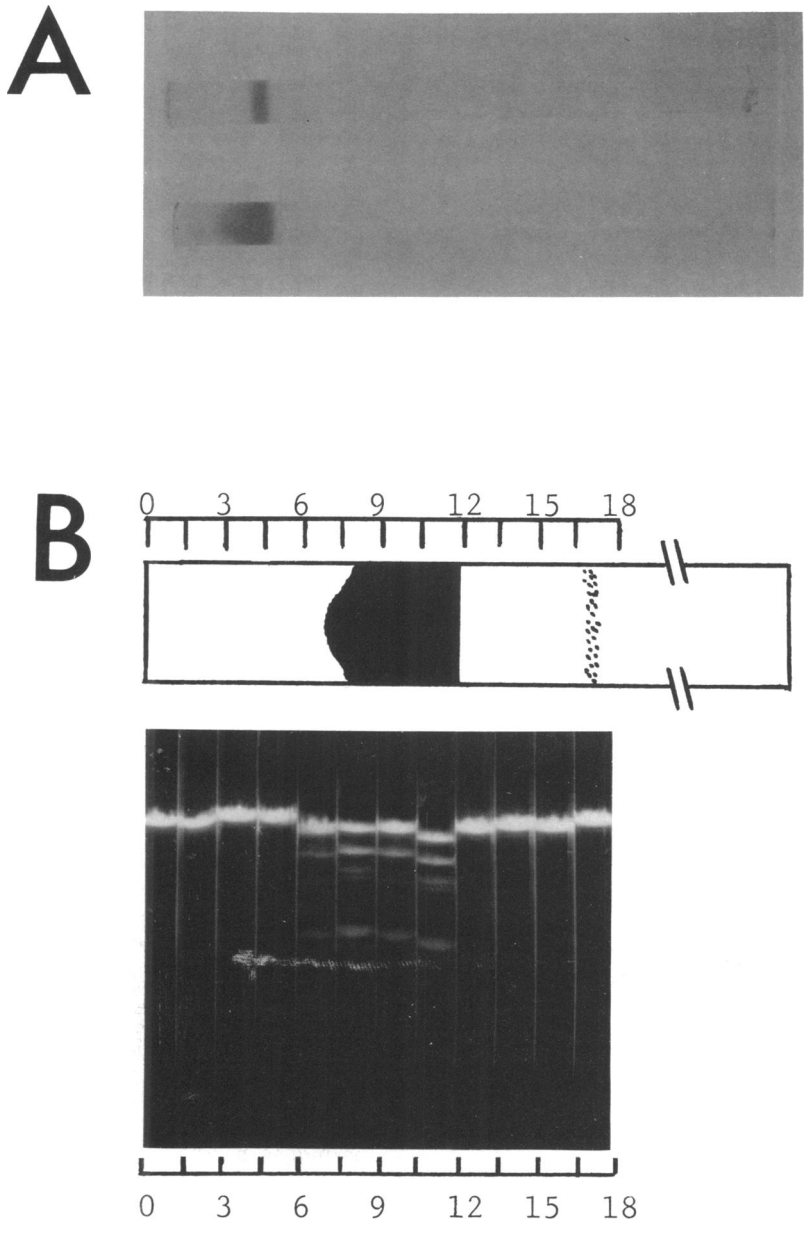

FIG. 1. *Polyacrylamide gel electrophoresis of Endo R·Bst1503. (A) Gels were stained with Coomassie brilliant blue. The amount of Bst1503 protein per gel was 3 to 5 μg (upper) and 10 to 12 μg (lower). (B) A duplicate gel containing 10 to 12 μg of Bst1503 was cut into 1.5-mm segments. Bst1503 activity was measured in each segment by agarose-ethidium bromide-gel electrophoresis. Gels were photographed under ultraviolet illumination with Polaroid type 55 P/N film through a Tiffen series 6 no. 15G orange filter. The drawing (upper portion only) of a Coomassie brilliant blue-stained gel (10 to 12 μg of protein) shows the correspondence between Bst1503 activity and the protein-staining band.*

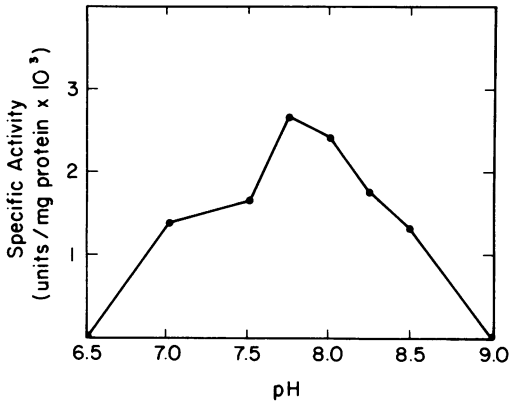

FIG. 2. *Effect of pH on Endo R·Bst1503 activity. Bst1503 (0.5 μg of protein) activity was measured in assay buffer (pH 6.5 to 9.0) at 55°C.*

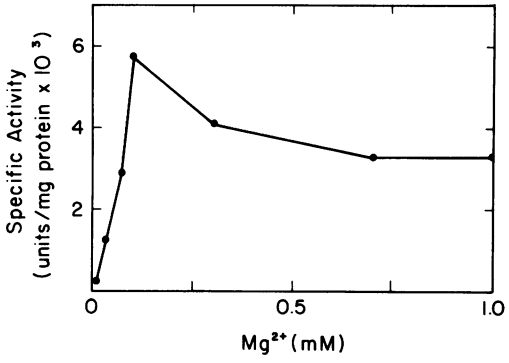

FIG. 3. *Effect of Mg^{2+} on Endo R·Bst1503 activity. Bst1503 (0.3 μg of protein) activity was measured in assay buffer (pH 7.8) in which the Mg^{2+} ion concentration was varied between 0 and 1 mM. The reactions were run at 55°C.*

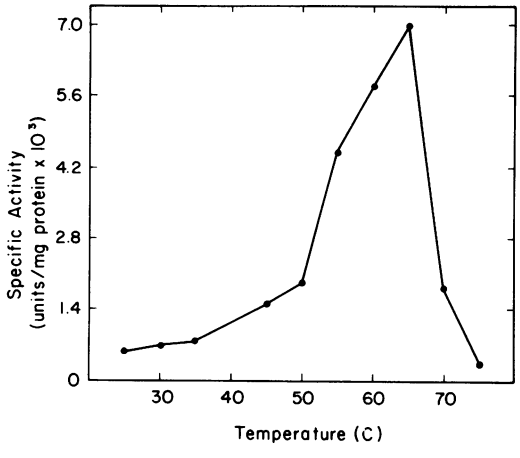

FIG. 4. *Effect of temperature on Endo R·Bst1503 activity. Bst1503 (0.3 μg of protein) activity was measured in assay buffer (pH 7.5) at various temperatures.*

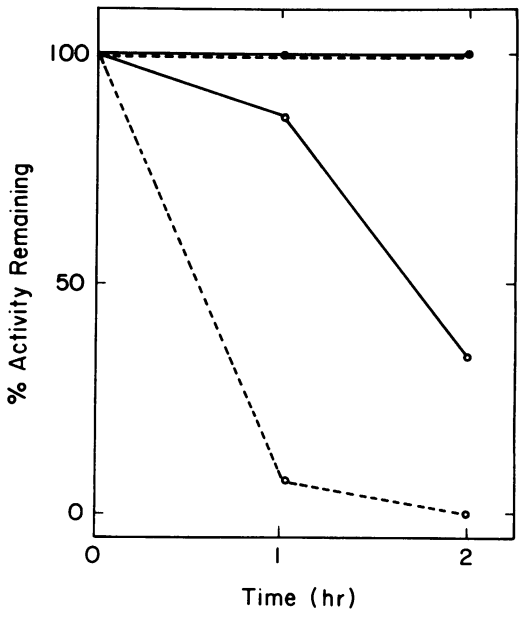

FIG. 5. *Thermostability of Endo R·Bst1503. Bst1503 (50 μg of protein per ml) in P buffer (pH 6.8) containing approximately 0.3 M KCl was incubated for 1 and 2 h at 55 (—●—), 65 (- -●- -), 70 (—○—), and 75°C (- -○- -). Bst1503 (0.25 μg of protein) activity was measured in assay buffer (pH 7.8) at 55°C.*

of 133,000, 136,000, and 147,000, for an average of 139,000 ± 7,000. If the two forms of *Bst*1503 having molecular weights of 180,000 and 96,000 are present, one would calculate the weight-average molecular weight to be 138,000. The dissociation of tetramer to dimer may occur at the meniscus region as it is depleted of protein. The dissociation may also be favored by other conditions. In the initial stages of this investigation, *Bst*1503 was prepared by pooling all of the active phosphocellulose fractions. This one enzyme preparation contained only the dimer as judged by glycerol gradient sedimentation and polyacrylamide gel electrophoresis.

The conditions that favor the dimer-tetramer transition were not determined. In all experiments, fractions containing mostly the 8.3*S* form were used.

Endo R·*Bst*1503 cleavage patterns on various DNA substrates. The cleavage patterns of *Bst*1503 on TP-1C, λ*vir*, SV40, and plasmid pSC101 DNAs were determined by agarose-ethidium bromide gel electrophoresis. *Bst*1503 recognized six sites in TP-1C DNA (linear genome; Catterall, unpublished data), one site in

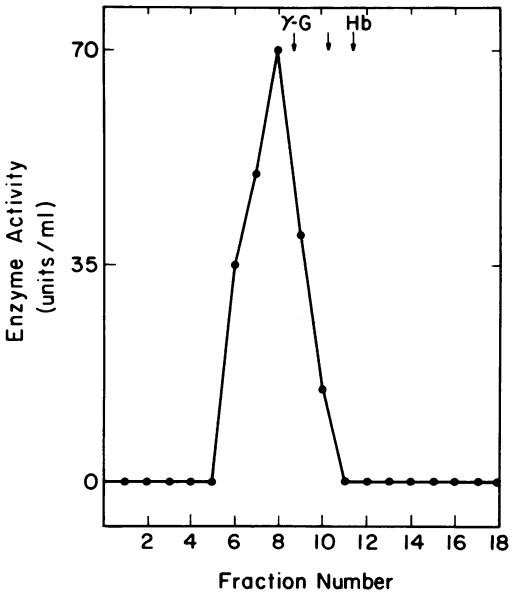

FIG. 6. *Glycerol gradient sedimentation of Endo R·Bst1503. Fractions were assayed for Bst1503 activity. The arrows show the position of human gamma globulin (γ-G; $s_{20,w}$ = 7.18), human hemoglobin (Hb; $s_{20,w}$ = 4.56), and the second peak of Bst1503 activity (arrow), which was detected in some preparations. The direction of sedimentation is from right to left.*

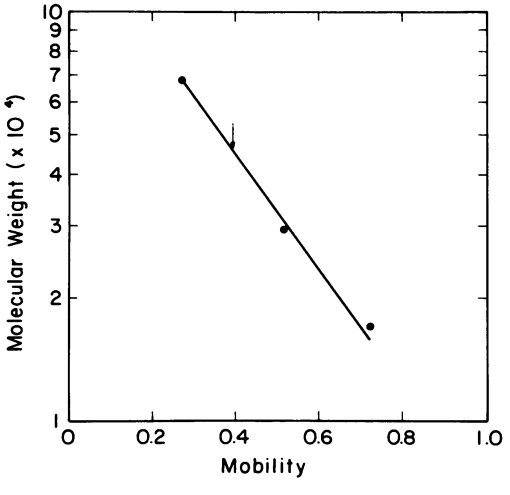

FIG. 7. *Determination of subunit molecular weight by SDS-polyacrylamide gel electrophoresis. A semilogarithmic plot of the molecular weights of standard proteins (10 μg) and Bst1503 (3 to 5 μg) versus their mobility is shown. Protein markers used were bovine serum albumin (68,000), carbonic anhydrase (29,000), and myoglobin (17,000). The arrow indicates the mobility of Bst1503.*

pSC101 and SV40 DNAs, and three sites in λ*vir* DNA (Fig. 8).

Only a few DNA genomes have been exam-

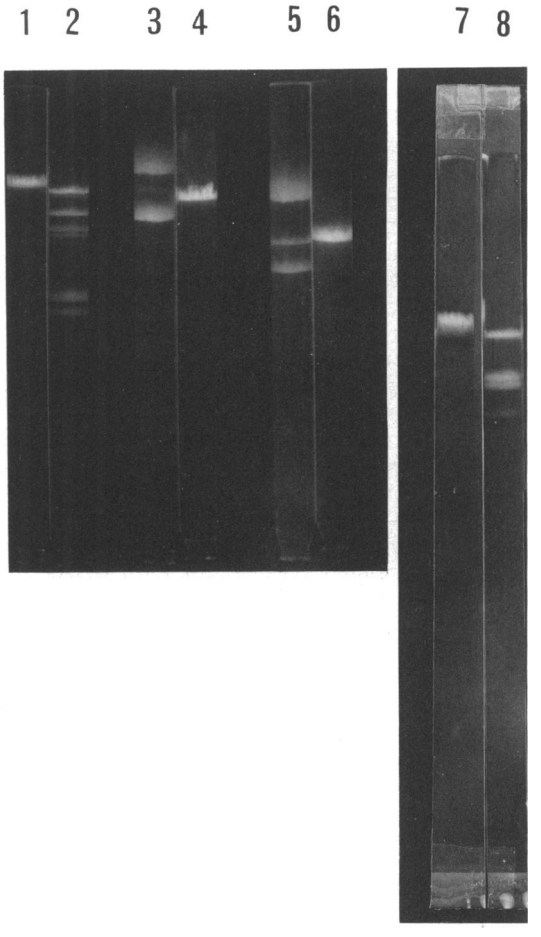

FIG. 8. *Agarose-ethidium bromide-gel electrophoresis of TP-1C, pSC101, SV40, and λvir DNAs after digestion with Bst1503. Approximately 1 μg each of TP-1C (gels 1 and 2), pSC101 (gels 3 and 4), SV40 (gels 5 and 6) and λvir (gels 7 and 8) DNAs was digested for 30 min at 55°C (assay buffer, pH 7.8) with Bst1503. Conditions for electrophoresis are described in Materials and Methods, except that in gels 7 and 8 the length of the gel was increased to 10.5 cm and electrophoresis was carried out for 4 h. The modified procedure was necessary to separate bands 2 and 3 of the λvir digest. Gels were photographed as described in the legend of Fig. 1. The even-numbered gels contain DNA that was treated with Bst1503. Undigested pSC101 (gel 3) and SV40 (gel 5) DNAs contained 85 and 30%, respectively, of the supercoil configuration. Under the conditions of electrophoresis used, supercoiled (form I) pSC101 and SV40 DNAs had the highest mobility, followed by linear (form III) and open circular (form II) forms.*

ined as substrates for *Bst*1503. Thermophilic phage TP-12 DNA is cleaved, whereas DNAs from phages ϕX174 (form I) and f1 (form I), *Bacillus subtilis* phages ϕ25 and SPL11, and thermophilic phage TP-8 are not cleaved.

Incubation of TP-1C DNA with *Bst*1503 for 10 h at 55 or 65°C did not change the number of DNA fragments or the relative amounts of each fragment. These results indicate that the *Bst*1503 preparations were free of contaminating nucleases.

Although phage ϕX174 DNA was not cleaved by *Bst*1503, incubation with the enzyme did result in a conversion of some ϕX174 form I (closed circles) DNA to form II (open circles). These results indicate that either the *Bst*1503 preparations contain trace amounts of a contaminating nuclease not detected with the larger DNA genomes or that *Bst*1503 is capable of making single-strand scissions in this DNA.

Prolonged incubation of native or Endo R·*Bst*1503-treated TP-1C DNA at 65°C (longer than 10 h) resulted in DNA degradation as judged by the disappearance of fluorescent bands in agarose gels.

The conversion of SV40 and pSC101 DNAs to linear forms after incubation with *Bst*1503 was judged by a comparison of the product with the linear form of SV40 and pSC101 DNAs produced by the action of *Eco*RI.

*Bst*1503 cleaves λ*vir* into four fragments and λ into five fragments (R. Roberts, personal communication). These results suggest that the immunity region of the λ genome contains a *Bst*1503 cleavage site.

Preliminary studies by R. Roberts (personal communication) demonstrated that *Bst*1503 and *Bam*HI recognize identical palindromic sites in various DNA genomes. Restriction enzymes having this property are designed isoschizomers. We have verified this finding by comparing *Bst*1503 and *Bam*HI limit digests of TP-1C, λ*vir*, SV40, and pSC101 DNA. Sequential digestion of TP-1C DNA with *Bst*1503 and *Bam*HI did not alter the DNA fragment pattern. Since *Bam*HI recognizes and cleaves DNA containing the sequence GGATCC (G. Wilson, R. Roberts, and F. Young, unpublished data), we conclude that *Bst*1503 recognizes and cleaves the identical nucleotide sequence.

Effect of temperature on Endo R·*Bam*HI. Although *Bst*1503 and *Bam*HI are isoschizomers, *Bst*1503 may be potentially more useful in some studies because of its inherent thermostability. The effect of temperature on *Bam*HI activity and stability was examined by the same procedure described for *Bst*1503. *Bam*HI was active over a temperature range of 25 to 65°C, with maximal activity between 30 and 40°C (Table 1). *Bam*HI was not inactivated after a 2-h exposure at 37 or 40°C. After 1 h at 45°C or above, however, the enzyme was completely inactivated. Increasing the concentration of *Bam*HI (fivefold) did not protect the enzyme from thermal inactivation.

There was a distinct difference in the thermostability between *Bst*1503 and *Bam*HI. *Bst*1503 was inactivated at temperatures above 65°C, whereas *Bam*HI was inactivated at temperatures above 40°C.

Although *Bst*1503 and *Bam*HI have similar catalytic properties, no studies on the subunit composition of *Bam*HI have been reported.

DISCUSSION

From the data presented, Endo R·*Bst*1503 possesses the characteristics of a type II restriction endonuclease: it requires Mg^{2+} as the only cofactor, produces limit digests with DNA substrates, and is composed of subunits of identical size. Two active forms of *Bst*1503 were detected, one with a molecular weight of 180,000 (tetramer) and the other with a molecular weight of 96,000 (dimer). No dimer-tetramer interconversion was detected during storage. The dimer elutes from phosphocellulose at a lower salt concentration than the tetramer. We are unable to say at this time which is the active form in vivo.

Several single-step nonrestricting mutants of strain 1503-4R have been isolated. One-half of these mutants have the phenotype R^-M^-, and half have the phenotype R^-M^+. These results indicate that either the restriction and modification enzymes share a common polypeptide or there is a regulatory gene that controls the

TABLE 1. *Effect of temperature on BamHI endonuclease*

Enzyme activity[a]		Enzyme stability[b]	
Temp (°C)	Percent of maximum activity	Temp (°C)	Percent inactivation
25	75	37	0
30	75	40	0
37	90	45	100
40	100	55	100
45	15	70	100
55	5	75	100
65	2		
75	0		

[a] *Bam*HI (0.1 μg) activity was measured at various temperatures.

[b] *Bam*HI (20 μg/ml) in 10 mM KPO_4, pH 7.5, 0.1 mM EDTA, 10% (vol/vol) glycerol, and 10 mM 2-mercaptoethanol was incubated for 1 h at each temperature. *Bam*HI (0.1 μg) activity was measured at 37°C.

restriction and modification functions. The subunit composition of Endo R·*Bst*1503 argues against the former. It is possible, of course, that Endo R·*Bst*1503 is composed of two nonidentical subunits, each having a different primary structure but similar molecular weight. Alternatively, multiple, single-step mutations may occur in this region of the genome.

Cells of *B. stearothermophilus* 1503-4R $R^{-}{}_{1}M^{+}$ were subjected to the Endo R·*Bst*1503 purification procedure, and less than 5% of the *Bst*1503 activity obtained from strain 1503-4R $R^{+}M^{+}$ was recovered. We did not determine whether the R^{-} strain contained less *Bst*1503 protein or contained wild-type amounts of enzyme having a reduced specific activity.

In vivo studies have been published which demonstrate that *Bst*1503 is a restriction endonuclease (6). Strain 1503-4R also contains a corresponding modification methylase. The isolation of Meth M·*Bst*1503 from strain 1503-4R $R^{-}{}_{1}M^{+}$ has been accomplished, and an investigation of its properties is underway in this laboratory.

Although *Bst*1503 and *Bam*HI are isoschizomers, the enhanced thermostability of *Bst*1503 may prove to be useful in the analysis of high-molecular-weight (eukaryotic) DNA at elevated temperatures. Fragmentation of chromatin by restriction endonucleases has met with limited success due to the contamination of chromatin preparations with endogenous nucleases and by the production of a large number of DNA fragments (27, 28). Incubation of chromatin with *Bst*1503 at 60 to 65°C should eliminate or reduce the activity of endogenous nucleases and cause the melting of the adenosine-thymidine-rich regions of the DNA. The reduction in the number of double-stranded regions containing cleavage sites would result in a smaller number of DNA fragments. Low melting regions in chromatin are associated with transcribable or template-active chromatin (22, 29, 34). The chromatin fractions that elute late from ECTHAM-cellulose (29, 34) and the early fractions from high-temperature hydroxyapatite chromatography (22) are enriched in low-melting sequences.

Fragmentation of these DNA fractions, enriched in adenosine and thymidine, by *Bst*1503 at elevated temperatures may result in a specific subpopulation of chromatin DNA fragments that can be separated and analyzed.

There have been several reports concerning the thermal stability of enzymes and other proteins isolated from thermophiles. If we accept the concept that thermophilic and mesophilic microorganisms have a common origin, we can also postulate that the various cell constituents must also have evolved from a common source. It follows from this, therefore, that an enzyme from a mesophilic or thermophilic species of the same genus would have similar structural and functional properties, but that the thermophilic enzyme would be relatively more heat stable than the mesophilic enzyme. Although there are subtle differences in some of the physical properties and in amino acid composition and sequence (21), specific explanations for the difference in thermostability between homologous proteins of thermophiles and mesophiles require a determination of the amino acid sequence and their three-dimensional structure.

Endo R·*Bst*1503 and its counterpart isolated from a mesophile (Endo R·*Bam*HI) are excellent candidates to study the molecular basis for thermophily. An added bonus resulting from these studies is the information obtained on the specificity of the protein-DNA interaction.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Biomedical Sciences Support Grant 5 S05 RR07028 from the National Institutes of Health.

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