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Bacillus species LU4 Is an Effective Producer of Thermostable Site-Specific Endonuclease *Bsp*LU4I, an Isoschizomer of *Ava*I

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Abstract—The site-specific endonuclease *Bsp*LU4I was discovered in the thermophilic *Bacillus* species LU4 strain and purified to functionally pure state by chromatography on blue agarose, hydroxyapatite HTP, and heparin-Sepharose columns. Analysis of cleavage patterns of different DNAs with known nucleotide sequences demonstrated that the enzyme recognizes the CPyCGPuG site on the DNA. Cleavage points in the sequence were determined with the elongated primer method. It was shown that the endonuclease is an isoschizomer of *Ava*I. The final yield of the enzyme is $2.25 \cdot 10^6$ units per g wet biomass.

Key words: restriction endonucleases, isoschizomers, *Ava*I, DNA size markers

Site-specific endonucleases play a key role in methods used in modern molecular biology. This demands an intensive search for new strains producing such enzymes. The aim of such work is not only the search for enzymes with novel specificity, but also the finding of strains that produce enzymes with already known specificity and of high yield and/or stability.

This study describes the isolation and some characteristics of the thermostable endonuclease *Bsp*LU4I that is an isoschizomer of *Ava*I [1, 2]. The strain producing the endonuclease does this with a very high yield of $2.25 \cdot 10^6$ units per g wet biomass.

MATERIALS AND METHODS

DNAs (phages T7, λ, M13mp18, plasmids pUC18, pBR322, and pHC624) utilized in the study were obtained using standard techniques [3]. Enzymes (restriction endonucleases, Klenow fragment, phage T4 DNA ligase, and Taq polymerase) were also prepared by us.

Isolation of site-specific endonuclease. *Bacillus* species strain LU4 was isolated from soil by growing in dishes with the LB medium (1% bactotrypton, 0.5% yeast extract, 1% NaCl, pH 7.0) and subsequent incubation at 50°C with subsequent thrice growing of individual colonies in fresh dishes. Cells were grown in 4 liters of a SOC-medium (2% peptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) at 50°C to the late logarithmic phase with intensive aeration. Then the cells were pelleted, suspended in a TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), and centrifuged once again [4]. From 4 liters of the bacterial suspension, 20 g of wet biomass were obtained and kept at −20°C.

To isolate the endonuclease, we added 70 ml of a lysis buffer containing 20 mM Tris-HCl, pH 8.3, 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 7 mM 2-mercaptoethanol, to the cells thawed to 0°C and stirred the mixture to obtain a homogeneous mass. Then 2 ml of lysozyme prepared on the lysis buffer at concentration of 10 mg/ml were added, the suspension was incubated for 20 min at 4°C and processed in an UZDN-1A ultrasonic disintegrator for 5 min with 30-sec sonications and 30-sec pauses. Subsequent to 40-min centrifugation of the lysate at 35,000 rpm in a Ti-45 rotor (Beckman, USA), the supernatant (60 ml) was loaded on a 30-ml column with blue agarose (Institute of Chemistry, Tallinn, Estonia). The column was equilibrated with the

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starting buffer containing 200 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 7 mM 2-mercaptoethanol. The column was eluted using 180 ml of NaCl gradient (0.2-3 M) at 24 ml/h collecting 8.4-ml fractions. Active fractions containing the endonuclease were pooled and loaded on a 130-ml column with hydroxyapatite HTP (BioRad, USA) equilibrated with buffer containing 10 mM potassium phosphate, pH 7.0, 50 mM NaCl, and 7 mM 2-mercaptoethanol. The column was eluted with 640 ml gradient of the potassium phosphate buffer (0.01-1 M) at a rate of 15 ml/h. Ten-milliliter fractions were collected. The active fractions were pooled and dialyzed against the buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 7 mM 2-mercaptoethanol) and then loaded on a 4-ml heparin-Sepharose column (Pharmacia, Sweden) equilibrated with the dialysis buffer. The column was eluted with 60 ml NaCl gradient (0.05-0.7 M) in buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 7 mM 2-mercaptoethanol) at a rate of 10 ml/h. Fractions of 1.6-ml each were collected. The active fractions were pooled, albumin (qualified for molecular biology, Olaine, Latvia) was added to the final concentration of 100 μg/ml, and the sample was dialyzed against the storage buffer (10 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol). The preparation was stored at −20°C.

Determination of endonuclease activity. Endonuclease activity was determined in 50 μl of HRB buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$) containing 1 μg of phage λ DNA, grown on *E. coli* dam^- dcm^- strain, and 1.5 μl of diluted fractions from the columns or 2 μl of the final diluted preparation. The reaction mixture was incubated for 1 h at 60°C. One unit of the restriction endonuclease activity is defined as the amount of the enzyme required to completely digest 1 μg of phage λ DNA in 60 min. The hydrolysis products were analyzed using electrophoresis in 1.5% agarose gel in 1× TBE buffer (0.089 M Tris, 0.089 M boric acid, 1 mM EDTA, pH 8.3).

Determination of endonuclease functional purity. The absence of nonspecific nuclease contamination in the preparation was monitored with the digestion–ligation–digestion test. Phage λ DNA (3 μg) was digested with a 10-fold excess of the enzyme in 100 μl of buffer HRB for 1 h at 60°C (digestion). The enzyme was removed by extraction with phenol with subsequent precipitation of DNA fragments with ethanol. The precipitate was dissolved in the ligation buffer (60 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP), phage T4 DNA ligase was added, and the mixture was incubated for 14 h at 16°C. The reaction was terminated by heating the mixture for 10 min at 60°C. A half of the preparation was used to demonstrate ligation of phage λ DNA fragments. *Bsp*LU4I was added to the remaining half of the ligated preparation, and the mixture was incubated in the HRB buffer for 1 h at 60°C.

Determination of molecular mass. The molecular mass of the endonuclease was determined by its mobility in 12% denaturing polyacrylamide gel [5]. To clarify the subunit structure of the endonuclease, we performed gel chromatography on a Sephacryl S200 HR column (Pharmacia) preliminarily calibrated by marker proteins: BSA (66 kD), ovalbumin (45 kD), and chymotrypsinogen A (25 kD). The column was eluted with 20 mM of Tris-HCl, pH 8.0, 75 mM NaCl, 1 mM EDTA, 1 mM DTT at a rate of 8 ml/h.

Determination of cleavage points. Cleavage points on DNA were determined by restriction endonuclease *Bsp*LU4I with the elongated primer method [6]. Single-stranded DNA of phage M13mp18 was used as a template for the DNA-polymerase synthesis. The labeled primer was obtained using [γ-^{32}P]ATP (Cluster Scientific Production Association, Russia) and phage T4 polynucleotide kinase (Boehringer Mannheim, Germany) as described earlier [3], annealed with the single-stranded DNA of phage M13mp18, and then the second complementary DNA strand was synthesized by Klenow fragment in the presence of all four deoxynucleoside triphosphates. The double-stranded DNA was purified with phenol deproteinization and reprecipitated with ethanol. The precipitate was dissolved in water and incubated with endonuclease under optimal conditions. A half of the preparation was pooled and incubated with Klenow fragment. The portion treated with endonuclease was used to determine the cleavage point on the labeled strand. The other half treated first with endonuclease and then with Klenow fragment allows determining the cleavage point on the complementary strand. In parallel, the method of Sanger [7] modified by Promega [8] was used for sequencing the single-stranded DNA of phage M13mp18. Radioactive products both of DNA hydrolysis and sequencing were analyzed by electrophoresis in 6% polyacrylamide gel containing 7 M urea at 50°C on a Macrophor instrument (LKB, Sweden).

Purification using Silica. To remove endonuclease from the restriction mixture, purification with Silica quartz powder (Sigma, USA) was used. Silica suspension (15 μl, 100 mg/ml) and five volumes of 5 M guanidine thiocyanate (ICN, USA) prepared with 100 mM Tris-HCl, pH 7.0, were added to 1 μg of the pUC18 plasmid digested with *Bsp*LU4I in 50 μl. The mixture was processed in a microcentrifuge at 12,000 rpm. The pellet was washed thrice with 750 μl of washing buffer (60% ethanol, 80 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0) and vacuum dried. DNA was eluted in 50 μl of elution buffer containing 10 mM Tris-HCl, pH 8.5.

Conditions of ligation for formation of plasmid pHC624ΔBglII multimers. DNA ligase (2 μl, 1000-, 2000-, and 4000-fold dilutions) with the starting activity of 80 units (the activity was determined by connecting the sticky ends of DNA fragments) was added to 35 μg of plasmid pHC624ΔBglII linearized with *Bsp*LU4I in 65 μl

of buffer containing 66 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.1 mM spermine, 0.1 mg/ml of autoclaved gelatin, 13% polyethylene glycol 6000 (w/v). The sample was incubated for 1 h at about 20°C.

Pulse electrophoresis with polarity inversion. To separate plasmid multimers, 1% agarose gel on 0.5× Tris-borate buffer, pH 8.8, was used. The gel length was 30 cm and its thickness was 4 mm. Electrophoresis conditions were as follows: forward direction, 260 V, 1 sec; reverse direction, 26 V, 3 sec. Electrophoresis continued for 22 h at room temperature with constant stirring of buffers in the cathode and anode cells.

RESULTS

Determination of dependence of endonuclease content on cell growth phase. The medium for bacterial growth was chosen prior to the isolation of endonuclease. To grow the biomass, LB and SOC media were used. A 1.2-ml sample of overnight culture was mixed into 100 ml of the medium, grown at 50°C with vigorous aeration, and the culture turbidity was measured at 590 nm every growth hour. Curves of growth in the media showed that in the SOC medium the culture density is twice as large as that in the LB medium; therefore, later the SOC medium with glucose was used to grow the bacteria. To determine the dependence of endonuclease content on the cell growth phase, after measuring the culture turbidity the cells were precipitated from such a bacterial culture volume that contained 3 units (A_{590}) of optical density. The cells were frozen and stored at −20°C. After thawing, the cells were suspended in 2.2 ml of the lysis buffer; 100 μl lysozyme was added at a concentration of 10 mg/ml. The culture was incubated in an ice bath for 20 min and processed in the ultrasonic disintegrator three times with 30-sec sonications and 1-min pauses, the temperature being no higher than 10°C. Then the lysate was centrifuged for 20 min at 4°C and the enzyme activity in the supernatant was measured. The results demonstrated that the enzyme content in the cells depends little on the growth phase: at the stationary growth phase the endonuclease content in the culture is 1.5 times greater than at the logarithmic phase.

Isolation of *Bsp*LU4I endonuclease. The preliminary screening of *Bsp*LU4I showed its high activity, so the enzyme was diluted to cleave substrate DNA.

To obtain a *Bsp*LU4I preparation, three stages of column chromatography were used. The general scheme of isolation is given in Fig. 1. The first stage of purification was chromatography with blue agarose. Endonuclease activity was revealed to have a wide front, from fraction 4 to fraction 39, with the maximum being displayed in fractions 4-11 at 100-fold aliquot dilution (Fig. 2). Fractions 4-11 were pooled and loaded on a hydroxyapatite HTP column. The enzyme was eluted from the column at the concentration of the potassium phosphate buffer from 0.29 to 0.81 M. Fractions 11-20 with the maximum activity at 125-fold dilution (Fig. 3) were pooled, and after dialysis against the starting buffer they were loaded on a heparin-Sepharose column for further purification. Endonuclease was also eluted from the column in a wide front (100-fold dilution) (Fig. 4a). To reveal peak fractions containing maximal amounts of enzyme *Bsp*LU4I, substrate DNA was digested with aliquots from fractions 5-19, which were diluted 2500-fold. Complete hydrolysis of substrate DNA was produced by diluted aliquots from fractions 7-11 (Fig. 4b). These fractions were pooled and dialyzed against the storage buffer.

The final enzyme yield was determined after testing the activity of the final preparation in several sequential dilutions (up to 25,000 times) by phage λ DNA cleavage. The activity of *Bsp*LU4I was 12,800 units/ml and the final yield of endonuclease was $2.25 \cdot 10^6$ units per g wet biomass.

Determination of substrate specificity of endonuclease *Bsp*LU4I. To determine substrate specificity of endonuclease, purified enzyme was used to cleave several DNAs (those of phage λ and T7, plasmids pBR322 and pUC18) whose nucleotide sequences are known.

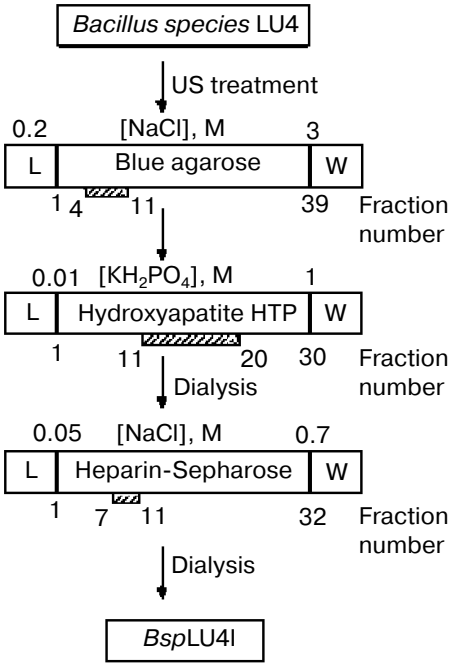

Fig. 1. General scheme of endonuclease *Bsp*LU4I isolation. Rectangles show the steps of purification. Numerals above them indicate initial and final molarity of NaCl and elution buffer. L is the step of column loading and washing; W is column washing after the gradient. Under the rectangles are fractions (hatched) containing the enzyme.

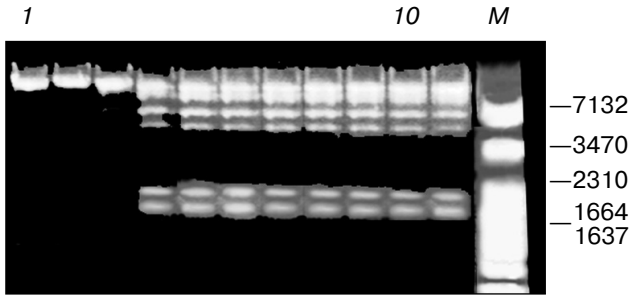

Fig. 2. Analysis of *Bsp*LU4I activity (by cleavage of phage λ DNA) in fractions (1-10) eluted from the column with blue agarose. M, fragment size markers (bp) (phage T7 DNA cleaved with *Bli*736I).

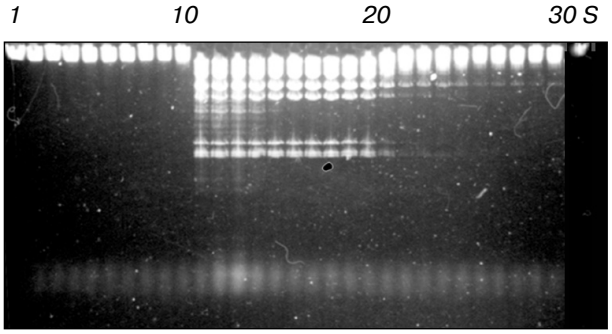

Fig. 3. Analysis of *Bsp*LU4I activity (by cleavage of phage λ DNA) in the fractions eluted from the column with hydroxyapatite HTP. S, uncleaved DNA.

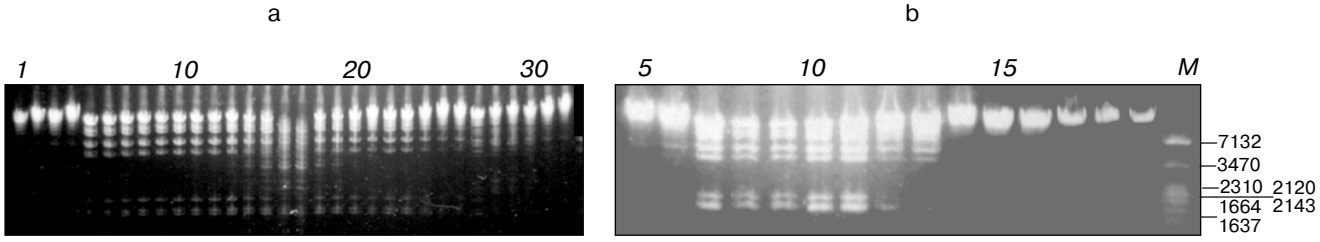

Fig. 4. Analysis of *Bsp*LU4I activity (by cleavage of phage λ DNA) in fractions (1-30) eluted from the column with heparin-Sepharose: a) 100-fold dilution; b) 2500-fold dilution; M, fragment size marker (bp) (phage T7 DNA cleaved with *Bli*736I).

*Bsp*LU4I cleaves pUC18 and pBR322 in the single site and two linear DNAs are formed of 2686 and 4361 bp, respectively. Phage λ DNA is cleaved into fragments of 14677, 13334, 6888, 4716, 3730, 1881, 1674, and 1602 bp. Phage T7 DNA is cleaved with the formation of four fragments of 20367, 10212, 5390, and 3963 bp. The analysis of hydrolysis products showed that *Bsp*LU4I cleaves the substrate DNAs in a way specific for hydrolysis of these DNAs with endonuclease *Ava*I (Fig. 5) that recognizes site 3′-CPyCGPuG-5′.

To verify this assumption and determine cleavage points, the method of Brown and Smith was used [6]. As a substrate we used bacteriophage M13mp18 DNA whose polylinker has a site for endonuclease *Ava*I located close to the universal primer. The radioautograph in Fig. 6 shows that endonuclease *Bsp*LU4I does recognize the same site as endonuclease *Ava*I and cleaves it analogously to *Ava*I, within the palindrome recognition site after the first nucleotide 5′-C↓CCGGG-3′.

Thus, endonuclease *Bsp*LU4I is an isoschizomer of *Ava*I.

Removal of endonuclease *Bsp*LU4I from the reaction mixture. Inasmuch as endonuclease *Bsp*LU4I is a thermostable enzyme and is not inactivated upon heating, it is necessary to remove the enzyme after incubation with DNA. One of the fast and efficient ways to do this is

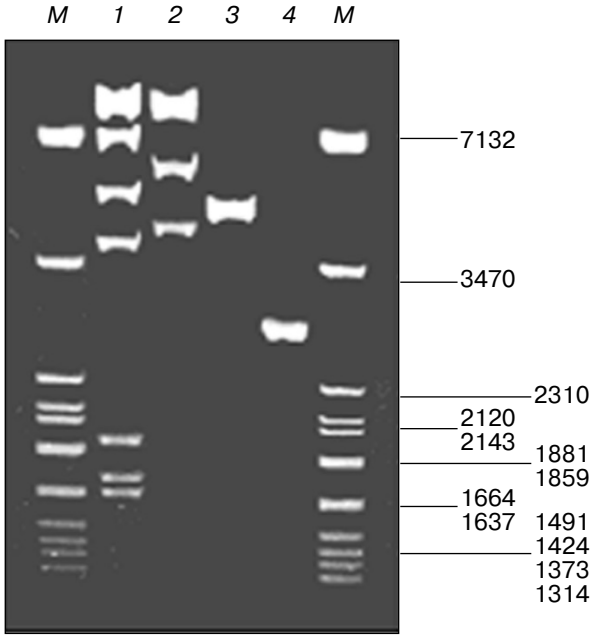

Fig. 5. Determination of the *Bsp*LU4I recognition site by cleavage of different DNAs: *1*) phage λ; *2*) phage T7; *3*) pBR322; *4*) pUC18; M) fragment size marker (bp) (phage T7 DNA, cleaved with *Bli*736I).

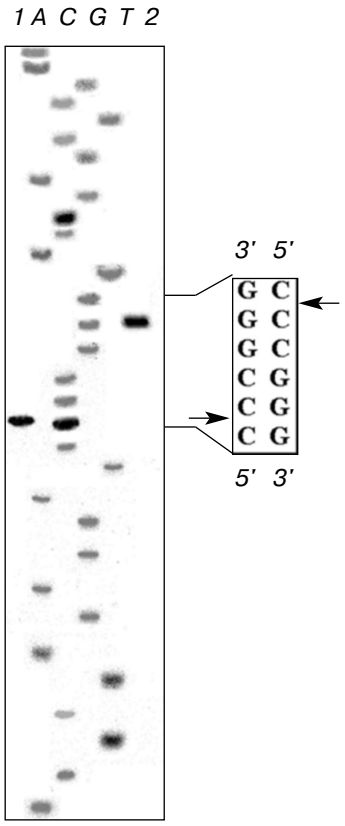

Fig. 6. Determination of cleavage points produced by *Bsp*LU4I within the recognition site using phage M13mp18 DNA as a template. A, C, G, and T are "sequencing" lanes; *1*) γ-^{32}P-labeled M13mp18 DNA cleaved with *Bsp*LU4I; *2*) the same product after treatment with Klenow fragment. The *Bsp*LU4I recognition site is framed. Arrows indicate DNA cleavage points.

purification using Silica suspension. To test this method of removing endonuclease *Bsp*LU4I from the reaction mixture, plasmid pUC18 digested with *Bsp*LU4I was purified using Silica suspension. The purified solution was incubated with phage λ DNA. The results showed that this DNA was not subjected to hydrolysis. The fact that Silica treatment did not lead to inhibiting endonuclease *Bsp*LU4I was monitored by phage λ DNA preliminarily treated with Silica suspension and then incubated with *Bsp*LU4I. The fragments formed corresponded completely to the expected pattern of hydrolysis. Thus, endonuclease *Bsp*LU4I can be removed from the reaction mixture by purifying with Silica suspension.

Determination of molecular mass of *Bsp*LU4I. The molecular mass of endonuclease was determined using electrophoresis in 12% polyacrylamide gel in the presence of SDS. Proteins of 94, 67, 43, 29, and 18 kD from the DiaProt set (Dia-M, Russia) were used as molecular mass standards. Gel staining revealed one band. The absence of other bands demonstrates high purity of the preparation obtained. The molecular mass of endonuclease *Bsp*LU4I is 33 kD (Fig. 7a).

To clarify the subunit structure of endonuclease *Bsp*LU4I, it was subjected to gel-filtration under nondenaturing conditions. The analysis of the fractions showed that restriction endonuclease *Bsp*LU4I is eluted as a protein with a molecular mass of 33 kD (Fig. 7b).

Comparison of the electrophoresis and gel-filtration data allowed us to conclude that endonuclease *Bsp*LU4I is a monomer protein with a molecular mass of 33 kD.

Functional purity of *Bsp*LU4I preparation. The use of a standard digestion–ligation–digestion test showed that

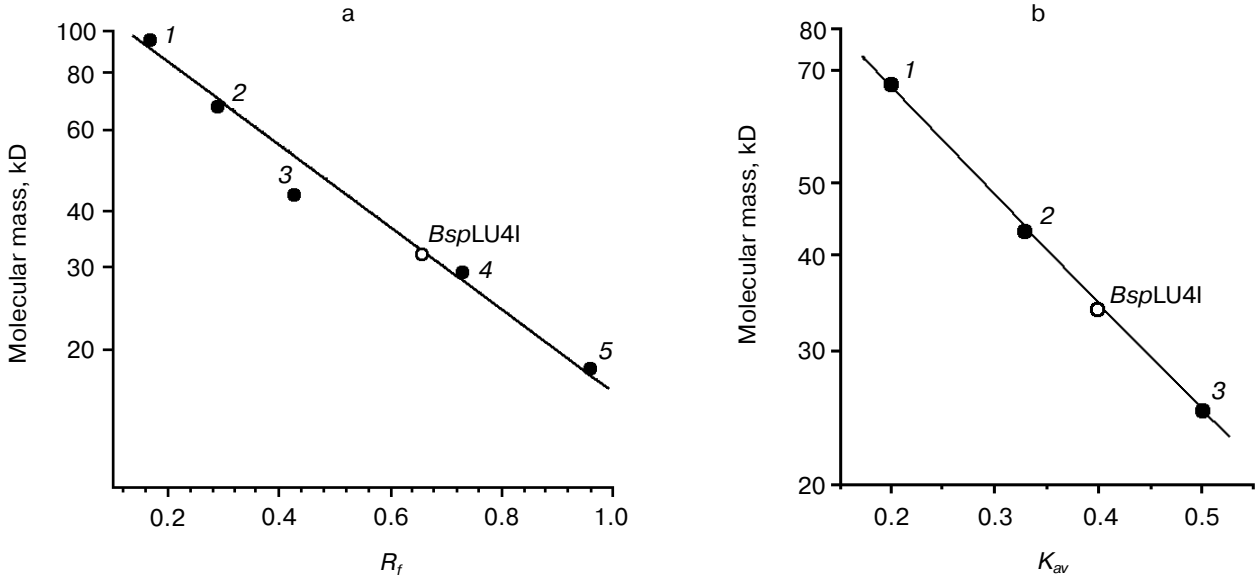

Fig. 7. Determination of molecular mass of endonuclease *Bsp*LU4I. a) Molecular mass determined by migration in denaturing 12% polyacrylamide gel, marker proteins: *1*) 95; *2*) 67; *3*) 43; *4*) 29; *5*) 18 kD; b) molecular mass determined by the volume eluted from the Sephacryl S200 HR column, marker proteins: *1*) 66; *2*) 45; *3*) 25 kD.

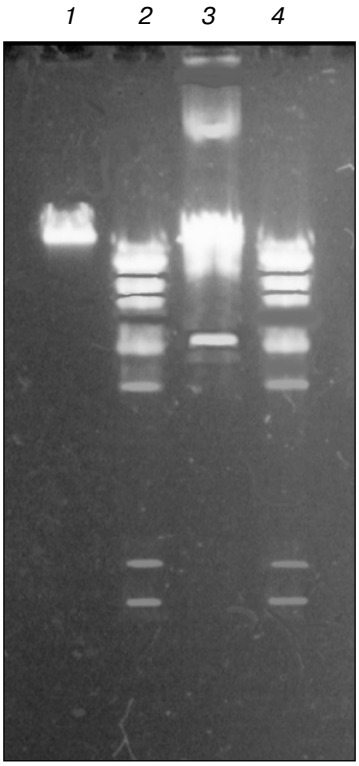

Fig. 8. Digestion–ligation–digestion test: *1*) phage λ DNA; *2*) DNA cleaved with *Bsp*LU4I; *3*) ligated DNA after cleavage with *Bsp*LU4I; *4*) ligation product cleaved with *Bsp*LU4I.

the obtained enzyme is functionally pure. This is confirmed by: 1) the absence of additional fragments after prolonged incubation of substrate DNA with a 100-fold enzyme excess; 2) virtually 100% ligation of DNA fragments upon ligation, and 3) recovery of the initial cleavage pattern after incubation of the ligated preparation with endonuclease (Fig. 8).

Moreover, when the endonuclease is used in sequencing experiments upon cleavage of the 5′-end labeled DNA, only one band is observed, which shows the purity of the preparation.

Optimal conditions for enzyme activity. The optimal conditions for the enzyme activity were determined by varying the components of the reaction mixture: the ionic strength of the solution (0, 50, 100 mM NaCl), pH of the Tris-HCl buffer (7.5, 8.0, 8.5, 8.9, 9.2), and incubation temperature (48, 50, 55, 60, 65, 70°C). It was found that at 50 and 100 mM NaCl the enzyme activity is nearly the same, depends little on pH at 7.2 to 8.5, but drops twofold at pH 8.9-9.2. The enzyme activity is maximal at 60 and 65°C, while at other temperatures it drops from 2 to 4 times. On overnight incubation (16-18 h) the hydrolysis of the substrate phage λ DNA was only twice higher.

The preparation is stable. After storage at room temperature (about 20°C) for two months the enzyme activity was no lower than that of the enzyme stored at –20°C.

Obtaining "ladders" of molecular sizes of DNA fragments. The high purity of the enzyme preparation is useful for obtaining size markers of DNA fragments of the ladder type. If a circular plasmid DNA with one site for *Bsp*LU4I is cleaved with this enzyme and the obtained linear monomers are connected with the DNA ligase under conditions suitable for the formation of mainly linear multimer molecules, a mixture of linear molecules that form a regular ladder upon electrophoresis will be obtained.

The possibility to prepare multimers depends strongly on the quality of the enzyme used. If in a significant part of linear molecules one sticky end is damaged (shortened due to nuclease contamination), further growth of the multimer will be blocked when such molecules are attached with their ends to multimers. Thus, the proportion of fragments with damaged ends will determine the maximal size of the resulting multimers.

To get a ladder with a step of 2000 bp, we constructed a special plasmid pHC624ΔBglII using the known

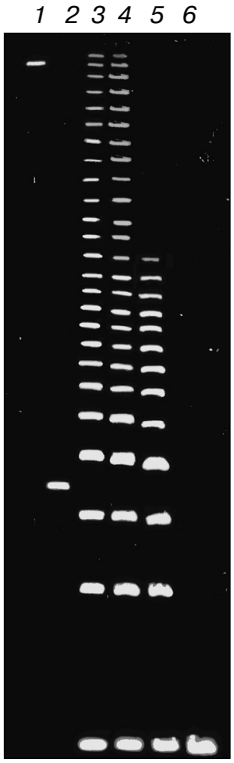

Fig. 9. Separation of tandem forms of plasmid DNA using pulse electrophoresis with polarity inversion in 1% agarose gel: *1*) phage λ DNA size (48,502 bp) marker; *2*) DNA size (7126 bp) marker; *3-5*) ligation products of cleaved plasmid pHC624ΔBglII (DNA ligase dilution is 1000, 2000, and 4000 times, respectively); *6*) initial plasmid pHC624ΔBglII of 1999 bp cleaved with *Bsp*LU4I.

multi-copy plasmid pHC624 [9]. Plasmid pHC624 is 2015 bp long and has two sites for endonuclease *Bgl*II with 20 and 40 bp coordinates. The plasmid was cleaved with *Bgl*II, the large fragment was purified by gel-filtration on a Sepharose 6CL column, and its sticky ends were filled with Klenow fragment of *E. coli* DNA-polymerase I in the presence of all four deoxynucleoside triphosphates. Circular molecules from this fragment were obtained by ligation at a low DNA concentration. After transformation of *E. coli*, a clone was obtained containing plasmid pHC624ΔBglII of 1999 bp and having a single site for *Bsp*LU4I.

The plasmid was cleaved with *Bsp*LU4I and after phenol treatment the linear molecules were used to prepare multimers. Prior to this, ligation conditions were chosen so as to prevent the appearance of circular DNAs. The results of the experiment are given in Fig. 9, which shows that a regular ladder up to 25-30 monomers can be obtained by selecting the amount of DNA ligase. Such ladders can be DNA size markers from 2 to 50 kb.

It should be noted that if non-self-complementary sticky ends (sites CTCGGG and CCCGAG) were formed during cleavage of the site, the monomers would be linked only "head-to-tail". Such multimers can be used to increase the number of gene copies in expression vectors, inasmuch as fused fragments have the same orientation.

There are no literature data on the yield of enzymes that are isoschizomers of *Ava*I. However, obviously strain *Bacillus* species LU4 is the most highly productive not only among the strains producing isoschizomers of *Ava*I but also among all other strains producing restriction endonucleases.

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REFERENCES

1. Murray, K., Hughes, S. G., Brown, G. S., and Bruce, S. A. (1976) *Biochem. J.*, **185**, 65-75.
2. Hughes, S. G., and Murray, K. (1980) *Biochem. J.*, **159**, 317-322.
3. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, New York.
4. Scopes, P. (1982) *Protein Purification*, Springer-Verlag, New York.
5. Laemmli, U. K. (1972) *Nature*, **227**, 680-685.
6. Brown, N. L., and Smith, M. (1980) *Meth. Enzymol.*, **65**, 391-404.
7. Sanger, F. (1981) *Science*, **214**, 1205-1210.
8. *fmol DNA Sequencing System Technical Manual*, Promega.
9. Bovos, I., and Posfai, G. (1984) *Gene*, **30**, 257-260.