*Asp*718 from a non-pathogenic species of the genus *Achromobacter:* a *Kpn*I isoschizomer generating DNA-fragments with 5′-protruding ends

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A new type II restriction endonuclease *Asp*718 has been isolated from the non-pathogenic species of *Achromobacter* 718. This novel enzyme, an isoschizomer of *Kpn*I, recognizes and cleaves specifically within the nucleotide sequence:

5′-G/GTAC-C-3′
3′-C-CATG/G-5′.

In contrast to *Kpn*I, *Asp*718 generates fragments with 5′-protruding single-stranded ends. These 5′-terminal extensions of the *Asp*718 fragments may be efficiently labeled with T4 polynucleotide kinase, whereas the recessed 3′-ends are suitable substrates for the terminal labeling reaction applying Klenow enzyme. The presence of only one restriction activity in this *Achromobacter* strain facilitates the preparation of *Asp*718 free of other contaminating site-specific nucleases which could interfere with the in vitro digestion of DNA.

Restriction endonuclease Kpn*I isoschizomer* *5′-Protruding terminus* *T4 polynucleotide kinase* *Klenow enzyme*

1. INTRODUCTION

Type II restriction endonucleases are fundamental tools for the elucidation of the structure and function of DNA as well as being prerequisites for molecular cloning.

Despite the fact that a great number of restriction endonucleases have already been purified and characterized [1,2], the availability of new enzymes is still valuable for experiments based on the site-specific digestion of DNA. Therefore, in addition to the genera *Lactobacillus, Pediococcus* and *Leuconostoc* we have screened 65 different non-pathogenic species of the genus *Achromobacter,* isolated from chickens of Bavarian origin, for the presence of potentially new type II restriction endonucleases. 20 of these strains were found to produce site-specific endonucleases including isoschizomers of *Ava*II, *Cla*I, *Hae*III, *Hpa*II, *Pst*I, *Sca*I, *Xho*I and *Xmn*I [1].

Another of the screened strains, *Achromobacter* species 718, contains a single type II restriction endonuclease, that is an isoschizomer of *Kpn*I isolated from the pathogenic strain *Klebsiella pneumoniae* OK8.

We have named the new restriction endonuclease as *Asp*718 in accordance to the proposal of Smith and Nathans [3].

*Asp*718 recognizes and cleaves the palindromic sequence 5′-G/GTACC-3′. In contrast to *Kpn*I, *Asp*718 generates DNA-fragments with 5′-protruding ends. These single-stranded 5-termini can be efficiently labeled using T4 polynucleotide kinase. The recessed 3′-ends are suitable substrates

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for the terminal labeling reaction applying Klenow enzyme.

Furthermore, the cohesive termini generated by *Asp*718 will be rapidly ligated to form recombinant DNA molecules. The ligation products may be re-digested with *Asp*718 resulting in the regeneration of the original *Asp*718-fragments. Because of the presence of unique *Asp*718 cleavage sites in pU18 and pUC19 [4], pEMBL18 and pEMBL19 [5], M13mp18 and M13mp19 [6] as well as M13tg130 and M13tg131 [7], the new type II restriction endonuclease *Asp*718 will be of considerable value in cloning and sequencing of DNA molecules.

2. MATERIALS AND METHODS

2.1. *DNAs and reagents*

λ*c*I857Sam7 DNA, pBR322 DNA, λ·*Eco*RI fragments (DNA M_r marker I), alkaline phosphatase from calf intestine, T4 polynucleotide kinase, T4 DNA ligase, Klenow enzyme and the restriction endonucleases *Hin*dIII, *Kpn*I and *Sca*I were products of Boehringer, Mannheim.

High specific activity [γ^{32}P]ATP (~3000 Ci/mmol) and [α^{32}P]dCTP (~3000 Ci/mmol) were obtained from New England Nuclear. Ultrogel AcA 54 was from LKB, heparin-Sepharose CL-6B from Pharmacia and cellulose-phosphate P11 from Whatman. Agarose was from Seaplaque, low melting temperature agarose (type VII) from Sigma.

2.2. *Strain culturing*

Achromobacter species 718 (DSM 2969) was grown in liquid medium containing Bactopeptone, 15.6 g/l; yeast extract (Difco), 2.8 g/l; NaCl, 5.6 g/l; glucose, 1.0 g/l; pH 7.5.

For fermentation the organism was cultivated in a 10-l Giovanola fermenter using a 10% inoculum and 0.08 Vvm aeration as well as 450 rpm agitation during cell growth. After 10 h the cells were harvested in the late-log phase by centrifugation in a Padberg 41G centrifuge at 20000 rpm and stored at −70°C. The yield of the cells was 0.2 g/l dry wt.

2.3. *Enzyme preparation*

The frozen cells (2 g dry wt) were suspended and thawed in 50 ml of buffer A (50 mM Tris-HCl, pH 7.6/4°C; 0.1 mM EDTA; 7 mM β-mercaptoethanol). They were disrupted at 4°C by two passages through a French pressure cel (Aminco J5-598AE) at 23000 lb/inch^2.

Polynucleotides were precipitated by addition of 10% (w/v) streptomycin-sulfate until the precipitation was complete. After stirring for 30 min at 4°C the precipitate was sedimented by centrifugation at 32000×*g* for 120 min.

The clear supernant was applied to a heparin-Sepharose CL-6B column column (2×15 cm) equilibrated with buffer B (20 mM Tris-HCl, pH 7.6/4°C; 0.1 mM EDTA; 7 mM β-mercaptoethanol; 10% (v/v) glycerol). After washing with two column-volumes of buffer B, a 400 ml linear NaCl gradient (0–1.0 M) in buffer B was applied. The active fractions, which eluted at 0.5–0.7 M NaCl, were combined and precipitated by adding a saturated solution of $(NH_4)_2SO_4$ until a saturation of 80% /w/v) was achieved. The precipitate was kept for 60 h at 4°C.

The $(NH_4)_2SO_4$ suspension was centrifuged for 30 min at 32000×*g*. The supernatant was discarded, whereas the pellet was re-dissolved in 1 ml buffer B. The resulting clear solution was fractionated using an Ultrogel AcA 34 column (0.5×100 cm) in buffer B, which contained 0.5 M NaCl.

The active fractions were combined, dialyzed against buffer B and applied to a cellulose-phosphate P11 column (1×10 cm) equilibrated with buffer B. After washing with two column-volumes of buffer B, the column was developed with 80 ml of a linear NaCl gradient (0–1.0 M) in buffer B.

The active fractions, which eluted at 0.4–0.6 M NaCl, were combined and dialyzed for 4 h against buffer B supplemented with 100 mM NaCl and 50% (v/v) glycerol. The enzyme preparation was stored at −20°C.

2.4. *Enzyme assay*

Assays during the enzyme purification were performed using 1–5 μl of the column fractions which were incubated with 1 μg λ·*Eco*RI-fragments in 25 μl incubation mixture (6 mM Tris–HCl, pH 8.5/37°C; 6 mM $MgCl_2$; 75 mM NaCl; 6 mM β-mercaptoethanol; 100 μg/ml bovine serum albumin) for 1 h at 37°C. The reactions were terminated by adding 5 μl of cold stop solution (7 M urea; 20% (w/v) sucrose, 60 mM EDTA and 0.01% (w/v) bromophenol blue). The complete reaction mixtures were resolved by electrophoresis for 3 h at 100 V on 1% (w/v) agarose gels using 40 mM Tris-

acetate (pH 8.2) 25°C; 2 mM EDTA and 1 μg/ml ethidium bromide as electrophoresis buffer.

For exact determination of the *Asp*718 activity, varying amounts of the final enzyme preparation were incubated with 1 μg λ DNA in 25 μl incubation mixture for 1 h at 37°C. The reactions were terminated by adding 25 μl of cold stop solution. 25 μl of the reaction mixtures were resolved by electrophoresis for 16 h at 40 V on 0.5% (w/v) agarose gels in electrophoresis buffer.

One unit of *Asp*718 is defined as the amount of enzyme which digests completely 1 μg of λ DNA within 1 h at 37°C under the stated assay conditions.

3. RESULTS AND DISCUSSION

3.1. *Characterization of the enzyme activity*

The activity of *Asp*718 was found to be optimal at a temperature of 37°C, a pH value of 8.5 (at 37°C), and salt concentrations between 50 and 100 mM NaCl. The enzyme activity is strictly dependent on the presence of Mg^{2+} but does not require *S*-adenosylmethionine or ATP.

Purified *Asp*718 was shown to be essentially free of contaminating nucleases, since λ DNA digested for 16 h with at least a 40-fold excess of enzyme gave sharp bands without any smearing after electrophoresis in agarose gels.

Ligation of 1 μg λ·*Asp*718-fragments with 0.5 unit T4 DNA Ligase for 16 h at 4°C resulted in more than 90% ligation products. Subsequent digestion with *Asp*718 re-generated more than 90% of the original *Asp*718 fragments.

The yield of enzyme was 3×10^5 units per 2 g cells (dry wt). The activity did not decrease after storage for at least 6 months at −20°C.

3.2. *Characterization of the recognition sequence*

Bacteriophage λ DNA [7] and λ·*Eco*RI-fragments were digested with *Asp*718 and the resulting *Asp*718-fragments separated by gel electrophoresis. From the analysis of the observed fragment patterns by applying computer programs designed to search for recognition sequences on the basis of physical mapping data, *Asp*718 was found to be specific for the sequence 5′-GGTACC-3′. Thus, *Asp*718 is an isoschizomer of *Kpn*I [1,2]. This could be confirmed by experiments, in which identical fragment patterns were obtained after digestion of both substrates with either *Kpn*I or *Asp*718 (fig.1). In addition, *Asp*I and *Kpn*I show identical cleavage specificities on the DNAs of the viruses Adeno2 and SV40, φX174 RF and M13mp8 RF, and the plasmids pBR322 and pBR328 [9–17].

3.3. *Identification of the cleavage positions*

The cleavage sites within both strands of the *Asp*718 recognition sequence were determined as described by McConnel et al. [18]. SV40 DNA, linearized with *Hpa*II at position 282, was terminally labeled with either T4 polynucleotide kinase and [γ-^{32}P]ATP at the 5′-ends or Klenow enzyme and [γ-^{32}P]dCTP at the 3′-ends. After digestion with *Bgl*I at position 5171, the 5′-labeled 351 bp *Hpa*II/*Bgl*I fragment (the length refers to the 5′-labeled (−)-strand) and the corresponding 3′-labeled 354 bp fragment (the length refers to the

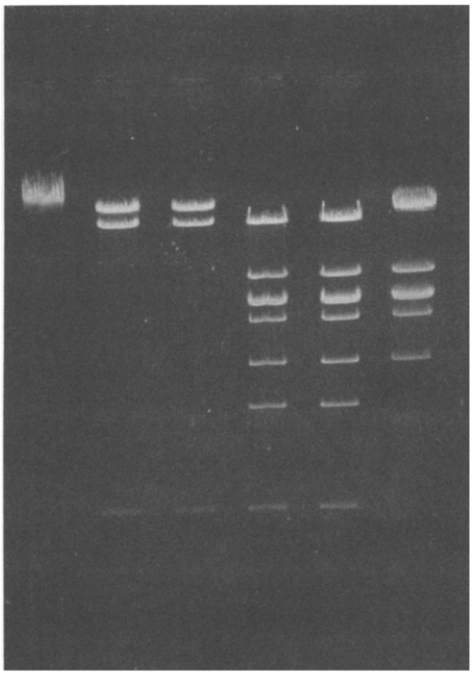

Fig.1. Digestion patterns of bacteriophage λ DNA and λ·*Eco*RI fragments obtained with *Kpn*I (lanes 2,4) and *Asp*718 (lanes 3,5), respectively. Undigested DNAs used as controls: λ DNA (lane 1), λ·*Eco*RI fragments (lane 6). The reaction mixtures were incubated for 1 h at 37°C and analyzed on a 0.5% (w/v) agarose slab-gel by electrophoresis for 16 h at 40 V.

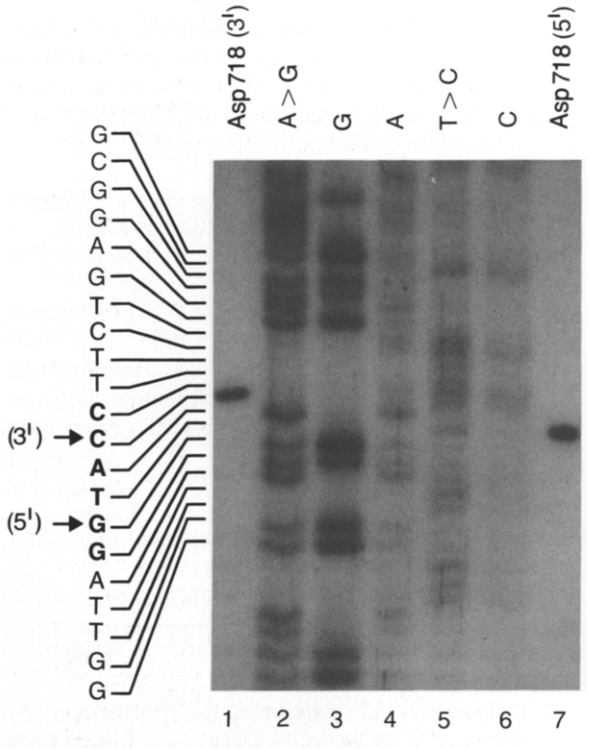

Fig.2. Determination of the cleavage position of *Asp*718 on both strands of SV40 DNA. The lengths of the 3'- as well as the 5'-labeled strand of the SV40 · *Asp*718/*Hpa*II fragment (lanes 1,7) were determined by comparing their migration distances with the positions of the various bands of the sequence ladder of the 5'-labeled (−)-strand of the SV40 · *Bgl*I/*Hpa*II fragment between positions 4892 and 284 (lanes 2–6). Position 1 of SV40 DNA is defined as the first G of the sequence 5'-GCGGAGTTA-3' within the (+)-strand near the origin of replication. Labeling of the protruding 5'-end of the (−)-strand of the SV40 · *Hpa*II fragment was performed with 2 units of T4 polynucleotide kinase and 10 pmol [γ-^{32}P]ATP (~3000 Ci/mmol) per μg DNA according to [19]; labeling of the recessed 3'-ends of the corresponding (+)-strand was achieved with 1 unit of Klenow enzyme and 3 pmol [α-^{32}P]dCTP (~3000 Ci/mmol) per μg DNA according to [20]. 3'-Endlabeling results in the elongation of the (+)-strand for a single deoxycytosine nucleotide. All labeled fragments were purified under native conditions by electrophoresis in 1% (w/v) low melting temperature agarose gels. The sequencing procedure followed the protocol of the chemical method of [19]. Electrophoresis under denaturing conditions was performed in 6% (w/v) polyacrylamide gels containing 7 M urea. The lengths of both the 3'- (lane 1) and the 5'-labeled strand (lane 7) of the SV40 · *Asp*718/*Hpa*II-fragment were determined by comparing their migration distances with the positions of the bands of the sequence ladder of the 5'-labeled (−)-strand of the SV40 · *Hpa*II/*Bgl*I fragment (lanes 2–6).

3'-labeled (+)-strand) were isolated. Samples of both terminally labeled *Hpa*II/*Bgl*I fragments were further digested with *Asp*718.

The lengths of the alternatively labeled (+)- and (−)-strands of the SV40 · *Asp*718/*Hpa*II fragment were analyzed on a sequencing-gel. The exact sizes of both strands were determined by comparing their migration distances with the positions of the various bands of a sequence ladder, which was obtained with the 5'-labeled (+)-strand of the SV40 · *Hpa*II/*Bgl*I-fragment by chemical modification and base-specific cleavage according to Maxam and Gilbert [19].

The 5'-labeled (−)-strand of the SV40 · *Asp*718/*Hpa*II fragment appears beneath the band of the 5'-sequence ladder, which represents the internal G-residue of the *Asp*718 recognition sequence 5'-GGTACC-3' at position 234. However, the fragment of the sequence ladder has lost its 3'-terminal nucleotide during chemical cleavage. Thus, this 5'-labeled (−)-strand of the SV40 · *Asp*718/*Hpa*II fragment actually ends with its 5'-neighbour, the G-residue at position 235.

The 3'-labeled (+)-strand of the SV40 · *Asp*718/*Hpa*II-fragment appears beneath the band of the 5'-sequence ladder, which represents the internal G-residue of the *Asp*718 recognition sequence 3'-CCATGG-5' at position 231. Because the elongation of the 3'-end for one C-residue during the Klenow-directed labeling reaction compensates the loss of one nucleotide during the chemical cleavage reaction, the 3'-labeled (+)-strand of the SV40 · *Asp*718/*Hpa*II fragment ends with the G-residue at position 231.

Therefore, the migration distances of the 5'- as well as the 3'-labeled strand of the SV40 · *Asp*718/*Hpa*II-fragment within the sequencing gel, show that the *Asp*718 cleavage positions within both strands are located between the two flanking G-residues of the recognition sequence. Thus, the complete *Asp*718 recognition sequence is defined as

5'-G/GTAC-C-3'
3'-C-CATG/G-5'

Both cleavage positions are confirmed by the fact, that the length of the 5'- and 3'-labeled strands differ for 3 nucleotides. This result is in agreement with the elongation of the 3'-end for one C-residue during the 3'-labeling reaction with Klenow enzyme.

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