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BpaI and BpnI: novel type II restriction endonucleases from *Bacillus pasteurii* and *Bacillus pantothenticus*

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Abstract

Two novel type II restriction endonucleases, designated as BpaI and BpnI, were isolated from *Bacillus pasteurii* strain1761 and *Bacillus pantothenticus* strain1639, respectively. They were partially purified and SDS-PAGE indicated Mr values of 28 and 67 kDa for BpaI, 28 and 48 kDa for BpnI. The partially purified endonucleases hydrolyzed DNA into discrete fragments: pUC18 (2.6 kb for BpaI; 1.8 and 0.8 kb for BpnI), pBR322 (2.5 and 1.8 kb for BpaI; 2.6 and 1.7 kb for BpnI) and Φx174 DNA (3.2 and 2.1 kb for BpaI; 4 and 1.3 kb for BpnI).

Introduction

Restriction endonucleases are widely used in molecular biology. Many are dimeric proteins that recognize palindromic DNA sequences comprising 4 or 8 defined nucleotides which can be continuous or interrupted, symmetric or asymmetric, unique or degenerate (Williams 2003). They require Mg^{2+} as cofactor and cleavage generally occurs on the 5′ side of the phosphate, leaving DNA fragments with 5′ phosphate and 3′ hydroxyl termini (Pingoud & Jeltsch 2001). The ability of the type II restriction endonucleases to cleave DNA *in vitro* only at their recognition site(s) has led to their use in many applications in analysis and manipulation of DNA. This has prompted extensive screening of bacteria for these enzymes by biochemical and by genome analysis (Kong *et al.* 2000). Some bacteria contain more than 20 different restriction-modification systems and currently more than 3500 restriction enzymes have been identified, encompassing 260 different specificities according to available data REBASE (Roberts *et al.* 2003). Type II restriction-modification systems comprise pairs of enzymes with matching DNA sequence specificity. The modification enzyme is a DNA methyltransferase that specifically methylates, either adenosyl or cytosyl residues, within the recognition sequence, thus making DNA resistant to the restriction activity. The restriction enzyme is an endodeoxyribonuclease that cleaves unmethylated DNA at a precise location within or around the recognition sequence. As a consequence, foreign double stranded DNA, unmethylated at the restriction sites recognized by the cell's restriction-modification systems, is quickly degraded (Rocha *et al.* 2001). In the present study, we describe isolation and partial purification of two restriction endonucleases: BpaI and BpnI from *Bacillus pasteurii* strain1761 and *Bacillus pantothenticus* strain1639 respectively and their activity on different substrate DNAs.

Materials and methods

Growth conditions

Bacillus pasteurii strain1761 and *Bacillus pantothenticus* strain1639 were obtained from MTCC culture collection, IMTECH, Chandigarh, India. pUC18, pBR322, Φx174 DNA and standard protein molecular markers were from Bangalore Genei, India. One kb DNA ladder was from MBI Fermentas. The strains were grown in liquid YBT (1.6% tryptone, 1% yeast extract, 0.8% beef extract and 0.5% NaCl) medium for

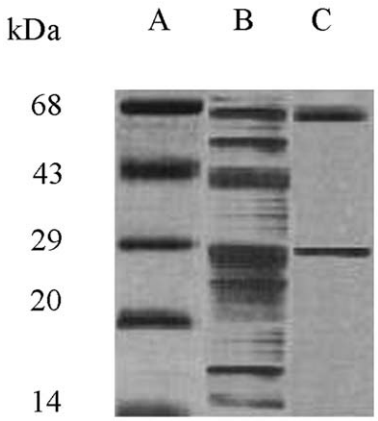

Fig. 1. Protein profile (kDa) of restriction endonuclease from *Bacillus pasteurii* strain1761. Protein was analyzed using 10% SDS-PAGE as described in Materials and methods. Molecular marker (lane A), crude extract (lane B), phosphocellulose fraction (lane C).

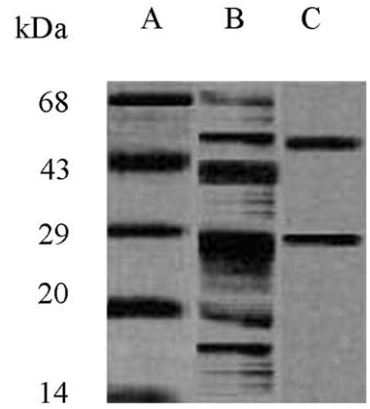

Fig. 3. Protein profile (kDa) of restriction endonuclease from *Bacillus pantothenticus* strain1639. Protein was analyzed using 10% SDS-PAGE as described in Materials and methods. Molecular marker (lane A), crude extract (lane B), phosphocellulose fraction (lane C).

16 h at 37 °C with shaking 130 rpm. The cells were collected by centrifugation at 8000 × *g* for 10 min and stored at −20 °C until use.

Purification of restriction endonucleases

All steps were carried at 4 °C. Frozen cells (16 g wet wt) of a mid-growth phase culture as described above, were thawed in 100 ml buffer A (8 mM NaH_2PO_4; 1 mM EDTA; 15 mM TrisHCl, pH 7.4; 10% (v/v) glycerol, 5 mM β-mercaptoethanol) containing 0.1 M NaCl and disrupted by sonication (Bronson Sonifier II, USA): 5 cycles of 2 min each with an interval of 5 min. After sonication, the slurry was centrifuged

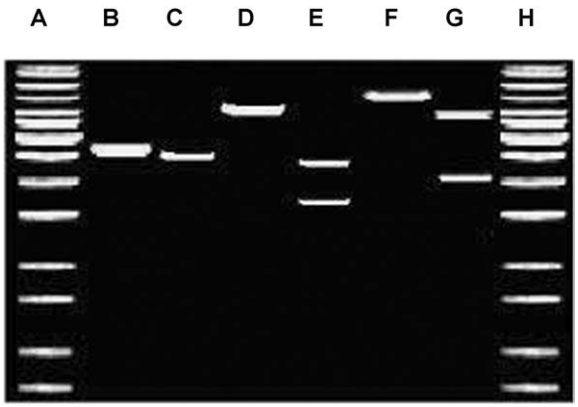

Fig. 2. Profile of digested fragments of pUC18, pBR322 and Φx174 DNA by restriction endonucleases isolated from *Bacillus pasteurii* strain1761 on 0.7% agarose gel. One Kb molecular ladder (lanes A, H), unrestricted pUC18 (lane B), restricted pUC18 (lane C), unrestricted pBR322 (lane D), restricted pBR322 (lane E), unrestricted Φx174 (lane F), restricted Φx174 (lane G).

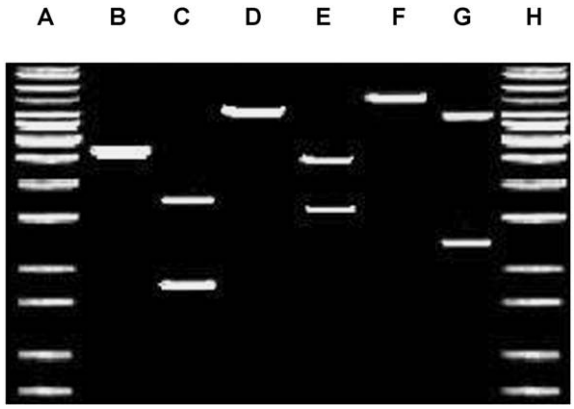

Fig. 4. Profile of digested fragments of pUC18, pBR322 and Φx174 DNA by restriction endonucleases isolated from *Bacillus pantothenticus* strain1639 respectively on 0.7% agarose gel. One Kb molecular ladder (lanes A, H), unrestricted pUC18 (lane B), restricted pUC18 (lane C), unrestricted pBR322 (lane D), restricted pBR322 (lane E), unrestricted Φx174 (lane F), restricted Φx174 (lane G).

at 12 000 × *g* for 2 h. The supernatant was applied on to a phosphocellulose column (1.2 × 8 cm), pre-equilibrated with buffer A containing 0.1 M NaCl. The column was washed with the same buffer and eluted with a linear gradient from 0.1 to 1 M NaCl. Fractions, 5 ml, were collected and assayed for endonuclease activity. The endonucleases were eluted at 0.2–0.5 M NaCl. Active fractions were pooled and was stored in 50% (v/v) glycerol at −20 °C. The fractions were analyzed by 10% SDS-PAGE.

SDS-PAGE analysis

SDS-PAGE on 10% (w/v) polyacrylamide gels was conducted according to Laemmli. Samples from crude extract and phosphocellulose were solubilized in 2× SDS sample buffer containing 62.5 mM Tris/HCl (pH 6.8), 5% (w/v) SDS, 1 mM PMSF, 2% (v/v) β-mercaptoethanol, 10% (w/v) sucrose and 0.01% Bromophenol Blue and heated at 70 °C for 3 min. The apparent molecular masses of proteins were estimated by comparison with the mobility of standard proteins. Proteins on the gel after SDS-PAGE were visualized by Coomassie Brilliant Blue staining following standard protocols (Sambrook & Russell 2001).

Endonuclease activity

The restriction endonuclease activity was assayed in 20 μl reaction mixture containing 2 μl pUC18 or pBR322 or Φx174 DNA (250 μg ml^{-1}) and restriction buffer (10 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiotheritol, 0.1 mg BSA ml^{-1}, 100 mM NaCl). Column samples (4 μl) were added to the reaction mixture and incubated at 37 °C for 90 min. The reaction was stopped by adding 2 μl of 10× loading dye, centrifuged for 30 s and an aliquot (10 μl) from each sample was then electrophoresed using 0.7% agarose gel.

Results and discussion

Restriction-modification systems occur ubiquitously among bacteria and algae (Roberts *et al.* 2003). A wide variety of restriction-modification systems have been discovered and characterized during the past 30 years. Our main objective was to isolate restriction endonucleases from different *Bacillus* species and determine their sites of restriction on different DNAs. We have now standardized a simple protocol for this purpose (Jutur & Reddy 2002). According to suggested nomenclature (Roberts *et al.* 2003) we tentatively named these enzymes as BpaI from *Bacillus pasteurii* strain1761 and BpnI from *Bacillus pantothenticus* strain1639 respectively. These partially purified endonucleases were found to be suitable for producing restriction digests. Our study also suggests that partial purification of these enzymes is better done by phosphocellulose chromatography. Our data indicate that the subunit molecular weights of these partially purified enzymes were around 28 and 67 kDa for the BpaI (Figure 1), 28 and 48 kDa for the BpnI (Figure 3).

A type II enzyme will cleave a supercoiled (SC) plasmid with one site first in one strand to give the open circle (OC) form of the DNA and then in the other strand, to give the full-length linear form (FLL). The cutting of both strands is often faster than the dissociation of enzyme from the DNA (Gormley *et al.* 2000). A plasmid with two sites is usually cleaved by an enzyme that acts independently at each site in sequential steps, leading first to the transient formation of FLL DNA as one site is cut and then, after a lag phase, both sites are cut into the final products.

Our restriction data also suggest that pUC18, pBR322 and Φx174 DNA are cleaved at two sites producing discrete fragments: pUC18 [2.6 kb for BpaI; 1.8 and 0.8 kb for BpnI], pBR322 [2.5 and 1.8 kb for BpaI; 2.6 and 1.7 kb for BpnI] and Φx174 DNA [3.2 and 2.1 kb for BpaI; 4 and 1.3 kb for BpnI] (Figures 2 and 4). This indicates that these two endonucleases have cleavage sites on pUC18, pBR322 and ϕx174 DNA. These enzymes however required Mg^{2+} as co-factor suggesting that they belong to type II class endonucleases (Pingoud & Jeltsch 2001).

In conclusion, restriction digestion of different DNAs by partially purified enzymes suggests that BpaI and BpnI are new restriction endonucleases from *Bacillus pasteurii* strain1761 and *Bacillus pantothenticus* strain1639 respectively, according to the available published data in REBASE (http://rebase.neb.com/).

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