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A novel restriction endonuclease *Unb*I, a neoschizomer of *Sau*96I from an unidentified psychrofilic bacterium from Antarctica is inhibited by phosphate ions*

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A novel type II restriction endonuclease *Unb*I was isolated from an unidentified psychrofilic bacterial strain from Antarctica. *Unb*I recognizes and cleaves the sequence 5′-GGNCC-3′, producing 5 nucleotide long sticky ends. In this respect it differs from its neoschizomer *Sau*96I and all other restriction enzymes recognizing this sequence. *Unb*I has a relatively low temperature optimum of 15°C to 20°C and its activity is completely inhibited by inorganic phosphate.

Organisms living in extreme environmental conditions are valuable objects for research. They were subjected to unusual evolutionary pressure and must have evolved specific molecular mechanisms in order to survive and colonize their respective ecological niches. Studies in Antarctica have shown that cold-tolerant microorganisms (psychrofilic) are abundant on land and in water. Their optimal temperature for growth is below 15°C and they play an important role in the Antarctic sea ecosystem. Polish Antarctic expeditions collected a few hundred different bacterial strains from seawater, ice, land and animals, mostly crustaceans (Donachie, 1995). We have tested several isolates from this collection for the presence of restriction enzymes. In this paper we report isolation and characterization of a novel restriction endonuclease called *Unb*I.

MATERIALS AND METHODS

Strains. All bacterial strains tested for the presence of restriction enzymes were obtained from the collection of the Institute for Antarctic Biology of the Polish Academy of Sciences. Strain No. 8 is a Gram-negative bacterium, rod-shaped, aerobic, isolated from seawater at approximately 5 m depth.

Culture conditions. Standard LB medium was used.

Determination of the cleavage site. PCR-amplified DNA fragment of the synthetic proinsulin gene (Kraszewski *et al.*,

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1986) which contained two sequences recognized by the enzyme, was partially cleaved by *Unb*I and subjected to cyclosequencing using primers from the 3′ end and the 5′ end. Cyclosequencing was performed using reagents and procedures supplied by Amersham USB.

Standard biochemical procedures were according to Sambrook *et al.* (1989).

RESULTS AND DISCUSSION

Screening tests

30 different strains from the Antarctic collection were grown in liquid LB culture at 15°C. Bacteria were sonicated and crude lysates were tested for the presence of restriction enzyme activity using phage lambda DNA as a substrate. After preliminary tests isolate No. 8. was selected because of the highest activity in the crude lysate.

Preparation of crude extracts

Bacteria were grown in a total volume of 3000 ml of LB for 30 h at 15°C. Bacterial pellets after centrifugation were suspended in 50 ml of buffer: 50 mM Tris/HCl, pH 8.0, 0.5 mM EDTA, 50 mM KCl, 5 mM mercaptoethanol, and sonication was performed in ice using 15 pulses 30 s each.

Protein chromatography

Contaminating endogeneous DNA was removed from the bacterial lysate using streptomycin sulphate precipitation (10%) and the resulting supernatant was subjected to column chromatography on Blue-Sepharose. *Unb*I activity did not bind to the column, but in this step a significant amount of contaminating nuclease activity was removed. The eluate was further purified on a DEAE-Sephadex column using a 20–1000 mM KCl gradient. Fractions containing the restriction activity were pooled, dialysed and subjected to DEAE-Sephadex chromatography using a 20–1000 mM KCl gradient. A broad peak of enzyme activity was detected at KCl concentrations between 0.5 M and 0.8 M. Fractions with the lowest activity of contaminating nucleases, eluting at lower KCl concentrations were pooled, dialysed and subjected to CM-Sephadex chromatography. Proteins were eluted using a 20–1000 mM KCl gradient. The enzymatic activity eluted as a single peak at approx. 300 mM KCl. Pooled fractions were dialyzed and concentrated using PEG 2000 dialysis. Finally the enzyme preparation was dialysed overnight and stored in 10% glycerol, 20 mM Tris/HCl, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 2 mM mercaptoethanol. The enzyme preparation was stored at –20°C without any significant loss of activity. The enzyme was named *Unb*I.

Temperature optimum

Activity of the purified enzyme was tested at different temperatures. Optimal activity was found between 10°C and 15°C; at 20°C some partial digestion products were visible. At 37°C and higher temperatures the activity was completely blocked.

Optimal reaction conditions

The enzyme was found to be more active in potassium salts than in sodium salts. Optimal salt concentration was found to be around 100 mM KCl, while a broad pH optimum was between 6.4 and 8.6.

Religation assay

In order to check if the DNA cut by the *Unb*I enzyme can be religated, we cut the plasmid Bluescript KS+. After the restriction reaction DNA was phenol-purified and incubated with DNA ligase in standard conditions. More than 50% of the DNA bands were found as ligation products. This indicates that a significant fraction of the 5′-end phosphate groups are intact after the restriction cleavage, and the enzyme does not exhibit a phosphatase activity.

Phosphate inhibition

During our tests for an optimal column chromatography purification procedure we have found that during chromatography on

phosphocellulose P the enzyme activity is lost. This indicated that the enzyme could be sensitive to inorganic phosphate. In order to study this phenomenon we checked the effect of different concentrations of phosphate on the activity of our purified enzyme preparation. The results shown in Fig. 1 indicate that at the concentration of inorganic phosphate reaching 150 mM the enzyme activity is completely blocked. Our data do not allow to distinguish if the inhibition is competitive or noncompetitive. We favor the hypothesis of noncompetitive inhibition, since the only known enzymes that are competitively blocked by phosphate are phosphatases. More experiments are required to elucidate this problem.

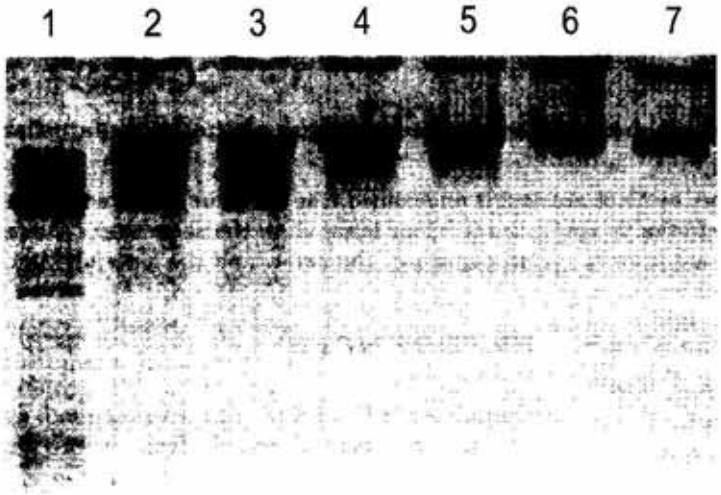

Figure 1. Influence of potassium phosphate buffer on the activity of *Unb*I in standard conditions.

Concentrations of KP_i: lane 1: zero, 2: 50 mM, 3: 100 mM, 4: 150 mM, 5: 200 mM, 6: 250 mM, 7: 300mM.

Identification of the recognition sequence

Initial studies to identify the recognition sequence of *Unb*I involved digestion of the plasmid Bluescript KS+ with the purified enzyme preparation and with other restriction enzymes: *Bgl*I, *Eco*RI, *Pvu*I and *Pvu*II. Analysis of restriction fragments was performed using the DNASIS computer program. We assumed that the enzyme belongs to the class II restriction enzymes and that it cleaves within an approximately six base pairs long palindrome. The obtained data indicated that the enzyme recognizes the same DNA sequence as *Sau*96I. This has been confirmed by restriction analysis of three different plasmids cut with the enzyme and with *Sau*96I. Figure 2 shows that DNA fragments cut by the enzyme or with *Sau*96I migrate at the same respective positions. Therefore the recognition sequence seems to be 5′-GGNCC-3′.

In order to identify the cleavage site of the enzyme we used a synthetic DNA fragment coding for human proinsulin, which contains two sequences recognized by the enzyme. PCR-amplified DNA fragment was cleaved partially by the *Unb*I enzyme, and then subjected to PCR cyclosequencing. In this way the products of the sequencing reaction would show frequent termination at the

Figure 2. Comparison of restriction activity of *Sau*96I and *Unb*I towards different DNA substrates.

Lane 1: pGEM3/*Sau*96I; lane 2: pGEM3/*Unb*I; lane 3: Yep351/*Sau*96I; lane 4: Yep551/*Unb*I; lane 5 pKS+/*Sau*96I; lane 6: pKS+/*Unb*I.

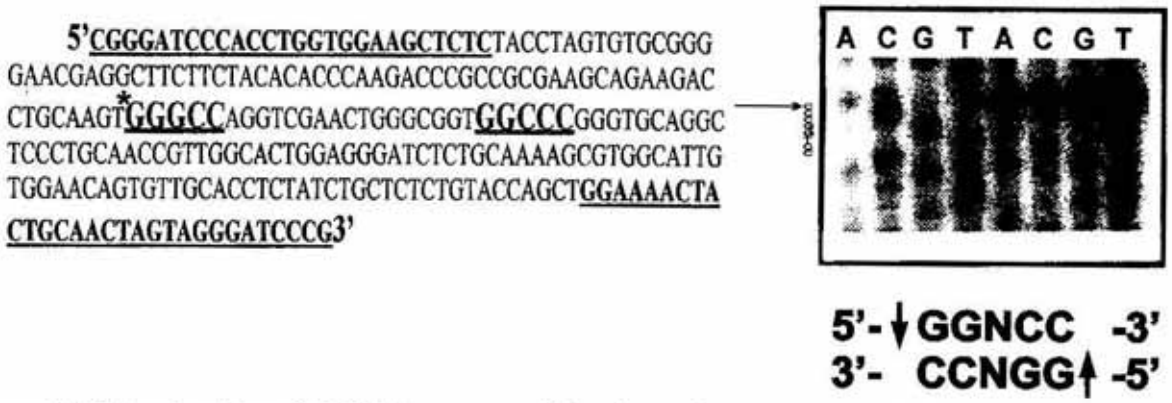

Figure 3. Determination of *Unb*I cleavage position by cyclosequencing of the PCR-amplified DNA fragment.

Primers are underlined, sequences recognized by *Unb*I are double underlined. The first four lanes on the sequencing gel show the control experiment without addition of *Unb*I, the last four lanes show the sequence of the template which was partially cleaved with the enzyme prior to cyclosequencing. The arrow and the asterisk indicate the cleavage position of *Unb*I.

cleavage site. Using primers from the 5′ end or 3′ end (not shown) we were able to demonstrate both 3′ and 5′ cleavage sites of the enzyme. The sequencing gel is shown in Fig. 3. The position of bands prematurely terminated due to the cleavage by the *Unb*I enzyme indicates that the enzyme cuts in the following way: 5′-↓GGNCC↑-3′. Therefore the *Unb*I restriction endonuclease is not the isoschizomere of any of the previously described enzymes recognizing the GGNCC sequence.

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