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*BPU*AMI: A NOVEL *SAC*I NEOSCHIZOMER FROM *BACILLUS PUMILUS* DISCOVERED IN AN ISOLATE FROM AMAZON BASIN, RECOGNIZING 5'-GAG↓CTC-3'

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ABSTRACT

A strain of *Bacillus pumilus* was isolated and identified from water samples collected from a small affluent of the Amazon River. Type II restriction endonuclease activity was detected in these bacteria. The enzyme was purified and the molecular weight of the native protein estimated by gel filtration and SDS-PAGE. The optimum pH, temperature and salt requirements were determined. Quality control assays showed the complete absence of "nonspecific nucleases." Restriction cleavage analysis and DNA sequencing of restriction fragments allowed the unequivocal demonstration of 5´GAG↓CTC3´ as the recognition sequence. This enzyme was named *Bpu*AmI and is apparently a neoschizomer of the prototype restriction endonuclease *Sac*I. This is the first report of an isoschizomer and/or neoschizomer of the prototype *Sac*I identified in the genus *Bacillus*.

Key words: type II restriction endonuclease, *Bpu*AmI, *Sac*I, neoschizomers, *Bacillus pumilus*

INTRODUCTION

Restriction endonucleases are enzymes which recognize short DNA sequences and cleave DNA in both strands. Depending on the enzymological properties, different types can be distinguished. Type II restriction endonucleases are homodimers which recognize short palindromic sequences (17). The recognition sequence for the majority of the ~3600 type II (with ~250 prototypes and 588 commercially available) restriction endonucleases identified to date are symmetrical palindromes of 4-8 bp in length, and in the presence of Mg2+, cleave DNA within or next to the recognition site (18,19).

In addition to being a valuable tool for molecular biology, type II restriction endonucleases have been used as a model system for studying aspects of specific DNA-protein interactions (16) and mechanisms of phosphodiester hydrolysis (12). These useful properties of type II restriction endonucleases have prompted many laboratories to screen thousands of taxonomically diverse bacteria for enzymes with new characteristics. However, to our knowledge, there has not been any report to date of an isoschizomer and/or neoschizomer of the prototype *Sac*I identified in the genus *Bacillus*.

This paper describes the isolation and identification of *B. pumilus* found in a bacterial collection of water samples from a small affluent of the Amazon River, and the detection of a type II restriction endonuclease activity, along with the purification, biochemical characterization, and cleavage site determination of a novel neoschizomer of the RE *Sac*I.

MATERIALS AND METHODS

Bacterial strain isolation and cultivation

B. pumilus bacteria were isolated from water samples from a small affluent of the Amazon River in the region of Itacotiara (58º27´W, 3º 9´S). At the time of sampling the temperature was

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36ºC. The water samples and dilutions of 10^{-1} and 10^{-2} in sterile distilled water, 50 µL of each, were plated on LB-agar, and incubated for 24 h at 30ºC in order to obtain isolated colonies. Cells were grown for 14-16 h at 37ºC (250 rpm) in LB broth and harvested by centrifugation (6,000 x *g* for 20 min). The cell pellet was washed once with phosphate buffered saline and stored at -70ºC. A yield of approximately 12 g (wet weight) was obtained from a 2-L culture.

Identification of the bacterial strain

The identification of the bacterial isolates was performed by biochemical tests (Gram stain, the GNI card/Vitek Systems, Hazelwood, Mo., and the API 20NE identification system/ bioMériuex, France) and by small-subunit rRNA gene (SSU rDNA) DNA sequencing (3,15), described elsewhere (5). The sequences were analyzed by multiple sequence alignments using the computer program Clustal X (11). The consensus sequence derived from the multiple alignment was used to search the GenBank database using BLAST (1).

Procedure for purification of type II restriction endonuclease

The purification was based on a protocol described elsewhere (9) with minor modifications. Typically, 12 g of cells were suspended in 80 mL of PC Buffer (100 mM K_2HPO_4/KH_2PO_4 pH 7.5, containing 0.1 mM EDTA, 10 mM β-mercaptoethanol), 150 mM NaCl, and 0.02 mM phenyl-methyl-sulfonyl-fluoride (PMSF). The cell suspension was incubated for 30 min in the presence of lysozyme (0.2 mg/mL), disrupted by sonication (10 pulses of 30 sec each) at 4ºC, and centrifuged at 29,000 x *g* for 1h at 4ºC. Streptomycin sulfate was slowly added to the resulting supernatant to a final concentration of 1.8%, which was then centrifuged at 28,000 x *g* for 1h at 4ºC. The supernatant was dialyzed against PC buffer containing 100 mM NaCl and clarified at 28,000 x *g* for 20 min at 4ºC. At this step of the protocol, the detection of restriction activity was measured in "One for all" buffer (Pharmacia, UK) with l DNA as substrate. The clear supernatant showing restriction activity was loaded onto a 2 x 12 cm phosphocellulose (P11) column (Whatman Inc, USA) previously equilibrated with PC buffer, washed with ten column volumes with the same buffer and eluted with a NaCl linear gradient (100 - 800 mM). Active fractions were identified by digestion of bacteriophage λ DNA. Fractions showing restriction activity were pooled, dialyzed against PC buffer containing 100 mM NaCl, and clarified by centrifugation (28,000 x *g* for 20 min at 4ºC). The resulting supernatant was loaded onto a 4.8 x 2.6 cm heparin-Sepharose column (Pharmacia-UK) and eluted with a 10-volume NaCl linear gradient (100 - 800 mM) in the same PC buffer. Fractions containing the peak of enzyme activity were pooled, dialyzed against PC Buffer and concentrated (Ultracel – YM 10.000 NMWM Amicon Bioseparations, Bedford MA, USA) to 5 mL, adjusted to 50% (v/v) with glycerol and stored at -20ºC.

Apparent molecular weight determination

Gel filtration was carried out on a 1 x 30-cm FPLC Superdex-75 column (Amersham Pharmacia Biotech), previously calibrated with the following molecular weight markers [79.5 kDa, human serum transferrin; 65 kDa, bovine serum albumin; 45 kDa, ovoalbumin; 29 kDa, bovine carbonic anhydrase; and 12.4 kDa, horse heart cytochrome]. The target enzyme peak activity was eluted at an elution volume. In addition, the determination of the molecular mass of this type II RE was estimated by polyacrylamide gel electrophoresis (12.5%) in the presence of sodium-dodecyl sulfate (SDS) carried out by the method of Laemmli (13). A HMW- SDS Calibration Kit 11B06413 (Amersham Pharmacia Biotech) and Mid-Range Protein MW markers (V523A - Promega, USA) were used during electrophoresis.

Protein concentration determination

Protein concentration was determined by the Bradford method (4) using the Bio-Rad protein assay kit (Bio-Rad, USA) and bovine serum albumin as standard.

Enzyme characterization, quality control tests and restriction cleavage analysis

The optimum salt concentration, pH, temperature and restriction cleavage site were determined for the purified enzyme preparation as described elsewhere (5). The restriction cleavage analysis was performed by the digestion of DNAs of bacteriophages Ad2, λ and ϕX174 and plasmid pCI-neo. DNA fragments were analyzed by gel electrophoresis (20). A commercially available enzyme *Sac*I (R6061 – Promega , USA) was used as control.

Determination of the recognition sequence of *Bpu*AmI using pUC18 DNA

pUC18 plasmid DNA was cleaved with the purified restriction enzyme in the presence of Mg^{2+} at 37ºC in SuRE/Cut buffer A (2) for 1 h. In order to determine the cleavage site of this restriction enzyme, sequencing of the digested DNA solution was performed by chain termination method (21) using ^{33}P-dNTPs and the Thermo Sequenase radiolabeled terminator cycle sequencing Kit (Amersham Pharmacia Biotech – UK). The primers utilized were as follows: 40 M13 forward, 5'GTT TTC CCA GTC ACG AC 3'; and reverse M13, 5´CAG GAA ACA GCT ATG A 3' primers for sequencing (Invitrogen). The same protocols for DNA cleavage and sequencing were performed using commercially available *Sac*I.

RESULTS AND DISCUSSION

Bacteria were isolated from water samples of a small affluent of the Amazon River in the region of Itacotiara, and a strain of *Bacillus pumillus* was identified. The sequence of its SSU rDNA and sequence alignments to other 16S rRNA coding DNA

sequences revealed 100% homology with the *Bacillus pumillus* strain MI -9 -1 16S ribosomal RNA gene (partial sequence GenBank accession AB048252-GI:10129890).

The specific activity for type II restriction endonuclease was determined in cell-free extracts of the *Bacillus pumillus* isolate. The separation protocol utilized allowed the recovery of 15 000 units of the restriction enzyme from 2 liters of culture. The crude cell extract contained some nonspecific nucleases, the bulk of which were removed by phosphocellulose chromatography. The remaining nonspecific nuclease activities were further removed by heparin-Sepharose chromatography as demonstrated by both the absence of changes in the electrophoresis patterns of both λ DNA and pCI -neo "over-digestion" (up to 24 h) and the same sharpness of DNA bands as those obtained after 1 h digestion. The molecular weight of the purified restriction enzyme was estimated by gel filtration and the elution profile was consistent with a value of 38 kDa for the native protein and confirmed as measured by SDS-PAGE (Fig. 1).The optimum pH, temperature, and salt concentrations were estimated to be, respectively, 7.9 at 37ºC in SuRE/Cut Buffer A (33 mM Tris-acetate, 10 mM magnesium acetate, 10 mM potassium acetate, and 0.5 mM dithiothreitol).

The restriction fragment patterns produced by the purified enzyme and commercially available *Sac*I analyzed by digestion of DNAs of bacteriophages Ad2, λ and ϕX174 and plasmid pCI-neo were compared to allow the determination of the recognition sequence of the *B. pumilus* RE. It was found that for Ad2 DNA there were16 restriction fragments, and for the substrates λ DNA, ϕX174 DNA and pCI-neo there were 2, 0 and 2 restriction fragments, respectively (Fig. 2). The cleavage restriction patterns strongly suggest that the unknown restriction enzyme recognizes the same DNA sequence as *Sac*I does.

The chosen primers were designed to anneal specifically either to a DNA sequence starting from nucleotide 204 or from nucleotide 326, allowing the synthesis of single-stranded DNA of different lengths. This stretch of DNA sequence was chosen because it possesses only one *Sac*I digestion site. The nucleotide sequence determination by the dideoxy chain termination method of the amplified fragments, which were produced by pUC18 plasmid DNA digestion with the *B. pumilus* enzyme, clearly showed that 5´GAG↓CTC3´ is the cleavage sequence (Fig. 3), different from *Sac*I, which is 5´GAGCT↓C3´ (data not show). The DNA sequencing results confirm that the *B. pumillus* restriction enzyme is a neoschizomer of *Sac*I.

There have been only two isoschizomers described with the same cleavage site as that of the prototype *Sac*I from *Streptomyces achomogenes*, although only one, *Sst*I from S*treptomyces stanford* (8) is commercially available together with the prototype, the other one being from *Pseudomonas* sp (6). All three bacteria have an optimum growth temperature below 30ºC, but their optimum restriction activity temperature is at 37ºC. Four neoschizomers have been previously described with the same 5´GAG↓CTC3´ cleavage sequence; two have been described in *Escherichia coli* (7), and the two others in *Myxococcus xantus* (14) and *Enterobacter cloacae* (10).

Apparently, few restriction endonucleases have been described that recognize this 6-base pair sequence. On the other hand, there have been a number of different restriction enzyme

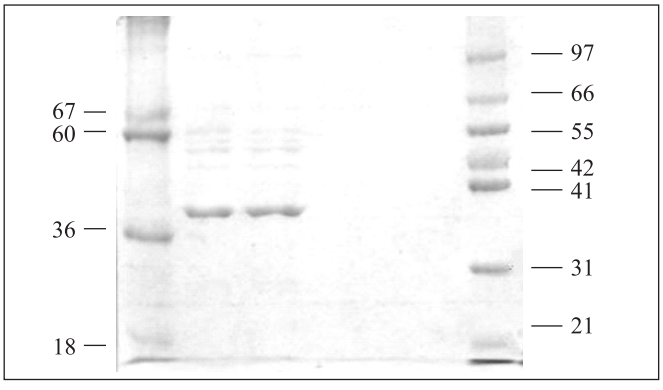

Figure 1. The purified *Bpu*AmI was analysed by SDS-PAGE; lane 1and 6, molecular mass standards; lanes 2-5, purified *Bpu*AmI RE.

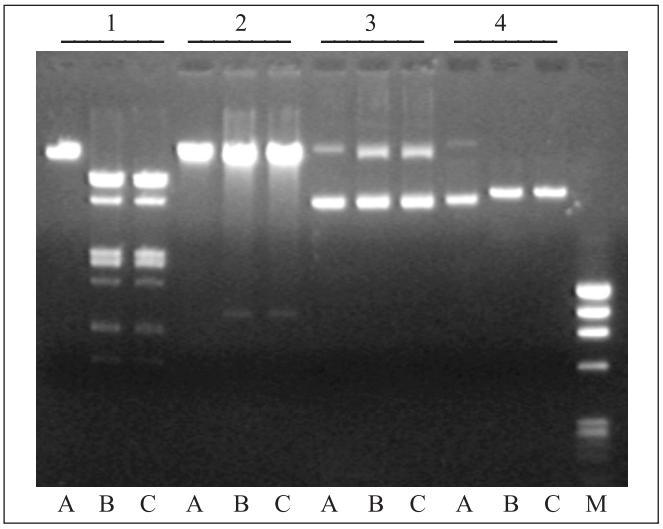

Figure 2. Agarose gel electrophoresis of restriction fragments produced by cleavage of four DNA substrates. The cleaved DNAs were separated by electrophoresis on 0.8% agarose gel and visualized under UV after ethidium bromide staining.(1) Ad2 phage DNA (2) λ phage DNA, (3) ϕX174 phage DNA, and (4) pCI-neo DNA. (A) undigested DNA, (B) *Bpu*AmI and (C) *Sac*I (R6061–Promega, USA) (M) 1mg ϕX174/*Hae*III Markers (G1761-Promega).

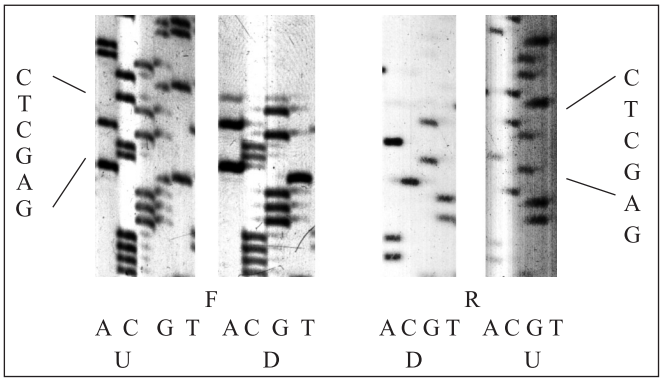

Figure 3. DNA sequencing gel showing the labelled product, approximately 20 bases either side of the putative cut site of *Bpu*AmI. R and F are the amplification products using, respectively, the Reverse and Forward primers. U and D are the same amplification products Undigested and Digested, respectively.

activities described in at least 20 strains of *Bacillus pumillus*. However, as far as we know, the work presented here describes the first neoschizomer and/or isoschizomer of the *Sac*I restriction enzyme found in the genus *Bacillus*.

The type II restriction enzyme reported here has been named *Bpu*AmI, *Bpu* as recommended by the official nomenclature (22), and the intervening letters "Am" as suggested by Dr. Richard Roberts (personal communication). The *Bpu*AmI enzyme has been deposited on the official REBASE web site (http://rebase.neb.com) under enzyme number 6408.

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RESUMO

*Bpu*AmI: Um novo neoesquisomero de *Sac*I de *Bacillus pumilus* descoberta em um isolado oriundo da Bacia Amazônica, reconhecendo 5'-GAG↓CTC-3'

Uma linhagem de *Bacillus pumilus* foi isolada e identificada de amostras de águas coletadas em um pequeno Igarapé do Rio Amazonas. Foi detectada atividade de restrição do tipo II nesta bactéria. A enzima foi purificada e o peso molecular da proteína nativa foi estimado por gel filtração e por eletroforese em gel de poliacrilamida. Foram determinados, o pH e temperatura ótimos e as necessidades de sais. Os ensaios do controle de qualidade mostraram uma ausência completa de "nucleases não específicas". As analises das clivagens e o seqüenciamento do DNA dos fragmentos de restrição permitiram uma demonstração inequívoca de que 5´GAG↓CTC 3´ é a seqüência de reconhecimento da enzima. Esta enzima foi denominada de *Bpu*AmI e aparentemente é um neoesquisômero da enzima protótipo *Sac*I. Este é o primeiro relato de um isoesquisômero e/ou neoesquisômero da enzima protótipo *Sac*I identificada no gênero *Bacillus.*

Palavras-chave: endonuclease de restrição do tipo II, *Bpu*AmI, *Sac*I, neoesquisômero, *Bacillus pumilus*

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