# Identification and characterization of a type II restriction endonuclease, *Str*I from *Streptomyces thermodiastaticus*

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**A new type II restriction endonuclease, *Str*I has been identified from *Streptomyces thermodiastaticus*. The enzyme has been purified using three column chromatography steps. The enzyme recognizes hexanucleotide sequence and cleaves DNA 5¢ -C TCGAG-3¢ as indicated. The optimum temperature, pH, and cation requirements for the enzyme activity were determined.**

RESTRICTION–modification (R–M) systems serve as primary defense mechanisms of bacteria against intruding DNA molecules[1,2]. They have been classified into three main groups according to their cofactor require-ments and the type of DNA cleavage[3]. The type II restriction enzymes require only $Mg^{2+}$ as a cofactor and are also the simplest ones with respect to other properties such as subunit structure and cleavage characteristics. They are composed of two separate enzymatic activities. One is a restriction endonuclease (Enase) that cleaves DNA at a specific recognition sequence. The second is a DNA methyltransferase (Mtase), which is able to methylate the same sequence and render it refractive to cleavage by the corresponding Enase[4]. Most of the enzymes of this group recognize palindromic sequences which generally vary between four and eight base pairs in length. These enzymes are the most intensively searched group of enzymes due to their wide usage in genetic manipulations. Moreover they also serve as useful model systems for studying protein–DNA interactions.

Because of their application potential and exquisite specificity, there have been constant efforts to isolate new type II Enases. As a result, more than 2800 Enases have been isolated mostly from bacterial sources[5]. While no Enase activity has been detected so far in some bacterial strains, multiple enzyme activities have been characterized in many others. Bacteria exhibiting up to two different Enases are of common occurrence. Strains exhibiting even four[6] and five specificities[7] have also been identified. Here, we report the identification and characterization of a new Enase, *Str*I from a *Streptomyces thermodiastaticus*.

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We have isolated a wide variety of bacteria from soil and screened for the DNA

cleavage activity as described by Schleif[8] using l DNA as substrate. Cell-free extracts from one of the isolates showed a consistent cleavage pattern, characteristic of type II Enases. The bacterium exhibiting restriction activity is a gram-positive, long thin, rod-shaped aerobic, which grows at 37° C and produces brown diffusible pigment (Figure 1). It produces an extensively branched mycelium, and forms discrete leathery, colonies (Figure 2). The bacterium is catalase positive, citrate and starch utilization positive, has the ability to reduce nitrate and liquify gelatin. Further, it hydrolyses a variety of sugars such as dextrose, inositol, insulin, mannitol, salicin, sorbitol, sucrose and trehalose. However, it is unable to hydrolyse adonitol, arabinose, dulcitol, fructose, maltose, melibiose and raffinose. Based on these microbiological and biochemical tests, the organism was identified to be *S. thermodiastaticus*[9] (accesion number MTCC 3299). The strain appears to be different from the type strain[10].

The enzyme was purified by successive chromatographic steps using phosphocellulose, hydroxyapatite

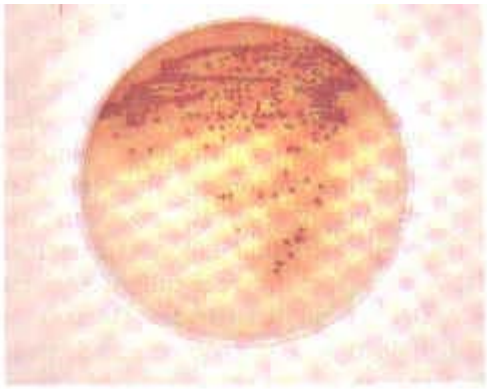


Figure 1. *S. thermodiastaticus* on LB-agar plate.

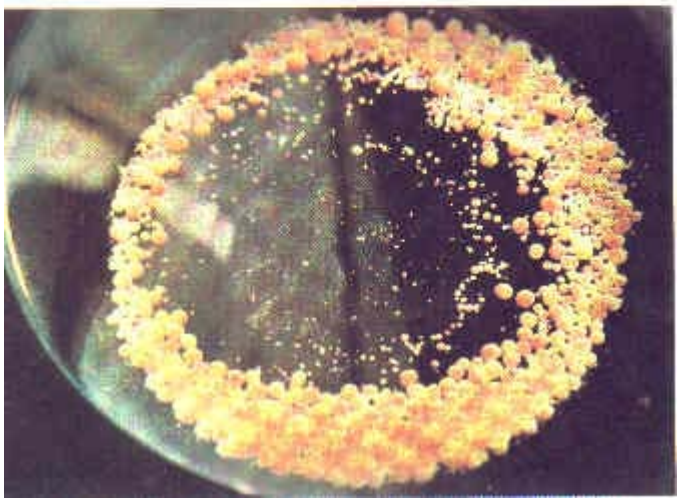


**Figure 1.** *S. thermodiastaticus* on LB-agar plate.

**Figure 2.** *S. thermodiastaticus* liquid culture.

and heparin sepharose columns. Twenty grams of cells was resuspended in 30 ml of buffer A (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 7 mM b -mercaptoethanol) with 2 mM PMSF. Cells were lysed by sonication and centrifuged at

16000 rpm for one hour. The supernatant was loaded on to phosphocellulose column which was preequilibrated with buffer A. The enzyme was eluted with a linear gradient of 0–1 M KCl. The fractions containing enzyme activity were pooled, diluted with buffer A and then loaded on to hydroxyapatite column. The enzyme was eluted using a linear gradient of 0.01–0.60 M potassium phosphate, the active fractions were pooled and dialysed against buffer B (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 7 mM b -mercaptoethanol) containing 50 mM NaCl. The dialysed sample was loaded on to heparin sepharose column and eluted with the linear gradient of 0.05–1.00 M NaCl. The active fractions were pooled and dialysed against buffer B containing 50% glycerol and stored at –20° C.

*Str*I digested I DNA was ligated and then redigested with the enzyme. The pattern is found to be same as that of the digestion pattern. Further, the DNA samples digested with an excess of enzyme for long periods gave sharp bands. Thus, the purified enzyme *Str*I seems to be free of any contaminating non-specific nucleases.

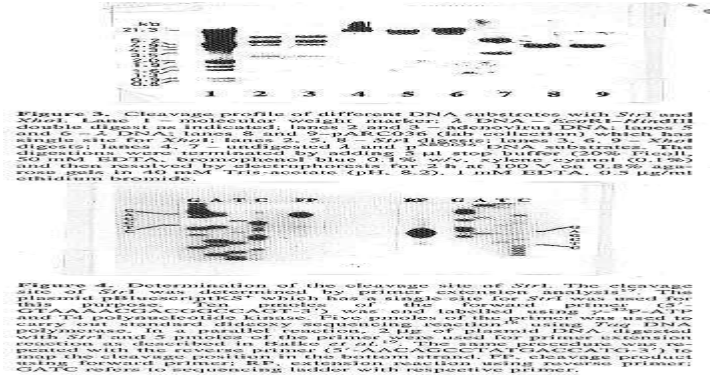
To analyse the cleavage pattern and also to determine the recognition sequence of *Str*I, different DNA substrates were used for the cleavage reaction. While I and pARC036 DNA (4586 bp) were cut once by the enzyme, adenovirus DNA had six sites (Figure 3) and mobility of the fragments resembled the cleavage pattern generated by *Xho*I. *Xho*I digestion of adenovirus DNA generated 7 fragments of sizes 9642, 6149, 5864, 5778, 4593, 2466, 1445 bp. Fragments of the same size were generated from adenovirus DNA with *Str*I. The exact cleavage site was determined by primer extension analysis using pBluescriptKS$^{+}$ which has a single *Str*I site. The enzyme recognized and cleaved the following sequence (Figure 4),

5¢ -C/TCGAG-3¢

3¢ -GAGCT/C-5¢

which is also the recognition sequence of *Xho*I (ref. 11). Thus, the enzyme *Str*I is an isoschizomer of *Xho*I.

Optimum temperature for the enzyme was determined by estimating the enzyme activity at different temperatures. The optimum pH for the enzyme was determined by estimating the per cent activity at different pH values such as 6.0, 6.5, 7.0, 8.0, 8.5 and 9.0. The enzyme exhibits maximum activity at 37° C and is active over a broad pH range (Table 1). The type II Enases have been shown to require divalent metal ions for their activity[2]. In the absence of divalent metal ions, some of the enzymes can bind to DNA at the recognition sequence but cannot cleave the DNA. The *Str*I enzyme was assayed for its activity in the presence of different divalent



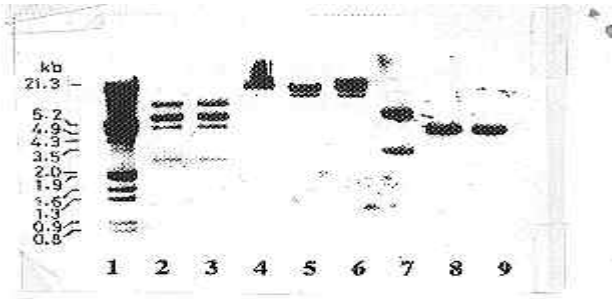



**Figure 3.** Cleavage profile of different DNA substrates with *Str*I and *Xho*I. Lane 1 – molecular weight marker; λ DNA – *Eco*RI–*Hin*dIII double digest as indicated; lanes 2 and 3 – adenovirus DNA; lanes 5 and 6 – λ DNA; lanes 8 and 9–pARC036 (lab collection) which has single site for *Xho*I; lanes 2, 5, 8 – *Str*I digests; lanes 3, 6, 9 – *Xho*I digests; lanes 4, 7 – undigested λ and $p^{ARC036}$ DNA substrates. The digestions were terminated by adding 5 µl stop buffer (20% Ficoll, 50 mM EDTA, bromophenol blue 0.1% w/v, xylene cyanol (0.1%) and then resolved by electrophoresis for 2 h at 100 V on 0.8% agarose gels in 40 mM Tris-acetate (pH, 8.2), 1 mM EDTA, 0.5 µg/ml ethidium bromide.

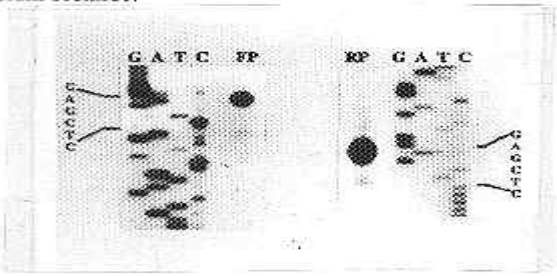



**Figure 4.** Determination of the cleavage site of *Str*I. The cleavage site of *Str*I was determined by primer extension analysis[17]. The plasmid pBluescriptKS+ which has a single site for *Str*I was used for this purpose. Ten pmoles of the forward primer (5′-GTAAAACGACGGCCAGT-3′) was end labelled using γ-$^{32}$P-ATP and T4 polynucleotide kinase. Five pmoles of the primer was used to carry out standard dideoxy sequencing reaction[18] using *Taq* DNA polymerase. In a parallel reaction, 2 µg of plasmid DNA digested with *Str*I and 5 pmoles of the primer were used for primer extension reaction as described in Balke *et al.*[19]. The same procedure was repeated with the reverse primer (5′-AACAGCCTATGACCATG-3′) to map the cleavage position in the bottom strand. FP, cleavage product using forward primer; RP, extension reaction using reverse primer; GATC refers to sequencing ladder with respective primer.

cations such as $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$ and $Cd^{2+}$. *Str*I exhibits enzyme activity both in presence of $Mg^{2+}$ and $Mn^{2+}$ while no detectable activity was obtained with other divalent cations. In the case of *Eco*RV, $Ca^{2+}$ is known to stimulate enzyme activity at lower concentrations but inhibits activity at higher concentration[12]. We found that $Ca^{2+}$ has no effect on the activity of *Str*I suggesting that $Ca^{2+}$ does not replace $Mg^{2+}$ at the active site of *Str*I enzyme. Many Enases exhibit star activity in the presence of $Mn^{2+}$ (ref. 13). We could not detect star ac-

**Table 1.** Relative activity of *Str*I at different temperatures, pH values and salt concentrations

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Temperature (°C) | 15 | 20 | 30 | 37 | 45 | 50 | 55 | 60 |
| % Activity | 20 | 20 | 50 | 100 | 55 | 35 | 15 | 5 |
| pH value | 6 | 6.5 | 7 | 7.5 | 8 | 8.5 | 9 | |
| % Activity | 20 | 65 | 80 | 100 | 100 | 100 | 80 | |
| NaCl (mM) | 0 | 50 | 100 | 150 | 200 | | | |
| % Activity | 100 | 60 | 35 | 20 | ND | | | |

The enzyme assays were performed by incubating the enzyme with λ DNA in 50 µl reaction buffer (10 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 50 mM NaCl, 5 mM β-mercaptoethanol) for 1 h at 37°C. The unit for the enzyme activity was estimated by incubating various amounts of enzyme with 1 µg of λ DNA under the standard assay conditions as mentioned above. One unit of the *Str*I is defined as the amount of enzyme required to digest 1 µg of λ DNA for 1 h at 37°C. Optimum temperature for the enzyme was determined by estimating the activity at different temperatures. The optimum pH for the enzyme was determined by estimating the per cent activity at different pH values such as 6.0, 6.5, 7.0, 8.0, 8.5 and 9.0. The ionic requirements for the enzyme activity were characterized by adding different concentrations of NaCl to the reaction mixture.

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tivity with *Str*I in the presence of $Mn^{2+}$, glycerol or other experimental conditions (not shown).

The purification of *Str*I was very simple and the purified enzyme is devoid of any other detectable Enase or nonspecific nucleases. Thus, the enzyme *Str*I could be a convenient alternative for its isochizomer *Xho*I, in which case there appears to be another Enase *Xho*II encountered during the different stages of purification[11].

A large number of R–M systems have been identified from the genus *Streptomyces*. A point worth noting here is the characterization of isoschizomers of *Xho*I from different *Streptomyces* species. Tetracycline producing strain of *S. aureofaciens* encodes a cryptic R–M system (*Sau*LPII) which gets activated only after actinophage infection[14]. Another strain, *S. aureofaciens* 3239I produces another isoschizomer *Sau*32391 (ref. 15). *Str*I is from *S. thermodiastaticus* and we are not dealing with another strain of *S. aureofaciens*. Further, unlike the above strains, no other Enase activity could be detected in this species at different stages of purification. A strong case has been made for horizontal gene transfer to account for wide distribution of type II R–M systems[16]. Occurrence of the same isoschizomers in different species/strains of *Streptomyces* might reflect efficient operation of gene transfer mechanism in the genus.


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