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R.*Ssp*D5I is a neoschizomer of *Hph*I producing blunt end DNA fragments

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Abstract The strain *Staphylococcus* species D5 produces a restriction enzyme. It is the neoschizomer of *Hph*I endonuclease, which cleaves DNA at a distance of eight nucleotides from the recognition sequences producing blunt end DNA fragments: 5′-GGTGA8N↓-3′ and 3′-CCACT8N↑-5′.

Key words: Site-specific endonuclease; Neoschizomer; Restriction

1. Introduction

Site-specific endonucleases are in the forefront of the enzymes used in genetic engineering. More than 2000 site-specific endonucleases recognizing more than 200 specific DNA sequences are identified now and the search for new enzymes continues [1]. Site-specific endonucleases type II (EC 3.1.21.4) are used particularly often. Thus, the identification of new site-specific endonucleases of this type or neoschizomers of known endonucleases possessing valuable new properties opens wider possibilities for the construction of recombinant DNA.

The present study describes the site-specific endonuclease *Ssp*D5I from the strain *Staphylococcus* species D5. A method is reported for the construction of recombinant phages that contain multiple *Ssp*D5I endonuclease recognition sites and some properties of this enzyme are described.

2. Materials and methods

Substrate DNAs (λCI857S7 [2], M13tg130 [3], M13tg131[3], pJRD184 [4], pUC19) used in this study were prepared in the Group of Molecular Genetics (Institute of Protein Research, Russian Academy of Sciences). Strain *Escherichia coli* XL-1 Blue (recA1, endA1, gyrA96, thi, hsdR17 (r_k^- m_k^-), supE44, relA1, lac/F′::Tn10, proA$^+$B$^+$, lac I^{q}Δ (lacZ) M15) was used for the transformation [5].

2.1. The isolation of site-specific endonuclease

To isolate the site-specific endonuclease *Ssp*D5I, we used a natural isolate of the soil bacterium *Staphylococcus* species D5. This endonuclease was purified to functional purity by sequential chromatography on blue-agarose, hydroxyapatite and heparine-agarose (manuscript in preparation).

2.2. Determination of the optimal conditions for the restriction reaction of endonuclease SspD5I

For elucidation of the optimal conditions for the restriction reaction, we varied the ionic strength, pH of the buffer for the restriction reaction and the incubation temperature. NaCl concentrations in the reaction buffer (10 mM Tris-HCl, pH 7.4, 100 μg/ml albumin, 10 mM $MgCl_2$) were 10, 50 and 100 mM for LRB, MRB and HRB, respectively. The pH values of the reaction buffer were 7.0, 7.4, 8.0. Incubation temperatures were 28, 37 and 48°C.

2.3. Determination of DNA cleavage points

Cleavage points on DNA were determined by the elongated primer method [6]. As a template, we used for the first time the recombinant phage DNAs specially constructed for this study (M13tg130: λCIHindIII and M13tg131: λCIHindIII) containing the small fragment of λCI857S7 DNA with coordinates 37459–37584 inserted in opposite orientations. These phage DNAs contained the recognition site for the *Ssp*D5I endonuclease near the universal primer.

To make DNA recombinants containing multiple recognition sites for the endonuclease *Ssp*D5I in different surrounding nucleotide sequences, we used a synthetic oligonucleotide: 5′-AGATCT11NTCACC3NGGATCC-3′, which contains the recognition site for *Ssp*D5I (underlined) neighboring random nucleotides. The oligonucleotides were annealed at 16°C. Due to self-complimentary of their 3′-OH ends, double-stranded dimer oligonucleotides were produced after synthesis of complimentary strands with the Klenow fragment (exo-) at 16°C for 20 min. They were phosphorylated with phage T4 polynucleotide kinase and then ligated to each other. The ligation products were cloned into vector M13tg131 cleaved with the *Sma*I endonuclease.

3. Results and discussion

In screening bacterial strains from soil samples for the production of new restriction enzymes, we have isolated the strain *Staphylococcus* species D5, which produces a restriction enzyme *Ssp*D5I. It was shown by the cleavage mapping and the primer extension method [6] that it is a neoschizomer of *Hph*I. Cleavage of substrate DNAs by *Ssp*D5I produces the following fragments: M13tg131 -2212, 1595, 1025, 589, 489, 271, 241, 194, 144 bp; pJRD184- 1343, 853, two 396–406, 282 and two 226–227 bp; pUC19- 1529, 396, 272 and two 226–227 bp (Fig. 1). These cleavage patterns are similar to those of DNAs with *Hph*I. Conditions of the restriction reaction for endonuclease *Ssp*D5I are as follows: (1) the optimal buffer for the restriction reaction is HRB, (2) the optimal pH is 7.4, (3) the optimal temperature of incubation is 37°C.

To determine the cleavage points of DNA with this enzyme, we constructed two M13tg130:λCIHindIII and M13tg131:λCIHindIII recombinants which contained the small *Hin*dIII DNA fragment of λCI857S7 DNA inserted in opposite orientations. It was shown by the primer extension method that the endonuclease cleaves DNA in both cases at distances of eight nucleotides from the recognition site, producing blunt end DNA fragments (Fig. 2), so it differs from *Hph*I which cleaves DNA usually at seven nucleotides from the site on one strand and eight nucleotides on the other, producing a one base 3′-OH overhang [7].

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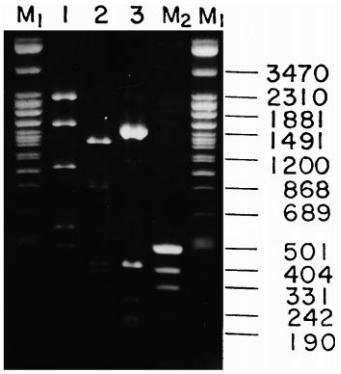

Fig. 1. Digestion of DNAs with *Bsp*D5I: (1) M13tg131+*Ssp*D5I, (2) pJRD184+*Ssp*D5I, (3) pUC19+*Ssp*D5I. DNA size markers: (M_1) T7+*Bli*736I, (M_2) pUC19+*Hpa*II.

It was shown that *Hph*I may cleave DNA at different distances from the recognition sites, depending on the sequence between the recognition and cleavage sites [8,9]. To check this possibility in the case of *Ssp*D5I, we constructed a recombinant phage M13tg131D12SmaI. It contained several *Ssp*D5I recognition sites with an opposite orientation separated by short variable nucleotide sequences. To make this recombinant, we used a synthetic oligonucleotide (31 nucleotides). It contained *Ssp*D5I recognition sites, surrounding random nucleotide sequences.

Oligonucleotides were annealed to each other using a conserved 3′-end region, then the complimentary strand was synthesized with the Klenow fragment.

The obtained dimers were ligated to each other building long fragments with multiple cleavage sites which were eventually cloned into *Sma*I-treated M13tg131. Fig. 3 shows the part of the M13tg131D12SmaI nucleotide sequence with four *Ssp*D5I recognition sites in opposite orientation. It was shown

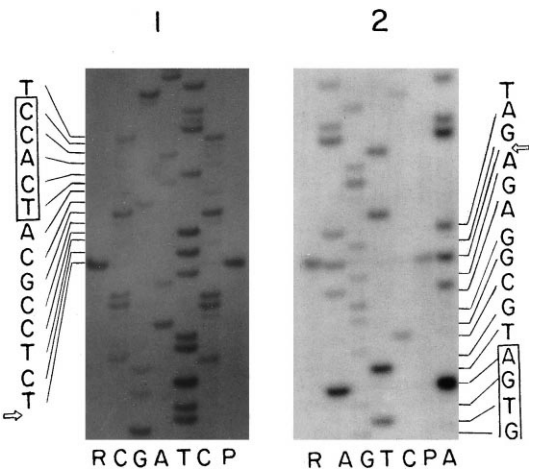

Fig. 2. The determination of *Ssp*D5I cleavage points. Single-stranded DNAs of the M13tg130: λCIHindIII recombinant (1) and M13tg131: λCIHindIII (2) were used in the primer extension method. Sequences in the boxes are recognition sites of *Ssp*D5I. (R) The product of the primed synthesis reaction was cleaved with *Ssp*D5I, (P) The product from lane R was treated with the Klenow fragment; G, A, T, C are the sequence ladders through the *Ssp*D5I recognition sequence using the termination method. Arrows are cleavage points.

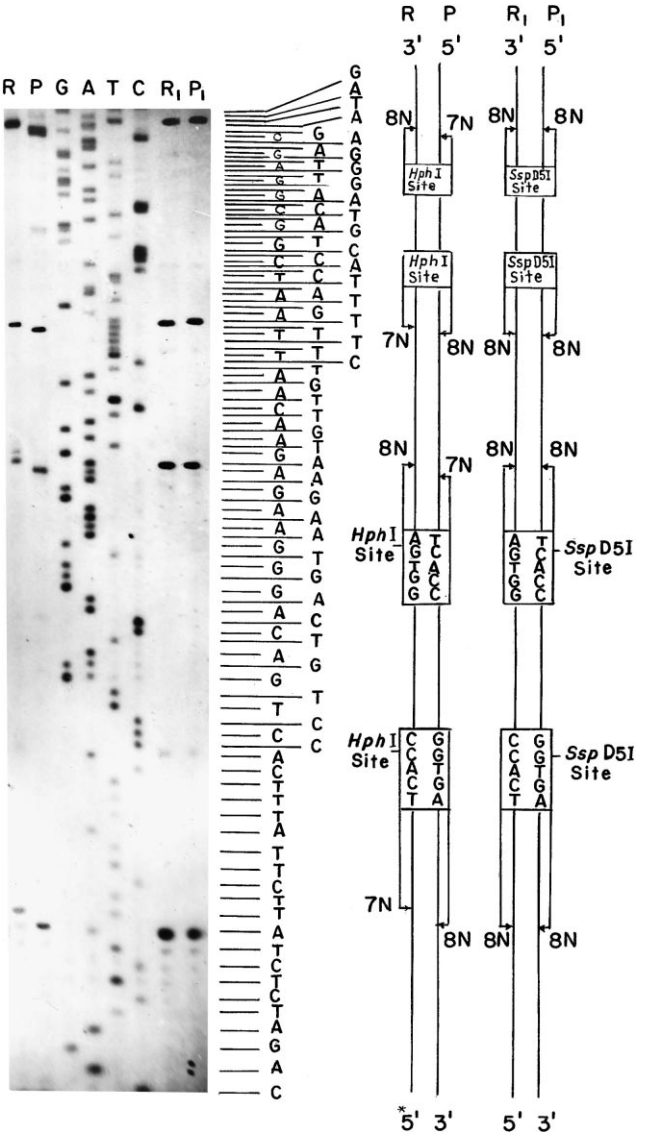

Fig. 3. Determination of *Ssp*D5I cleavage positions using the recombinant with multiple recognition sites. A single-stranded DNA of the M13tg131D12SmaI recombinant was used in the primer extension method. (R) The product of the primed synthesis reaction was cleaved with *Hph*I, (P) the product from lane R was treated with the Klenow fragment, (R_1) The product of the primed synthesis reaction was cleaved with *Ssp*D5I, (P_1) the product from lane R_1 was treated with the Klenow fragment; G, A, T, C are sequence ladders through the *Ssp*D5I and the *Hph*I recognition sequences using the termination method. Arrows are cleavage points.

by the primer extension method that endonuclease *Ssp*D5I cleaves both DNA strands at a distance of eight nucleotides from the recognition site independently of the sequence between the recognition and cleavage sites. Thus, the data on the additional four sites support our first inference that *Ssp*D5I is not an isoschizomer but a neoschizomer of endonuclease *Hph*I and therefore it is a new type II endonuclease. The advantage of the new enzyme is that blunt end DNA fragments produced with this enzyme can be inserted in many versatile vectors.

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