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Site-Specific Nickase from *Bacillus* Species Strain D6

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Abstract—Three site-specific endonucleases were found in thermophilic strain *Bacillus* species D6. One of them, *Bsp*D6II, is an isoschizomer of *Eco*57I. The second, *Bsp*D6III, is present in the strain in very small amount; therefore, it has not been characterized. This paper is devoted to the third, *Bsp*D6I, which recognizes pentanucleotide site 5′-GAGTC-3′ and cleaves only one DNA strand at a distance of 4 nucleotides from the site in the 3′-direction in the chain with the GAGTC sequence, i.e., it behaves as a site-specific nickase. Nickase *N.Bsp*D6I cleaves one DNA strand only in double-stranded DNA and does not cleave single-stranded DNA. Site-specific methylase *Ssc*L1I (an isohypectomer of M·HinfI) that methylates adenine in the sequence 5′-GANTC-3′ prevents DNA hydrolysis by nickase *Bsp*D6I.

Key words: site-specific endonucleases, site-specific nickases, DNA methyltransferases

Site-specific restriction endonucleases are important tools in construction of recombinant DNAs and are of use in studies of the mechanisms of DNA–protein interactions. In recent years, a new class of site-specific endonucleases, nickases *Bst*SE and *Bst*NBI, was found [1, 2]. The nickases recognize a strictly defined site on a double-stranded DNA but cleave only one DNA strand in a clearly determined manner. We have found a site-specific endonuclease in *Bacillus* species strain D6 with specificity identical to that of *N.Bst*SE and *N.Bst*NBI, cleaving only one strand in a double-stranded DNA. Site-specific methylase *Ssc*L1I [3], which methylates the recognition site of *N.Bsp*D6I, prevents the hydrolysis of the DNA by *N.Bsp*D6I. This supports the idea that *N.Bsp*D6I may be an integral part of a modification–restriction system.

MATERIALS AND METHODS

We prepared all the DNAs (of phages T7, λdam⁻dcm⁻, M13mp18, M13mp19, cytosine-containing DNA of phage T4, plasmid pJRD184) as well as enzymes (restriction endonucleases, DNA-methyltransferase *Ssc*L1I, Klenow fragment, Taq-polymerase, and DNA-ligase T4) used in the studies.

DNA cleavage. DNA was cleaved by various restriction endonucleases under their optimal conditions. The products of cleavage were analyzed by electrophoresis in agarose gel (0.8 and 1.2%) in 1 × TBE buffer (0.089 M Tris, 0.089 M boric acid, pH 8.3, 1 mM EDTA).

Determination of DNA cleavage points. Nickase *Bsp*D6I was used to determine DNA cleavage points using the elongated primer method [4]. DNA was sequenced by the Sanger method [5] as modified by Promega (USA) [6] using a ^{32}P-labeled primer and Taq-polymerase. Radioactive products of DNA hydrolysis and were analyzed and sequenced in 6% polyacrylamide gel containing 7 M urea at 50°C on a Macrophor instrument (LKB, Sweden).

Oligonucleotides. Oligonucleotides were synthesized by Sintol (Russia). The oligonucleotides were labeled using [γ-^{32}P]ATP (Cluster Scientific Production Association, Russia) and phage T4 polynucleotide kinases (Boehringer Mannheim, Germany) as described in [7]. The oligonucleotides were hybridized (annealed) by 10-min incubation at 60°C with subsequent incubation at room temperature for no less than 15 min in buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM dithiothreitol.

Methylation of oligonucleotide duplexes. Oligonucleotide duplexes were methylated with DNA methylase *Ssc*L1I in buffer containing 50 mM Tris-HCl (pH 7.5), 8 mM EDTA, 80 μM S-adenosyl-L-methionine, 100 μg/ml gelatin for 1 h at 37°C.

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Duplex analysis. Duplexes were analyzed on the Macrophor instrument using electrophoresis in 20% polyacrylamide gel containing 7 M urea, 1 × TBE buffer at 50°C. After the electrophoresis, the material was transferred onto a DEAE cellulose filter (BioRad, USA) with a Transblot cell (BioRad). Blotting was continued for 30 min at 250 mA and 50 V in a 0.05 × TAE buffer (1 × TAE: 50 mM Tris-Ac, pH 8.0, 2 mM EDTA). Radioautography was used to determine the position of bands on the filter, Retina X-ray film (Germany) being used for this purpose.

Preparation of deletion mutant M13mp19ΔAva. The deletion mutant of phage M13mp19 was obtained by cleaving the replicative form of DNA of phage M13mp19 with an isoschizomer of AvaI, the restriction endonuclease *Bsp*LU4I [8], followed by filling in the "sticky" ends with the Klenow fragment and ligation at low DNA concentration (0.5 μg/ml). After transformation of *E. coli* XL1, the deletion in one of the clones forming a colorless colony on a plate with Xgal (5-bromo-4-chloro-3-indolyl-β-galactoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) was confirmed by sequencing.

RESULTS

Three site-specific endonucleases—*Bsp*D6I, *Bsp*D6II, and *Bsp*D6III—were found in the thermophilic *Bacillus* species strain D6 (results will be published). One of them, *Bsp*D6II, is an isoschisomer of *Eco*57I. It was impossible to characterize *Bsp*D6III since it is present in the strain in very small amount. This paper is devoted to description of the new endonuclease *Bsp*D6I.

Determination of substrate specificity of endonuclease *Bsp*D6I. Upon treating various DNAs (cytosine-containing phage T4 DNA, DNAs of phages λdam⁻dcm⁻, T7, M13mp19, and plasmid pJRD184) with endonuclease *Bsp*D6I, it was found that only DNA of phage T7 is cleaved in four sites and DNA of phage M13mp19 in one site. This fact *per se* shows the unusual properties of this enzyme because phage T7 DNA is only one fourth the length of phage T4 DNA and plasmid pJRD184 contains unique sites for 46 restriction endonucleases.

Our primary goal was to obtain a map of the cleavage points of DNA of phages T7 and M13mp19. Figure 1 shows the results of cleavage of phage T7 DNA with endonuclease *Bsp*D6I and of the same DNA preliminarily cleaved with endonucleases that make only one cut of the DNA. The data obtained and the data on double cleavage of phage T7 DNA with endonuclease *Bsp*D6I and other endonucleases cleaving this DNA in several points established that phage T7 DNA is cleaved at points with coordinates 13,320, 20,610, 24,550, and 30,520. A similar analysis of M13mp19 DNA demonstrated that the single cleavage point is located close to 5,800. The analysis of nucleotide sequences near the cleavage points using the MicroGenie program did not reveal any homologous sequences of six or more nucleotides. However, it was found that in the vicinity of all the assumed cleavage points there are sequence 5′-GAGTC-3′ and complementary to it sequence 5′-GACTC-3′ separated by 10-17 nucleotides (Fig. 2). This means that two oppositely oriented sites 5′-GAGTC-3′/5′-GACTC-3′ are present in the vicinity of the cleavage points on the double-stranded DNA of phage T7. However, phage T7 DNA has more than 100 such sites, while endonuclease *Bsp*D6I cleaves T7 only in four sites. Therefore, we proposed that *Bsp*D6I recognizes this site but cleaves only one DNA strand, and the DNA fragmentation takes place only when the two sites with different orientations are located close to each other.

To test this assumption, two approaches were used. In the first approach, we used the fact that site GAGTC is present in the polylinker of DNA of phages M13mp18 and M13mp19, sequence 5′-GAGTC-3′ being present in the single-stranded DNA of M13mp18 and the complementary to it sequence 5′-GACTC-3′ being present in the single-stranded DNA of M13mp19. If two-stranded DNAs are synthesized using single-stranded DNAs of

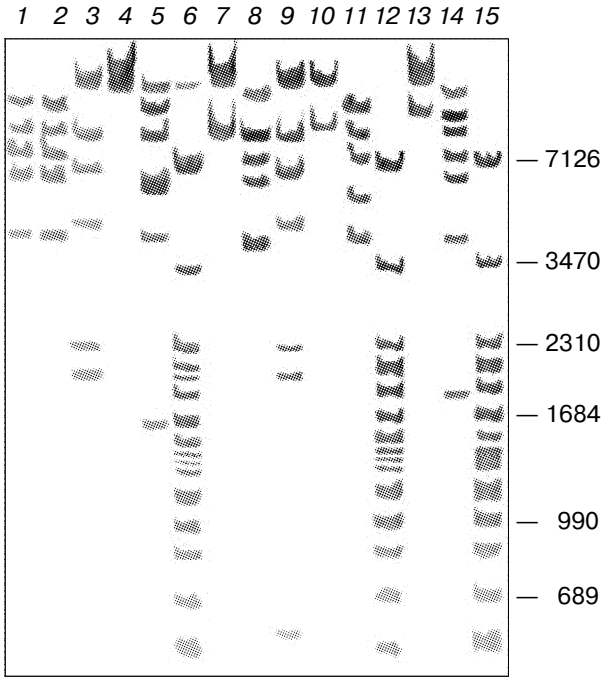

Fig. 1. Cleavage with *Bsp*D6I endonuclease of intact DNA of phage T7 and that after treatment with one-site endonucleases: *1*, *2*) T7: *Bsp*D6I (treatment for 1 and 4 h, respectively); *3*, *9*) λ: *Hind*III (fragment length markers); *4*) T7: *Stu*I; *5*) T7: *Stu*I + *Bsp*D6I; *6*, *12*, *15*) T7: *Bli*736I (markers of the fragment lengths); *7*) T7: *Mlu*I; *8*) T7: *Mlu*I + *Bsp*D6I; *10*) T7: *Nco*I; *11*) T7: *Nco*I + *Bsp*D6I; *13*) T7: *Bgl*II; *14*) T7: *Bgl*II + *Bsp*D6I. On the right, lengths (bp) of some fragments T7: *Bli*736I.

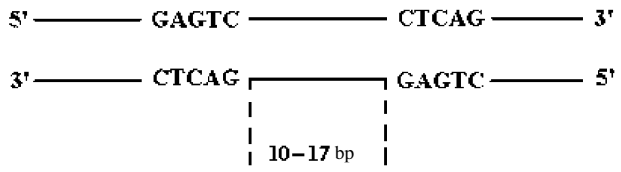

Fig. 2. Scheme demonstrating peculiarity of the nucleotide sequence in the vicinity of cleavage points of DNA of phages T7 and M13mp19 with endonuclease *Bsp*D6I.

these phages as templates and a labeled oligonucleotide complementary to the region adjoining the polylinker sequence as a primer, in a two-stranded DNA of M13mp18 the synthesized labeled strand would have sequence 5′-GACTC-3′ and the labeled strand of M13mp19 would have sequence 5′-GAGTC-3′. Then, if *Bsp*D6I cleaves only one strand, on incubation of labeled two-stranded DNAs of M13mp18 and M13mp19 with *Bsp*D6I the cleavage of the labeled strand would occur only in one of the two DNAs. As a result, during electrophoresis in denaturing polyacrylamide gel the radioautograph would display a cleaved fragment of the labeled single-stranded DNA whose size and correspondingly cleavage point could be determined relative to the "sequencing" ladders of the corresponding DNA. Additional evidence for the cleavage of only one DNA strand is the fact that the labeled fragment would "disappear" after incubation of the cleaved DNA with the DNA-polymerase (Klenow fragment) in the presence of four deoxynucleoside triphosphates (dNTP), because upon cleavage of one DNA strand the polymerase would synthesize DNAs from the cleavage point using as a template the uncleaved strand, displacing the earlier synthesized strand. As a result, the size of the labeled strand would increase so that the material would not leave the gel slot (would "disappear").

The data completely confirmed (Fig. 3) that *Bsp*D6I cleaves only one strand that contains sequence 5′-GAGTC-3′, and it is cleaved at a distance of four nucleotides from this sequence in the direction of the 3′-end. Indeed, the labeled fragment is found only in M13mp19 (Fig. 3a, lane *1*) and disappears after incubation with DNA polymerase (Fig. 3a, lane *2*). In M13mp18, no labeled fragment is revealed (Fig. 3b, lanes *1* and *2*).

In the other approach, a recombinant M13mp19ΔAva DNA was constructed in which the assumed cleavage region of DNA of phage M13mp19 with endonuclease *Bsp*D6I (5,800 nucleotides) is close to the universal primer. Cleavage points were determined as in the first approach, i.e., on the template of the single-stranded DNA a labeled two-stranded DNA was synthesized that was then cleaved with endonuclease *Bsp*D6I, and part of the cleaved DNA was incubated with Klenow fragment and dNTP mixture. The results of this experiment are given in Fig. 4, from which it is seen that in M13mp19ΔAva DNA, with the 5′-GAGTC-3′/5′-GACTC-3′ sites positioned as represented in Fig. 2, each of the strands containing sequence 5′-GAGTC-3′ is cleaved. Actually, upon DNA cleavage with endonuclease *Bsp*D6I a labeled fragment is revealed (Fig. 4, lane *1*) and on the addition of the Klenow fragment to the cleaved DNA this fragment extends the cut of the unlabeled strand (Fig. 4, lane *2*). It should be noted that the very fact of DNA synthesis with Klenow fragment from the cleavage point shows that the cleavage of the phosphodiester bond with endonuclease *Bsp*D6I leads to the formation of the 3′OH-end.

Thus, it is shown that endonuclease *Bsp*D6I recognizes site 5′-GAGTC-3′/5′-GACTC-3′ and cleaves one DNA strand that contains sequence 5′-GAGTC-3′ at a distance of four nucleotides from the sequence towards the 3′-end.

An enzyme with a similar specificity (*N.Bst*SE) was first discovered by Abdurashitov et al. [1]; they named it a site-specific nickase and designated it with the letter N. Accordingly, our enzyme should have the name *N.Bsp*D6I. Inasmuch as upon its disclosure and isolation nickase *Bsp*D6I behaved itself as a restriction endonucle-

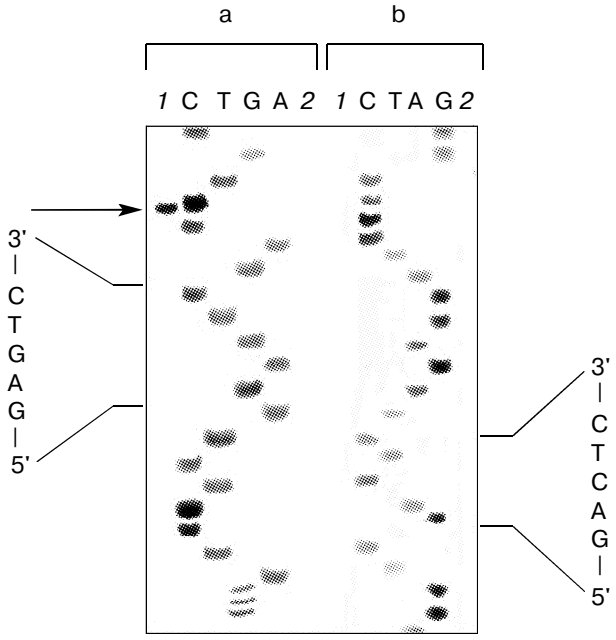

Fig. 3. Determination of DNA cleavage points with endonuclease *Bsp*D6I on the templates: a) M13mp19; b) M13mp18. C, T, G, A) "sequencing" ladders; *1*) labeled two-stranded DNA cleaved with *Bsp*D6I; *2*) the same DNA after treatment with Klenow fragment. The 5′-GAGTC-3′ sequence and the sequence complementary to it are marked, and the arrow indicates the point of DNA cleavage with the endonuclease.

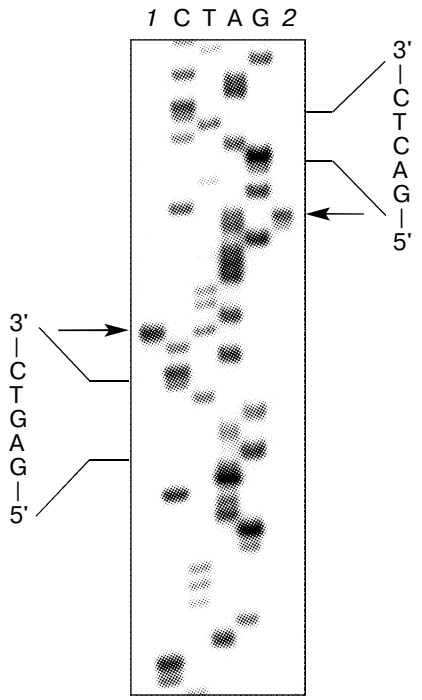

Fig. 4. Determination of DNA cleavage points with endonuclease *Bsp*D6I on template M13mp19ΔAva. Designations are the same as in Fig. 2.

ase, it is expedient to preserve the enzyme serial number (I) in its name as recommended for restriction endonucleases [9].

Properties of nickase *Bsp*D6I. To study properties of nickase *Bsp*D6I, duplexes of two complementary oligonucleotides were used, one of them (A) containing the sequence GAGTC:

A: 5′-CGTGGTCTC**GAGTC**TTCTCCTGG-3′,

B: 3′-GCACCAGAG**CTCAG**AAGAGGACC-5′.

The oligonucleotides were labeled with [γ-^{32}P]ATP and DNA-kinase; the labeled oligonucleotides were annealed with their complementary non-labeled oligonucleotides, and the duplexes obtained as well as labeled single-stranded oligonucleotides were treated with nickase *Bsp*D6I. As a control of cleavage, restriction endonuclease *Ssc*L1I (isoschizomer *Hinf*I) [10] was used that recognizes the site GANTC.

The data on cleavage of the labeled oligonucleotides (Fig. 5) corroborated that *N.Bsp*D6I as well as *N.Bst*SE cleaves only one strand of the duplex that contains sequence GAGTC.

The fact that restriction endonuclease *Ssc*L1I recognizes site GAGTC and consequently the site can be

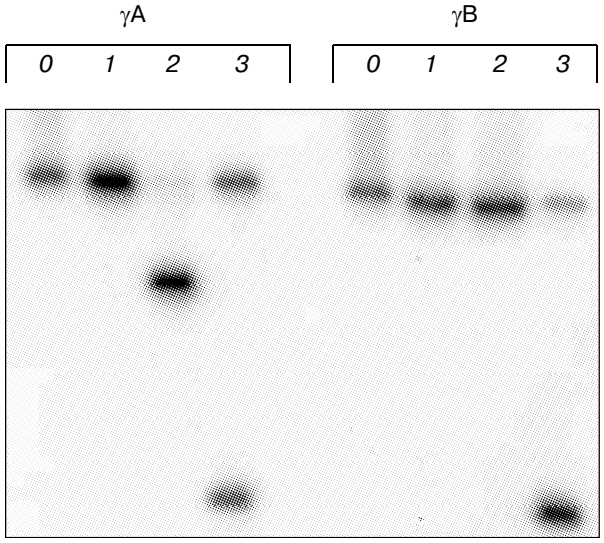

Fig. 5. Cleavage of oligonucleotides A and B and their duplexes with nickase *Bsp*D6I and endonuclease *Ssc*L1I: γA, γB) labeled oligonucleotides; *0*) initial labeled oligonucleotide incubated without enzymes; *1*) labeled oligonucleotide incubated with nickase; *2*) duplex of the labeled oligonucleotide with a complementary non-labeled one incubated with nickase *Bsp*D6I; *3*) the same duplex incubated with endonuclease *Ssc*L1I. Electrophoresis was conducted under denaturing conditions.

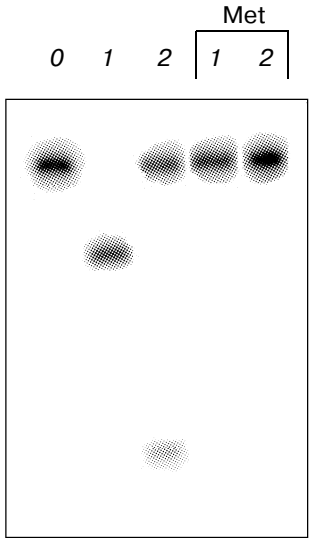

Fig. 6. Effect of methylation on cleavage by nickase *Bsp*D6I and endonuclease *Ssc*L1I of the duplex formed by labeled oligonucleotide A and non-labeled oligonucleotide B: *0*) initial duplex; *1*) duplex incubated with the nickase; *2*) duplex incubated with the endonuclease; Met) duplexes treated with methylase *Ssc*L1I. Electrophoresis was done under denaturing conditions.

methylated with methylase *Ssc*L1I at adenine residues was used to study the effect of methylation of the site on DNA cleavage by nickase *Bsp*D6I. The results (Fig. 6) showed that *N.Bsp*D6I does not cleave the methylated duplex, i.e., it is sensitive to methylation.

Determination of optimal conditions for functioning of *N.Bsp*D6I. By varying the pH of the Tris-HCl buffer, KCl concentration, and temperature, it was found that the optimal conditions for the enzyme action are 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$ at 48°C. The activity did not significantly depend on the type of the monovalent cation (Na^+ or K^+) in the reaction buffer.

DISCUSSION

It should be clarified why the three nickases (*N.Bst*SE, *N.Bst*NBI, and *N.Bsp*D6I) discovered so far recognize the same sequence. This may be connected with the fact that phage T7 DNA is widely used as a substrate DNA in the screening of strains for the presence of site-specific endonucleases. DNA of this phage contains closely positioned inverse sequences GAGTC (4 times), which in turn is explained by the fact that the sequence GAGTC is included in the sequences of phage-specific promoters [11]. Therefore, we speculate that a great number of nickases with other specificities exist which, however, cannot be detected with the substrate DNAs used.

The data obtained do not permit us to unambiguously ascribe nickase *Bsp*D6I to the modification–restriction enzymes. However the circumstance that methylation of adenine nucleotides of a DNA site with methylase that recognizes the sequence GAGTC prevents DNA cleavage with nickase *Bsp*D6I supports the likelihood that it is a modification–restriction enzyme.

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