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# Cloning and Sequencing of the Gene of Site-Specific Nickase N.*Bsp*D6I

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**Abstract**—A fragment of chromosomal DNA from *Bacillus species* D6 containing the gene of nickase N.*Bsp*D6I and the regions adjacent to its 5′- and 3′-ends was cloned and sequenced. The nucleotide sequence of the nickase gene, except of one neutral change, is homologous to the nicking endonuclease N.*Bst*NBI gene sequenced by Higgens et al. (2001). After integration of a PCR-copy of the nickase gene into an expression vector pET28b under the control of the phage T7 promoter, specific nicking activity was detected in the lysates of transformed *E. coli* cells.

*Key words*: site-specific endonuclease, site-specific nickase, cloning, sequencing

Site-specific nickases are a recently discovered class of enzymes that like type II restriction endonucleases recognize a short specific sequence (site) on the DNA and cleave it at a fixed distance from the site. However, in contrast to restriction nucleases, the nickases make a nick only in a definite DNA chain. At present four nickases isolated from bacterial strains are known [1-4]. Although all the nickases were isolated from different strains, they are isoschizomers, i.e., they recognize the same site (5′-GAGTC-3′/5′-GACTC-3′) on the double-stranded DNA and cleave it in the same way—just the chain containing the sequence 5′-GAGTC-3′ at a distance of four nucleotides from the site towards the 3′-end. The fact that all these nickases are isoschizomers might be explained by the techniques used for their discovery, which are usually employed in the screening of strains for the presence of restriction endonucleases in them. The presence of restriction endonucleases in a strain is detected by the cleavage of substrate DNAs in discrete fragments with a cell lysate of the strain, whereas site-specific nickases fragment DNA only if two sites of different orientations are at a distance of no less than 20 bp from each other [3].

Higgens and coworkers have cloned and sequenced the gene of nickase N.*Bst*NBI [5]. They have shown also that N.*Bst*NBI is very homologous to two restriction endonucleases of type IIS, *Ple*I and *Mly*I, which recognize the same site as the nickases and, like the nickases, cleave the DNA chain with the GAGTC sequence at a distance of four nucleotides from the site and the other chain at a distance of five (*Ple*I) and four (*Mly*I) nucleotides. The authors have compared the properties of N.*Bst*NBI, *Ple*I and *Mly*I and found that during the interaction with DNA, *Ple*I and *Mly*I dimerize and N.*Bst*NBI does not dimerize.

The aim of our study was cloning and sequencing of the gene of the site-specific nickase N.*Bst*NBI that we found and described earlier [3, 6].

## MATERIALS AND METHODS

*E. coli* Top10F′, TH5, and SURE strains were used in the studies. The enzymes phage T4 polynucleotide kinase, phage T4 DNA-ligase, *Taq* DNA polymerase, the Stoffel fragment of *Taq* DNA polymerase, *Pfu* DNA polymerase, *Sma*I, *Bsp*KT8I (isoschizomer *Hin*dIII) [7] and *Bst*MKI (isoschizomer *Sal*I) [8] endonucleases, as well as pUC19, pKF4, and pET28b vector DNAs were obtained in our laboratory. We also used restriction endonucleases *Bgl*II (Fermentas, Lithuania) and *Nco*I (New England Biolabs, USA), alkaline phosphatase from shrimps (Amersham Pharmacia Biotech, Germany), and [$\gamma$-$^{32}$P]ATP (Cluster, Russia). Oligonucleotides were synthesized by Syntol (Russia):

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3E8: 5′-CACC(2A/1T)(2C/1A)GAA(1G/2C)(2G/1T)(2C/1A)(2C/1T)(2T/1A)GAAAAAT

3E9: 5′-TT(2T/1C)(2A/1T)GG(1A/1C/1G)GC(2A/1T)AT(1A/2G)AATA(2A/1T)ACAATA

SNL: 5′-TCACCTAGAAGTCCAGAAAAAAT

SNR: 5′-TTCTGGCGCAATGAAACAATA

nic1: 5′-GCGCCATGGCTAAAAAAGTTAATTG

nic2: 5′-GCGGTCGACAAACCTTACCTCCTTG

B9: 5′-GTTTTCCCAGTCACGAC

B10: 5′- AACAGCTATGACCATG

T7 primer: 5′-TAATACGACTCACTATAGGG

T7 inverse: 5′-TGCTAGTTATTGCTCAGC

**Isolation of genomic DNA.** *Bacillus* species D6 cells were grown in 500 ml of the 2YT medium (1.6% bactotryptone, 1% yeast extract, 0.5% NaCl, pH 7.0) with intensive aeration at 65°C to reach the stationary growth phase ($A_{600}$ = 1.5). The cells precipitated by centrifugation were resuspended in 10 ml of STE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) and incubated for 10 min at room temperature. Cells were broken by incubation with 1% Triton X-100 at 55°C for 1 h. An equal volume of a mixture of chloroform and isoamyl alcohol (24 : 1 v/v) was added to the lysate and the mixture was placed in a stirrer for 2 h at room temperature, after which the aqueous phase was separated from chloroform by centrifugation. An equal volume of buffer QI (50 mM HEPES, pH 7.0, 0.2 M NaCl, 15% ethanol) was added to the aqueous phase and the mixture was applied to a Qiagen Tip500 column preliminarily equilibrated with the buffer. The bound nucleic acids were eluted with 150 ml of a linear gradient of the two buffers QI and QII (50 mM Tris-HCl, pH 8.5, 2 M NaCl, 15% ethanol), which ensured a linear gradient of the NaCl concentration (0.2-2 M) and pH (7.0-8.5). The fractions containing DNA were pooled, 2 volumes of ethanol were added, and the material was kept at −20°C for 1 h. The aggregated chromosomal DNA was taken out with a curved glass stick, washed in 70% ethanol, and lyophilized, and then the DNA was dissolved in buffer TE (10 mM Tris-HCl, pH 8.3, 0.1 mM EDTA).

**Phosphorylation of oligonucleotides** was carried out with the use of phage T4 polynucleotide kinase and unlabeled or labeled [$\gamma$-$^{32}$P]ATP as described in [9]. The non-incorporated [$\gamma$-$^{32}$P]ATP was eliminated with reverse-phase chromatography on an RC-18 column.

**DNA dephosphorylation** was performed using alkaline phosphatase from shrimps for 1 h at 37°C in buffer containing 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$. The enzyme was added as 0.1-0.5 activity unit per 1 pmol of the DNA 5′-ends. The phosphatase was inactivated by heating at 65°C for 15 min.

**Ligation** was done in a 20 μl volume containing the LIG buffer (25 mM Tris-acetate, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP). The molar ratio of the vector 5′-ends to the insert was 1 : 1. Phage T4 DNA-ligase was added as 5 activity units per 1 pmol of the DNA 5′-ends. The incubation was performed at 16°C for 12 h. After termination of the reaction, the DNA-ligase was inactivated by heating at 70°C for 20 min.

**Self-ligation of fragments** was done in a 2 ml volume of buffer LIG containing 0.04 μg of chromosomal DNA from *Bacillus* D6 cleaved with *Hin*dIII. After heating the ligase mixture at 70°C for 20 min, 5 μl of the mixture were added to the inverse PCR.

**Cells competent for electrotransformation** were prepared by the method suggested by Dower et al. [10]. *E. coli* Top10F′ cells were used for expression of the nickase gene cloned into plasmid pET28b under control of phage T7 promoter. Phage T7 RNA-polymerase was introduced by infecting the cells with phage λCE6 containing the polymerase gene under the control of the $\lambda_{pL}$-promoter.

**Preparative isolation of plasmid DNA.** Plasmid DNA was isolated from 800 ml of the bacterial mass. Alkaline lysis was used in the first stage of purification [9]. For a more thorough purification, half of the volume of 40% PEG was added to the solution that contained plasmid DNA freed of RNA by treatment with RNase. Then the mixture was incubated at 4°C for 30 min and centrifuged for 30 min at 9000*g*. The precipitate was washed with 70% ethanol, centrifuged in the same conditions, and resuspended in 20 ml of 3 M NaCl in 33 mM Pipes, pH 6.8. The resulting solution was placed on a Silica gel column (made from a plastic 15 ml centrifugation tube) connected to a water-jet pump, washed first with 5 ml of 3 M KCl in 33 mM Pipes, pH 6.8, and then with 10 ml of 70% ethanol in 33 mM Pipes, pH 6.8. The column was dried at room temperature for 30 min and then 5 ml of the TE buffer was added to it. The Silica gel was stirred-up and the column was centrifuged for 10 min at 1000*g*, the eluate being collected through a perforation in the bottom of the column.

**PCR using degenerate primers.** Primers 3E8 and 3E9 were used in the reaction. The reaction was performed in two rounds. In the first round, 1 ng of chromosomal DNA from *Bacillus* sp. D6 per reaction was used as a template. The reaction proceeded in 20 μl containing buffer MBI (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$, 0.8% Nonidet P40), 40 pmol of the primer (each), 200 μM deoxynucleoside triphosphate (each), and 2.5 units of *Taq* DNA polymerase. Amplification was done in a Mastercycler (Eppendorf, Germany) with a temperature gradient on annealing. The highest yield of the annealed

PCR product was in the sample with the annealing temperature of 48°C. Sample (1 μl) was dissolved in 100 μl of $H_2O$ and 1 μl of the resulting mixture was used as a template for the second PCR round under the same conditions.

**Inverse PCR.** For this reaction, the template was the fragments of chromosomal DNA from *Bacillus* sp. D6 that were obtained by hydrolysis with *Hin*dIII endonuclease and closed in a ring by preliminary ligation. Primers SNL and SNR (15 pmol each) and a mixture of DNA polymerases *Taq* and *Pfu* (with activity ratio 40 : 1) were used in the reaction. The other components were the same as in the PCR with degenerate primers.

**PCR of the complete gene *n.bspD6I*.** The reaction was done with primers nic1 and nic2 (15 pmol each) and with a mixture of DNA polymerases *Taq/Pfu*. Chromosomal DNA from *Bacillus* sp. D6 (10 ng) was used as a template; the other components were the same as in the PCR with degenerate primers.

**PCR products were purified** using a Qiagen PCR purification kit (Qiagen, Germany).

**DNA sequencing** was performed by the method of Sanger [11] in two ways: 1) using [γ-$^{32}$P]ATP labeled oligonucleotides and the Stoffel fragment of DNA polymerase *Taq*; 2) in an ABI Prizm 310 Genetic Analyzer using fluorescent dideoxyribonucleoside triphosphates.

## RESULTS AND DISCUSSION

To clone the nickase gene, we used the fact that, first, the gene of nickase N.*Bst*NBI has been sequenced [5] and, second, the amino acid sequence of N.*Bst*NBI showed remarkable homology with two restriction endonuclases of type IIS—*Ple*I and *Mly*I. As mentioned above, these endonucleases recognize the same site as the nickases do and, like the nickases, cleave the DNA chain containing the GAGTC sequence at a distance of 4 nucleotides from the site. The fact that N.*Bst*D6I and N.*Bst*NBI are isoschizomers suggested that N.*Bst*D6I can have homology with N.*Bst*NBI, *Ple*I, and *Mly*I. A comparison of the amino acid sequences of the three enzymes allowed selecting the most conservative regions at the N- and C-terminal sequences, which were used to synthesize degenerate primers 3E8 and 3E9.

**Cloning of fragments of the nickase gene.** A fragment of about 1500 bp (Fig. 1a) was prepared with PCR based on the degenerate primers 3E8 and 3E9 and using chromosomal DNA from *Bacillus* sp. D6 as a template. The fragment was cloned into the *Sma*I cleaved plasmid pUC19 and the fragment ends were sequenced. This allowed us to synthesize non-degenerate primers SNL and SNR (*1* and *2*, respectively, Fig. 1b) to the fragment ends. The primers were used for cloning the regions adjacent to the fragment of about 1500 bp in the chromosomal DNA. For this purpose, the strategy schematically shown in Fig. 1b was used. Chromosomal DNA was cleaved with *Hind*III and the resulting fragments were self-ligated. The ligase mixture and primers SNL and SNR were used to carry out an inverse PCR from which a fragment of about 3500 bp long was obtained (Fig. 2).

The fragment of 3500 bp was cloned into the *Sma*I cleaved plasmid pKF4 and its ends were sequenced. A part of the determined sequence of one of the fragment ends coincided with the 5′-end of the gene of nickase N.*Bst*NBI, whereas a part of the sequence of the other fragment end coincided with the 3′-end of the nickase N.*Bst*NBI gene. Therefore, it was concluded that the 3500-bp fragment contains the beginning and the end of the gene of nickase N.*Bst*D6I as well as the neighboring regions of chromosomal DNA from *Bacillus* sp. D6.

**Cloning of the complete gene of nickase.** To clone the complete gene, on the chromosomal DNA template a PCR product of about 1800 bp long was obtained with the use of primers to the beginning and the end of the gene

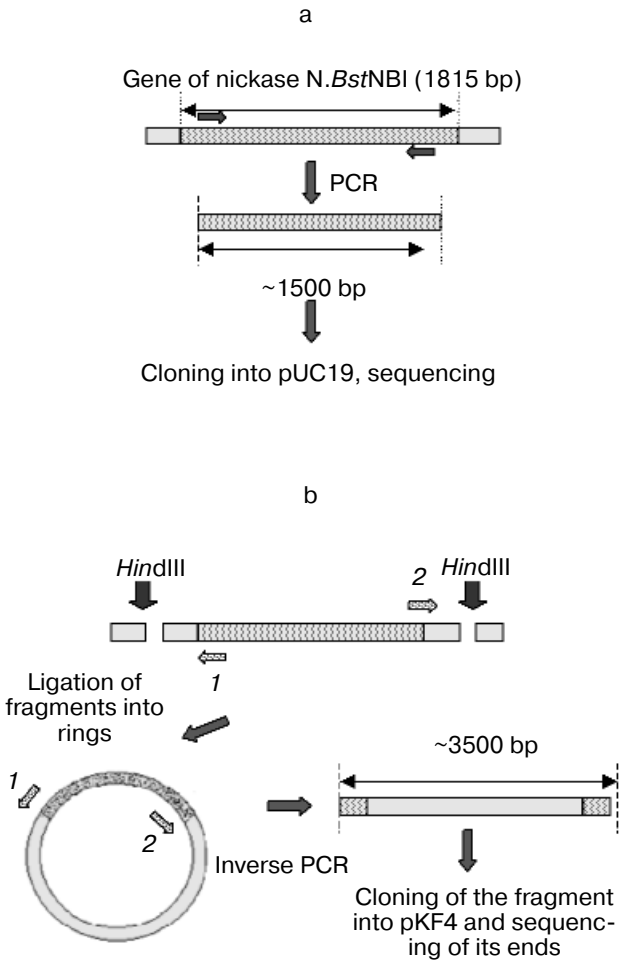


**Fig. 1.** Scheme of cloning of fragments of chromosomal DNA from *Bacillus* species D6 (see details in text).

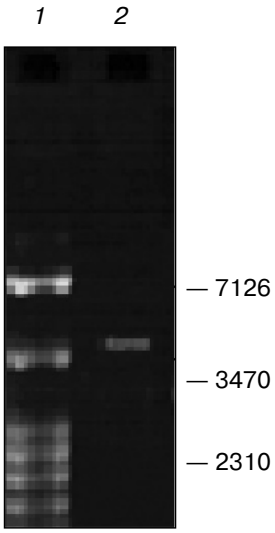


**Fig. 2.** Amplification of flanking regions of the nickase *Bsp*D6I gene: *1*) molecular weight marker (T7:*Bli*736I); *2*) products of inverse PCR. Numerals on the right show the lengths of some T7:*Bli*736I fragments (bp).

(nic 1 and nic 2). The PCR product was cleaved with *Sal*I and *Nco*I and inserted into the *Sal*I and *Nco*I cleaved and dephosphorylated expression vector pET28b. Sites of these endonucleases were introduced into the primers. Since any foreign nuclease is toxic for cells, the cells were preliminarily transformed with the earlier prepared recombinant plasmid p15Ssc containing the gene of methylase *Ssc*L1I [12]. The adenine-specific M. *Ssc*L1I recognizes the site GANTC, and previously we showed that nickase N.*Bst*D6I does not cleave DNA if the latter is methylated with M.*Ssc*L1I [6]. After induction, the lysate of the cells transformed with plasmid pET28b, containing the nickase gene, cleaved phage T7 DNA, the formed fragments being specific for the nickase. Thus, the analysis of the cell lysates supported the presence of specific nicking activity in the transformed cells. However, no intensive induced protein band at 60 kD was revealed during electrophoresis of the lysate proteins. This may be explained by different frequencies of codon usage in *E. coli* and bacillus cells.

**Sequencing the nickase gene and the neighboring regions.** A fragment of chromosomal DNA from *Bacillus* sp. D6 of 2500 nucleotides long was sequenced (Accession No. AJ534336). The fragment contains one open reading frame of 1812 nucleotides long that encodes the gene of nickase N.*Bst*D6I. The sequence of the frame, except for one substitution, was completely compatible with that of the nickase N.*Bst*NBI gene. The substitution of A915 for G in the gene of nickase N.*Bst*D6I does not result in a change of the amino acid residue.

Quite surprising is the fact of almost entire homology of the nucleotide sequences of genes of the nickases from the strains, isolated from distant ecological niches, because the genes of the restriction–modification systems are not an obligatory genetic element of cells and accordingly can quickly evolve.

It should be noted that the sequences adjacent to the 3′-ends of nickases N.*Bst*D6I and N.*Bst*NBI were compatible as well. The sequence adjacent to the 5′-end of the gene of N.*Bst*NBI is not available, and therefore it is impossible to say whether these regions in the two nickases are homologous. The search for a promoter in the non-coding sequence adjacent to the 5′-end of the N.*Bst*D6I gene by a comparison with the bank of *Bacillus* promoters (using the DBTBS program accessible on the server MNS search http://www.elmo.ims.u-tokio.ac.ip/dbtbs) did not reveal any homology with either of the promoters available in the bank. However, it was found that a region of 79 nucleotides (–233 to –309) has high homology (87%) with the beginning of the gene of arginase from *Bacillus coadovelox* (the GENBANK data base where the search was done with the BLAST program is http://www.ncbi.nih.gov/BLAST/) [13].

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