

N.BstSE, Site-Specific Nickase from *Bacillus stearothermophilus* SE-589

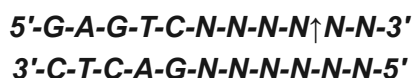
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Hits: 6539

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Translated from "Molecular Biology"(Russia) 1996, Vol. 30, No. 6, pp. 1261-1267

A site-specific nickase recognizing and cleaving the DNA site



*was isolated from *Bacillus stearothermophilus* SE-589 and named N.BstSE. Its properties indicate a possible relation with type II restriction endonucleases.*

To date, several types of enzymes are known to recognize certain DNA sequences and to cleave the phosphodiester bonds at strictly definite position of recognition site. These include first of all the type II restriction endonucleases from prokaryotes and the intron-encoded endonucleases of eukaryotes and archaeobacteria [1]. Much lower specificity is exhibited by topoisomerases, integrases and some other enzymes involved in recombination [2].

We have discovered a new enzyme, site-specific nickase named N.BstSE, which is close in properties to type II restriction endonucleases but cleaves only one strand in dsDNA. The nickase is strictly specific and can be used in gene engineering.

MATERIALS AND METHODS

Bacillus stearothermophilus SE-589 from the SibEnzyme collection of thermophilic microorganisms was grown in 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl (pH 7.6) at 50°C with vigorous aeration; in the late exponential growth phase the cells were harvested by centrifugation and suspended in buffer A (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, 200 mM KCl). The cells were disrupted in an MSE Soniprep 150 ultrasonic disintegrator, 5 x 45 sec with 1-min intervals in an ice bath. Before the last sonification DIFP (diisopropyl fluorophosphates) and Triton X-100 were added at concentration 0.001% and 0.1%, respectively. The debris was removed by centrifugation at 18,000 rpm.

The enzyme was obtained in three consecutive steps of chromatographic purification on

phosphocellulose P11, DEAE cellulose DE52 (Whatman), and heparin-sepharose (Sigma). Elution was performed with a linear 0.2-1.0 M KCl gradient in buffer A. For activity assays, 1 ml of the eluate was added to 50 ml of 20 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 mM NaCl, 1 mM dithiothreitol and 20 mg/ml phage T7 DNA; after 30 min at 50°C, 5 ml of solution containing 100 mM EDTA, 40% sucrose, 0.05% Bromphenol Blue was added. The reaction products were resolved electrophoretically in 1% agarose gel in TAE (50 mM Tris-acetate pH 8.0, 20 mM NaAc, 2 mM EDTA). After staining with ethidium bromide, the gels were photographed under UV light. One unit of activity was the amount of enzyme needed for complete fragmentation of 1 mg T7 DNA in 50 ml in 1 hour.

The products of enzymatic cleavage of radiolabeled oligonucleotides were resolved by electrophoresis in 20% PAG with 7 M urea.

DNA preparations, oligonucleotides, restriction endonucleases and other enzymes were from SibEnzyme.

RESULTS

In the course of studies on bacterial restriction-modification systems, strain *B. stearothermophilus* SE-589 was found to produce an enzyme specifically hydrolysing some common DNA substrates (Fig. 1a). This endonuclease, named N.BstSE (see Discussion), was isolated in three steps of column chromatography. The yield of purified enzyme was about 1000 units per gram of biomass. In standard tests [3] the N.BstSE preparation proved to be free of nonspecific nucleases or phosphatases.

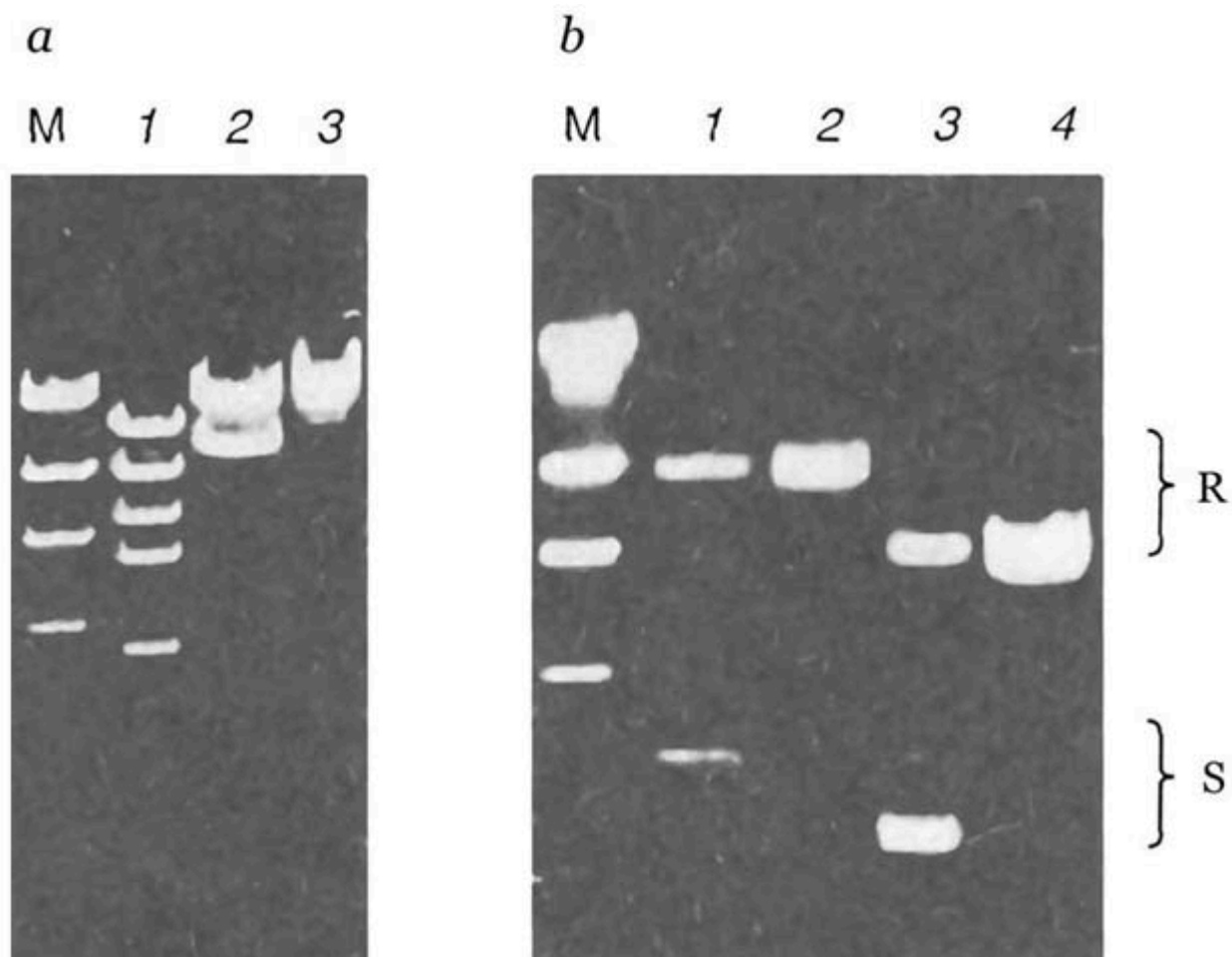


Fig. 1. Electrophoresis of the products of DNA cleavage by N.BstSE. Panel a. viral DNA: 1) phage T7, 2) Ad2, 3) phage lambda. Panel b, plasmids pBR322 (1, 2) and pUC19 (3, 4): odd lanes are nontreated controls, R for relaxed and S for supercoiled forms. M is lambda DNA/HindIII markers. Visible bands correspond to fragments of 23310, 9416, 6557, 4361, 2322 and 2027 bp.

The optimal composition of the reaction mixture for the new enzyme was determined. Figure 2 shows the effect of salts on the cleavage of phage T7 DNA by N.BstSE; as can be seen, KCl is preferred to NaCl, with an optimum of 100-200 mM. Similarly, the conditions were optimized in respect of MgCl_2 pH, and temperature. Among all possible cofactors tested (ATP, S-adenosylmethionine, Mg^{2+} , Mn^{2+}) the enzyme required only Mg^{2+} , whereas substitution Mg^{2+} by Mn^{2+} significantly decreased the enzyme's activity (not shown). Maximal activity was observed in 10 mM Tris-HCl, pH 8.5, 10 mM MgCl_2 , 150 mM KCl, 1 mM dithiothreitol, at 55°C.

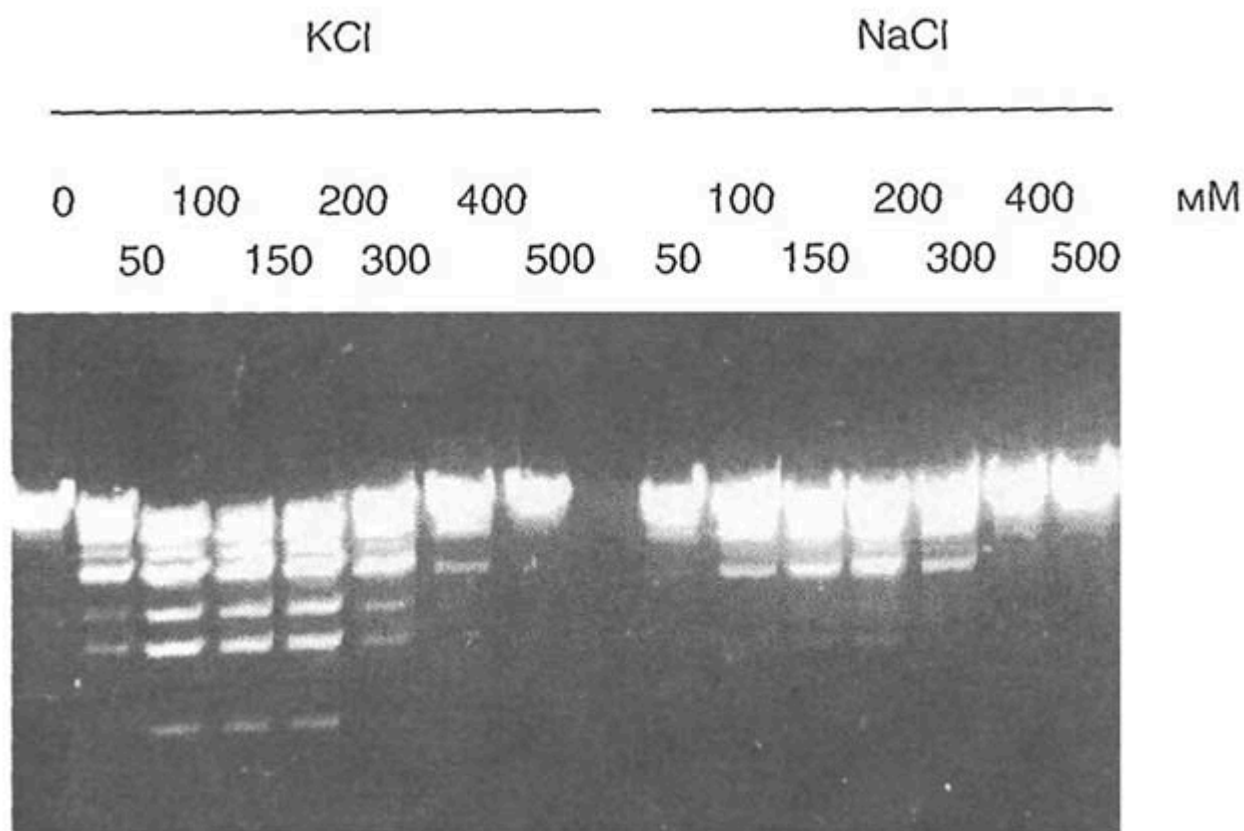


Fig. 2. N.BstSE activity at different salt concentrations in 20 mM Tris-HCL pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol, 20 mkg/ml T7 DNA.

The substrate specificity of the enzyme was determined in the standard way, mapping the cleavage sites on various substrates [4]. N.BstSE made four two-strand breaks in phage T7 DNA and two in adenovirus 2 DNA. Combined hydrolysis with N.BstSE and restriction endonucleases demonstrated that the enzyme cleaves T7 DNA approximately at positions 13,320, 20,610, 24,560, and 30,520, and Ad2 DNA at 23,910 and 35,290. Analysis of the corresponding DNA regions revealed (Table 1) that each contains stretches of nucleotides 5'-GAGTC-3' and 5'-GACTC-3', the former always preceding the latter and spaced by 9-17 bp. Considering that the second sequence is complementary to the first one, it could be supposed that N.BstSE recognizes sequence 5'-GAGTC-3' but cleaves only one DNA strand. Figure 1b shows N.BstSE action on pBR322 and pUC19 each containing four putative recognition sites; it is clearly seen that the enzyme relaxes the supercoiled plasmids and thus displays nicking activity.

DNA	Sequence
T7	13313 13330 5'-TTTCGCTGGA GAGTC CCATTCTAATAC GACTC ACTAAAGGAG-3'
	20602 20622 5'-GAACTACATT GAGTC TAACAGTTACCGCGT GACTC TCTTTGAGGC-3'
	24547 24566 5'-ATGACCGAAC GAGTC AATCAGACCGCTTT GACTC TGGTATTACT-3'
	30512 30534 5'-GCTGCTAAAC GAGTC CGAGAGAAACGTAAACA GACTC CTAAGTTCAT-3'
Ad2	23904 23922 5'-ATCTTGTGAC GAGTC TTCTTCGTCCTCG GACTC GAGACGCCGC-3'
	35286 35300 5'-GTCATGTCCG GAGTC ATAATGTAA GACTC GGTAACACA-3'

Table 1. Sequences of T7 and Ad2 DNA Undergoing Two-Strand Scission upon Exposure to N.BstSE

Note: Regions of homology are shown in boldface; numbers give positions in the total DNA sequence.

Thus, we made a conclusion that the observed fragmentation of viral DNA is due to the closeness of independently produced nicks in two strands. Computation confirmed this suggestion. The DNAs of three viruses and two plasmids were searched for regions where pairs of close nicks (within 30 nt) could be produced in two strands by an enzyme with the GAGTC recognition site, and no sequences other than those shown in Table 1 could be found.

To determine which strand is cleaved, we sequenced a cowpox virus DNA fragment containing the N.BstSE site by a modified Maxam-Gilbert technique [5] and also treated it with our enzyme. It follows from Fig. 3a that only the GAGTC-containing strand is cleaved as indicated by arrow: GAGTCNNNN[^], while the complementary strand in this region (about 80 nt) remains intact (Fig. 3b). This was strictly confirmed by the data on cleavage of labeled oligonucleotides specified in Table 2. N.BstSE was capable to cleave ssDNA as well (Fig. 4).

Oligonucleotide	Sequence
A	5-CGTGGTCTC GAGTC TTCACCTGG-3
B	5-CCAGGTGAA GACTC GAGACCACG-3

Table 2. Complementary Oligonucleotides Used as Substrates

Note: N.BstSE recognition site marked in bold.

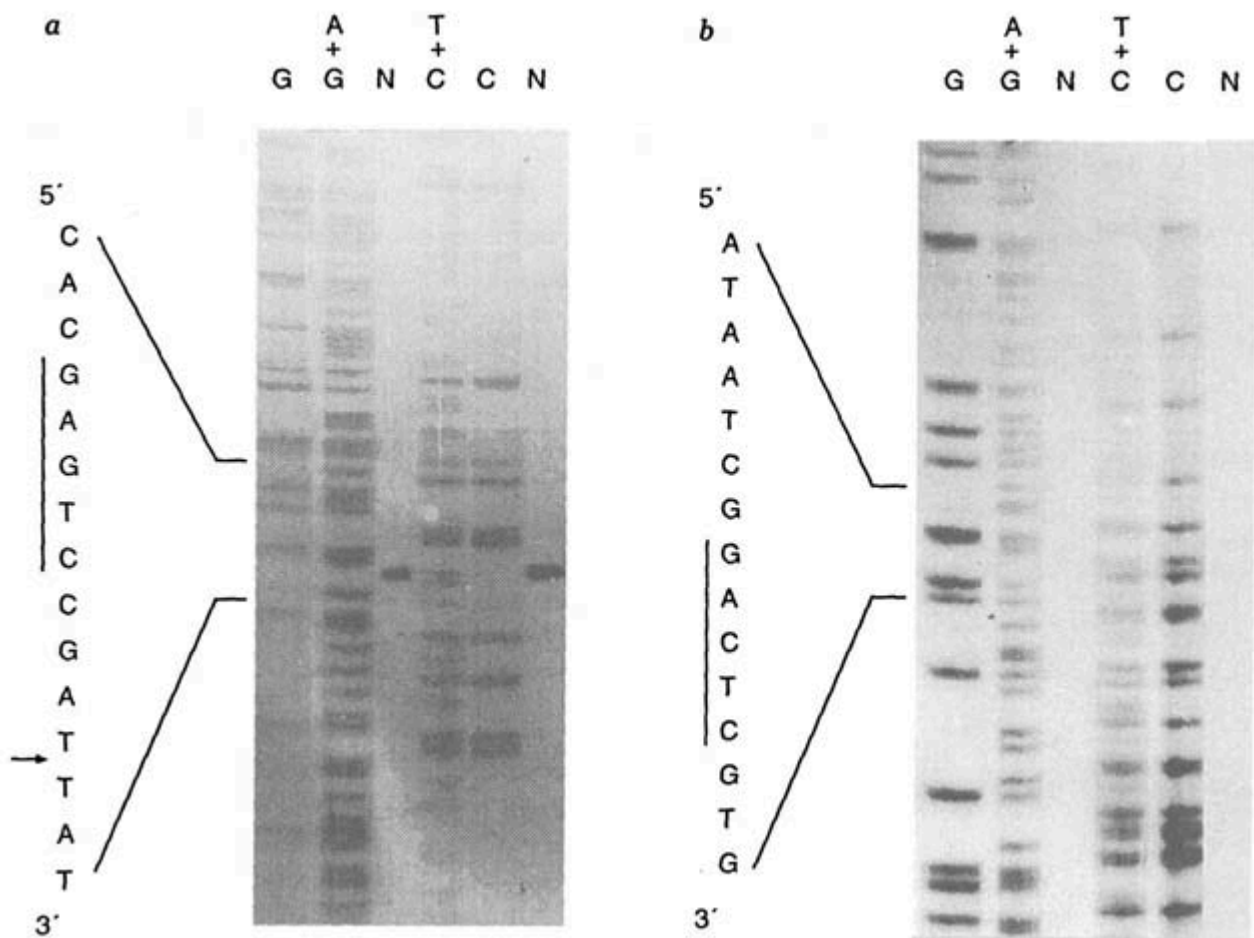


Fig. 3. Location of the bond split by N.BstSE: sequencing of (a) DNA strand containing GAGTC and (b) complementary strand: (N) N.BstSE cleavage products.

$[\gamma\text{-}^{32}\text{P}] \text{ A}$	+	+	+	+	+					
B	+	+	+							
A								+	+	+
$[\gamma\text{-}^{32}\text{P}] \text{ B}$						+	+	+	+	+
<i>Hinf</i> I		+							+	
N. <i>Bst</i> SE			+	+			+	+		

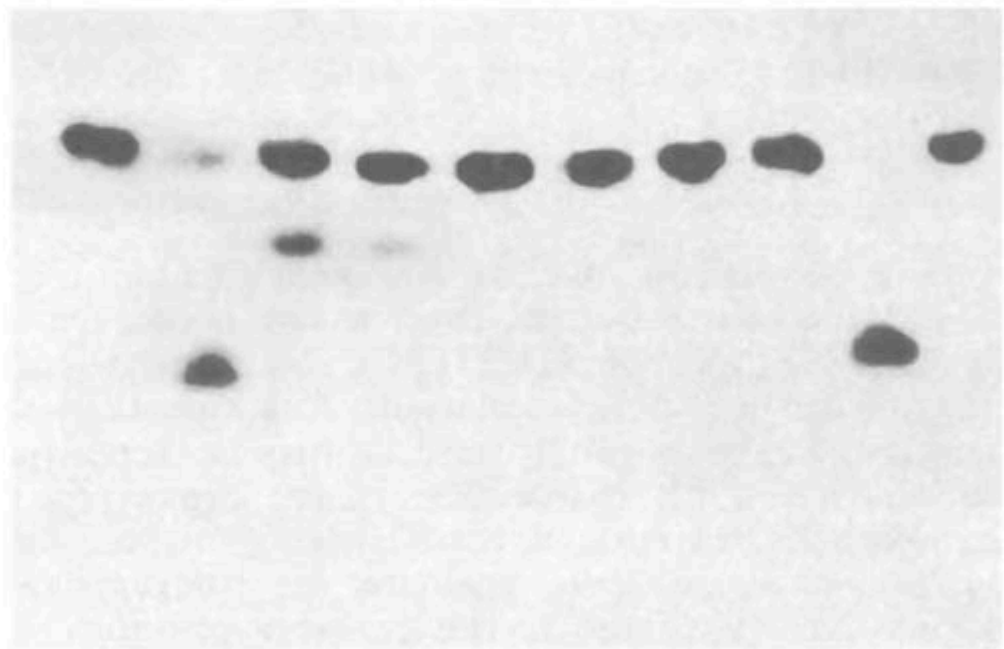


Fig. 4. Autoradiograph of the products of cleaving oligonucleotides A, B (Table 2) and their duplex with N.BstSE and *Hinf*I (G↑ANTC).

Figure 5 shows the results of standard cleavage-ligation-recleavage test for N.BstSE. One can see that the enzyme is pure enough and suitable for gene-engineering experiments. The very fact of enzymatic ligation together with the data on nickase product mobility testify that splitting of the phosphodiester bond by N.BstSE leaves the phosphate at the 5' end of the product.

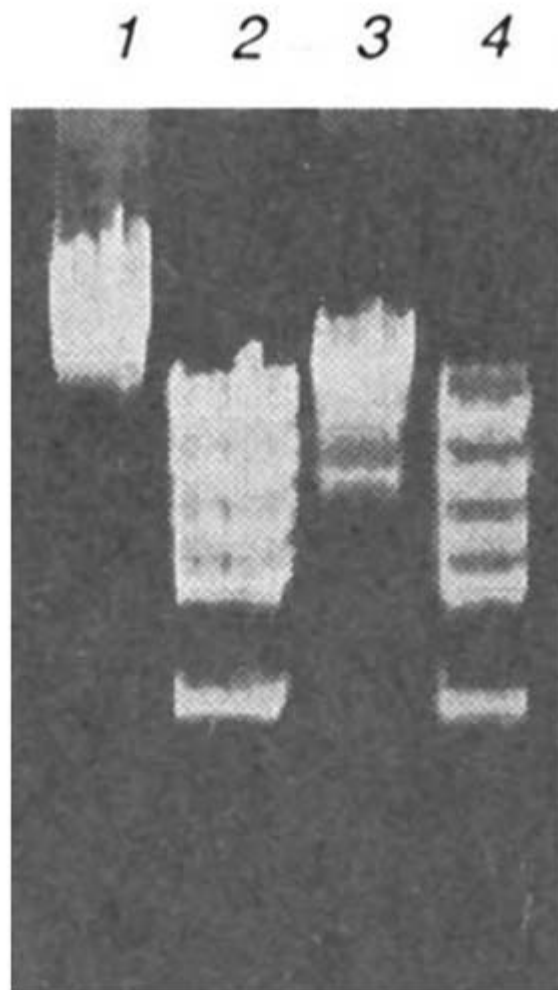


Fig. 5. Ligation test:

1. phage T7 DNA,
2. its fragmentation by N.BstSE,
3. treatment of products from lane 2 with T4 DNA ligase,
4. repeated cleavage of DNA from lane 3 with N.BstSE.

DISCUSSION

The enzyme reported here is distinct from those described in the literature. Although topoisomerase I from *Escherichia coli* and some other enzymes are capable of cleaving one strand in dsDNA, they usually also possess other enzymatic activities (e.g., DNA ligase) that are absent in N.BstSE, and do not have such clear-cut site specificity [2].

The enzymatic properties of N.BstSE are most close to type II restriction endonucleases. The typical

features of these enzymes are: (i) recognition of short (4-8 bp), mostly palindromic nucleotide sequences; (ii) cleavage of both strands in dsDNA within or close to the recognition site; (iii) requirement of Mg^{2+} for DNA cleavage. The novel site-specific endonuclease from the thermophilic bacterium *B. stearothermophilus* SE-589 possesses such properties except that it cleaves only one of the DNA strands. Furthermore, it is similar to restriction endonucleases [6] in its ability to cleave relatively short oligonucleotides. Comparison of specificities revealed that restriction endonuclease *PleI* has the same recognition and cleavage sites in the "upper" DNA strand [1] but, unlike *N.BstSE*, cleaves the other strand as well.

Based on above mentioned observation, we believe that *N.BstSE* has originated from a restriction endonuclease with recognition sequence GAGTC(4/5), by mutation that eliminated its ability to cleave the second DNA strand.

To date, two type of bacterial enzymes are known to interact in a highly specific way with short DNA regions: DNA methylases and restriction endonucleases. They are designated with letters M and R, e.g., *M.EcoRI* and *R.EcoRI*. Likewise, we propose to designate the new type of enzyme, site-specific nickases, with letter N; thus the site-specific nickase from *B. stearothermophilus* SE-589 is named *N.BstSE*.

The novel enzyme is easily available and simple in use, which allows us to hope that *N.BstSE* will find a broad application both in genetic engineering and in study of restriction endonucleases and other site-specific enzymes properties.

The authors wish to thank T. A. Madina (SibEnzyme) and N. A. Petrov (Institute of Molecular Biology, Vector) for help with experiments, and to V. S. Dedkov (SibEnzyme) for valuable criticism.

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