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(54) Title: CLONING AND PRODUCING THE N.*Bst*NBI NICKING ENDONUCLEASE AND RELATED METHODS FOR USING NICKING ENDONUCLEASES IN SINGLE-STRANDED DISPLACEMENT AMPLIFICATION

(57) Abstract: The present invention relates to recombinant DNA which encodes a novel nicking endonuclease, N.*Bst*NBI, and the production of N.*Bst*NBI restriction endonuclease from the recombinant DNA utilizing *PleI* modification methylase. Related expression vectors, as well as the application of N.*Bst*NBI and other nicking enzymes in non-modified strand displacement amplification, is disclosed also.

CLONING AND PRODUCING THE N.*Bst*NBI NICKING ENDONUCLEASE AND RELATED METHODS FOR USING NICKING ENDONUCLEASES IN SINGLE-STRANDED DISPLACEMENT AMPLIFICATION

BACKGROUND OF THE INVENTION

The present invention relates to the recombinant DNA which encodes the N.*Bst*NBI nicking endonuclease and modification methylase, and the production of N.*Bst*NBI nicking endonuclease from the recombinant DNA. N.*Bst*NBI nicking endonuclease is originally isolated from *Bacillus stearothermophilus*. It recognizes a simple asymmetric sequence, 5′ GAGTC 3′, and it cleaves only one DNA strand, 4 bases away from the 3′-end of its recognition site.

The present invention also relates to the use of nicking endonucleases in strand-displacement amplification application (SDA). More particularly, it relates to liberating such amplification from the technical limitation of employing modified (particularly α-thiophosphate substituted) nucleotides.

Restriction endonucleases are enzymes that recognize and cleave specific DNA sequences. Usually there is a corresponding DNA methyltransferase that methylates and therefore protects the endogenous host DNA from the digestion of a certain restriction endonuclease. Restriction endonucleases can be classified into three groups: type I, II, and III. More than 3000 restriction endonucleases with over two hundred different specificities have been isolated from bacteria (Roberts and Macelis, *Nucleic Acids Res.* 26:338-350 (1998)). Type II and type IIs restriction enzymes cleave DNA at a specific position, and therefore are useful in genetic engineering and molecular cloning.

Most restriction endonucleases catalyze double-stranded cleavage of DNA substrates via hydrolysis of two phosphodiester bonds on two DNA strands (Heitman, *Genetic Engineering* 15:57-107 (1993)). For example, type II enzymes, such as *Eco*RI and *Eco*RV, recognize palindromic sequences and cleave both strands symmetrically within the recognition sequence. Type IIs endonucleases recognize asymmetric DNA sequences and cleave both DNA strands outside of the recognition sequence.

There are some proteins in the literature which break only one DNA strand and therefore introduce a nick into the DNA molecule. Most of those proteins are involved in DNA replication, DNA repair, and other DNA-related metabolisms (Kornberg and Baker, DNA replication. 2nd edit. W.H. Freeman and Company, New York, (1992)). For example, gpII protein of bacteriophage fI recognizes and binds a very complicated sequence at the replication origin. It introduces a nick in the plus strand, which initiates rolling circle replication, and it is also involved in circularizing the plus strand to generate single-stranded circular phage DNA. (Geider *et al.*, *J. Biol. Chem.* 257:6488-6493 (1982); Higashitani *et al.*, *J. Mol. Biol.* 237:388-400 (1994)). Another example is the MutH protein, which is involved in DNA mismatch repair in *E. coli*. MutH binds at dam methylation sites (GATC), where it forms a protein complex with nearby MutS which binds to a mismatch. The MutL protein facilitates this interaction and this triggers single-stranded cleavage by MutH at the 5' end of the unmethylated GATC site. The nick is then translated by an exonuclease to remove the

mismatched nucleotide (Modrich, *J. Biol. Chem.* 264:6597-6600 (1989)).

The nicking enzymes mentioned above are not very useful in the laboratory for manipulating DNA due to the fact that they usually recognize long, complicated sequences and usually associate with other proteins to form protein complexes which are difficult to manufacture. Thus none of these nicking proteins are commercially available. Recently, we have found a nicking protein, N.*Bst*NBI, from the thermophilic bacterium *Bacillus stearothermophilus*, which is an isoschizomer of N.*Bst*SEI (Abdurashitov *et al.*, *Mol. Biol.* (Mosk) 30:1261-1267 (1996)). Unlike gpII and MutH, N.*Bst*NBI behaves like a restriction endonuclease. It recognizes a simple asymmetric sequence, 5' GAGTC 3', and it cleaves only one DNA strand, 4 bases away from the 3'-end of its recognition site (Fig. 1A).

Because N.*Bst*NBI acts more like a restriction endonuclease, it should be useful in DNA engineering. For example, it can be used to generate a DNA substrate containing a nick at a specific position. N.*Bst*NBI can also be used to generate DNA with gaps, long overhangs, or other structures. DNA templates containing a nick or gap are useful substrates for researchers in studying DNA replication, DNA repair and other DNA related subjects (Kornberg and Baker, DNA replication. 2nd edit. W.H. Freeman and Company, New York, (1992)). A potential application of the nicking endonuclease is its use in strand displacement amplification (SDA), which is an isothermal DNA amplification technology. SDA provides an alternative to polymerase chain reaction (PCR), and it can reach 10^6-fold amplification in 30 minutes without thermo-cycling (Walker *et al.*, *Proc. Natl. Acad. Sci.*

USA 89:392-396 (1992)). SDA uses a restriction enzyme to nick the DNA and a DNA polymerase to extend the 3'-OH end of the nick and displace the downstream DNA strand (Walker *et al.*, (1992)). The SDA assay provides a simple (no temperature cycling, only incubation at 60°C) and very rapid (as short as 15 minutes) detection method and can be used to detect viral or bacterial DNA. SDA is being introduced as a diagnostic method to detect infectious agents, such as *Mycobacterium tuberculosis* and *Chlamydia trachomatis* (Walker and Linn, *Clin. Chem.* 42:1604-1608 (1996); Spears *et al.*, *Anal. Biochem.* 247:130-137 (1997)).

For SDA to work, a nick has to be introduced into the DNA template by a restriction enzyme. Most restriction endonucleases make double-stranded cleavages. Therefore, modified α-thio deoxynucleotides (dNTPαS) have to be incorporated into the DNA, so that the endonuclease only cleaves the unmodified strand which is within the primer region (Walker *et al.*, 1992). The α-thio deoxynucleotides are eight times more expensive than regular dNTPs (Pharmacia), and are not incorporated well by the *Bst* DNA polymerase as compared to regular deoxynucleotides (J. Aliotta, L. Higgins, and H. Kong, unpublished observation).

Alternatively, in accordance with the present invention, it has been found that if a nicking endonuclease is used in SDA, it will introduce a nick into the DNA template naturally. Thus the dNTPαS is no longer needed for the SDA reaction when a nicking endonuclease is being used. This idea has been tested, and the result agreed with our speculation. The target DNA can, for example, be amplified in the presence of the nicking endonuclease N.*Bst*NBI, dNTPs, and *Bst* DNA

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