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Engineering BspQI nicking enzymes and application of N.BspQI in DNA labeling and production of single-strand DNA

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ABSTRACT

BspQI is a thermostable Type IIS restriction endonuclease (REase) with the recognition sequence 5′GCTCTTC N1/N4 3′. Here we report the cloning and expression of the *bspQIR* gene for the BspQI restriction enzyme in *Escherichia coli*. Alanine scanning of the BspQI charged residues identified a number of DNA nicking variants. After sampling combinations of different amino acid substitutions, an Nt.BspQI triple mutant (E172A/E248A/E255K) was constructed with predominantly top-strand DNA nicking activity. Furthermore, a triple mutant of BspQI (Nb.BspQI, N235A/K331A/R428A) was engineered to create a bottom-strand nicking enzyme. In addition, we demonstrated the application of Nt.BspQI in optical mapping of single DNA molecules. Nt or Nb.BspQI-nicked dsDNA can be further digested by *E. coli* exonuclease III to create ssDNA for downstream applications. BspQI contains two potential catalytic sites: a top-strand catalytic site (Ct) with a D-H-N-K motif found in the HNH endonuclease family and a bottom-strand catalytic site (Cb) with three scattered Glu residues. BlastP analysis of proteins in GenBank indicated a putative restriction enzyme with significant amino acid sequence identity to BspQI from the sequenced bacterial genome *Croceibacter atlanticus* HTCC2559. This restriction gene was amplified by PCR and cloned into a T7 expression vector. Restriction mapping and run-off DNA sequencing of digested products from the partially purified enzyme indicated that it is an EarI isoschizomer with 6-bp recognition, which we named CatHI (CTCTTC N1/N4).

Introduction

Restriction endonucleases (REases) with 4–8 bp recognition sequences are indispensible tools in creating recombinant DNA molecules [1]. Nearly 300 unique Type II specificities have been found so far from bacterial and viral sources. DNA nicking endonucleases (NEases) which bind DNA sequence specifically and cleave only one strand, however, are only available in small numbers. The first two natural NEases, Nt.CviPII and Nt.CviQII, were found from infected cells of Chlorella viruses [2,3]. Other natural NEases are Nt.BstNBI/Nt.BspD6I/N.BstSEI, Nb.BsrDI, and Nb.BtsI, which are the large subunits of their respective REases [4–7] (Wilson, unpublished results). NEases have also been engineered from heterodimeric REases BbvCI and Bpu10I by inactivation of the top-strand or bottom-strand catalytic site [8] (Janulaitis et al. (2005) Strand-specific polynucleotide nickases, US Patent number 6,867,028). A number of NEases were derived from protein engineering of Type IIS REases. For example, nicking variants have been engineered from AlwI, BsmAI, BsmBI, BsaI, BsmI, FokI, MlyI, and SapI either by site-directed mutagenesis of a few amino acid residues or by protein domain swapping [9–13] (Nb.BsmI, Zhu and S.Y.X., unpublished results). In addition, nicking variants have been constructed from homing endonucleases with large recognition sequences useful for gene targeting [14,15].

NEases are useful to prepare pre-nicked DNA substrates for DNA repair study [16]. In addition, NEases have been employed in DNA amplification in conjunction with a DNA polymerase with strand-displacement activity. For example, the isothermal exponential amplification reaction (EXPAR) exponentially amplifies short oligonucleotides called “triggers” by primer extension and regeneration through DNA polymerase and NEase activities [17,18]. Nt.CviPII can be used in random DNA amplification (NEMDA, nicking endonuclease mediated DNA amplification) without addition of any

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primers since DNA can be directly amplified from partially nicked duplexes [19]. Target-specific DNA amplification can also be achieved by a set of specific primers, NEases, single-strand DNA binding protein such as gp32, and a DNA polymerase with strand-displacement activity (Kucera and Raleigh, unpublished results). Another example of NEases in DNA-based diagnostic application is termed nicking endonuclease signal amplification (NESA) [20]. A fluorescent probe and target DNA anneal to create a recognition site for a strand-specific NEase. The NEase cleaves the probe into two pieces while leaving the target intact. Fluorescence coupled with capillary electrophoresis can be used to measure the nicked products.

NEases can also be used to label DNA using the nicked sites for nick translation with fluorescent-labeled dNTPs and a suitable DNA polymerase. The labeled DNA molecules can be physically stretched out by passing through nanochannels or nanoslit devices and the fluorescent "dots" on single DNA molecules are visualized by fluorescent imaging and optical mapping [21,22] (BioNanomatrix platform technology: www.bionanomatrix.com). The distance of the fluorescent "dots" can be measured to reveal particular kind of genetic rearrangement (deletion or insertion). For DNA nicking and single DNA molecule labeling and detection, it would be useful to have a collection of strand-specific NEases with 5–7 bp specificities. Here we report the successful engineering of strand-specific and sequence-specific BspQI nicking enzymes and the application of Nt.BspQI in DNA nicking and single molecule labeling.

During the cloning and expression of the BspQI R–M system,[1] we also found a putative R–M system from the sequenced bacterial genome *Croceibacter atlanticus* HTCC2559 that shows significant amino acid sequence identity to BspQI and SapI. We report here the characterization of this R–M system.

Materials and methods

Bacterial strains, plasmid DNA, restriction and modification enzymes

T7 Express (ER2566) was used as the expression strain (New England Biolabs, NEB). BspQI nicking enzymes were expressed in M1.EarI and M2.EarI-modified host ER2566 [pLG339-*earIM1M2*] (EarI recognition sequence 5′CTCTTC3′ overlaps with the BspQI site, the plasmid carries Km^R selection marker and pSC101 origin). Site-directed mutagenesis was carried out by inverse PCR using either Vent DNA polymerase or Phusion DNA polymerase (NEB). The T7 expression vector pET21a was purchased from Novagen. Restriction and modification enzymes, pUC19 and pBR322 were from NEB. Plasmid pACYC-T7-ter is a low-copy number expression vector with a T7 promoter and a transcription terminator. IPTG-induced cell extracts were prepared as described [23]. One unit of BspQI REase is defined as the amount of enzyme to completely digest 1 μg of pUC19 DNA in 1 h at 50 °C in NEB buffer 3. One unit of Nt.BspQI is defined as the amount of enzyme to completely nick pUC19 (covalently closed circular form) into nicked circular DNA in 1 h at 50 °C in NEB buffer 3.

Construction of genomic DNA libraries and shot-gun sequencing

Bacillus sphaericus genomic DNA was partially digested with BfuCI (5′/GATC3′). Genomic DNA fragments in the range of 1.5–10 kb were gel-purified and ligated into pBR322 (BamHI/CIP), pUC19 (BamHI/CIP) or pACYC-T7-ter (BamHI/CIP). Ligated DNA was transferred into *Escherichia coli* ER2683 or ER1992 competent cells (endo-blue indicator strain) by transformation. Plasmid pUC19 or pBR322 with genomic DNA insert library was plated on LB agar plates plus Amp (100 μg/ml) and X-gal (80 μg/ml). Genomic DNA fragments inserted in plasmid pACYC-T7-ter was selected on LB agar plates plus Cm (30 μg/ml) and X-gal (80 μg/ml). Blue colonies were screened for possible clones with *bspQIR* gene insert. The inserts in the dark blue colonies were amplified in PCR by forward and reverse universal primers from pUC19 derivatives or T7 forward and reverser primers from pACYC-T7-ter derivatives. The PCR DNA was sequenced by using the BigDye terminator cycle sequencing kit (Applied Biosystems). In the methylase selection procedure, primary genomic DNA library (pBR322 plus genomic inserts) was challenged by 10-fold over-digestion with concentrated SapI and the digested plasmid DNA was used to transform *E. coli* ER2683 competent cells. Transformants were plated on LB agar plates supplemented with Amp. Plasmid DNA was extracted from overnight culture of individual transformants and analyzed for resistance to SapI digestion.

Bacillus sphaericus genomic DNA was shot-gun sequenced using the 454 method (454 Inc.) [24]. Mutant *bspQIR* alleles were confirmed by DNA sequencing (Applied Biosystems).

Protein purification of BspQI and BspQI nicking enzymes

T7 Express strains (ER2566) containing the *bspQIR* or *Nt.bspQIR* gene were protected in vivo by the EarI M1 & M2 methyltransferases. Cells were grown to OD_{600} 0.5 and protein production was induced by addition of IPTG to a final concentration of 0.5 mM for 3–4 h. Harvested cell pellets were resuspended in a buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol) and lysed by sonication. The clarified lysate was loaded onto a Heparin Hyper D column (Pall Corp.). Protein was eluted by applying a NaCl gradient (0.05–1.0 M). Active fractions were collected and diluted to a final salt concentration of 100 mM. The diluted pool was then passed through a Source TM15Q column (GE Healthcare). The flow-through was collected and loaded onto a Heparin-TSK column (Tosoh Bioscience). The fractions with enzyme activity were collected and dialyzed into a storage buffer (10 mM Tris–HCl, pH 7.4, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 50% glycerol). Nb.BspQI was expressed from a pUC19 derivative (pUC19-*Nb.bspQIR*) in a pre-modified host and partially purified by chromatography through Heparin Sepharose and SP Sepharose HiTrap columns (GE Healthcare).

Site-directed mutagenesis and molecular biology techniques

Mutagenic primers with desired mutations were used in inverse PCR mutagenesis. Typically, 20–25 cycles of inverse PCR were performed using Vent DNA polymerase or Phusion DNA polymerase (NEB). Following DpnI digestion of the template DNA, the amplified DNA was transferred into M.EarI-modified *E. coli* host by transformation or electroporation. Plasmid extraction kits from Qiagen were used to prepare plasmid DNA.

DNA labeling and single molecule imaging

A DNA labeling procedure and two-color fluorescence imaging followed a previously described protocol [21] with modifications including provisions for mounting labeled molecules on optical mapping surfaces, in place of nanoslits [25]. As such, dialysis steps were obviated for reducing solution ionic strength. T7 DNA (Yorkshire Biosciences Ltd., UK) embedded in gel inserts was nicked with Nt.BspQI (2.5 U) in NEB buffer 3, prior to nick translation with *E. coli* DNA polymerase I (5 U, endonuclease free grade, Roche Applied Sciences) and one fluorochrome label (Alexa Fluor 647-aha-dUTP, Invitrogen) in the reaction mix. Nicked, labeled DNA

[1] *Abbreviations used:* R–M, restriction–modification system; REase, restriction endonuclease; NEase, nicking endonuclease; Nt, top-strand nicking; Nb, bottom-strand nicking.