Novel Subtype of Type IIs Restriction Enzymes

*BfiI ENDONUCLEASE EXHIBITS SIMILARITIES TO THE EDTA-RESISTANT NUCLEASE Nuc OF SALMONELLA TYPHIMURIUM*

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The type IIs restriction enzyme *BfiI* recognizes the non-palindromic nucleotide sequence 5’-ACTGGG-3’ and cleaves complementary DNA strands 5/4 nucleotides downstream of the recognition sequence. The genes coding for the *BfiI* restriction-modification (R-M) system were cloned/sequenced and biochemical characterization of *BfiI* restriction enzyme was performed. The *BfiI* R-M system contained three proteins: two N4-methylcytosine methyltransferases and a restriction enzyme. Sequencing of bisulfite-treated methylated DNA indicated that each methyltransferase modifies cytosines on opposite strands of the recognition sequence. The N-terminal part of the *BfiI* restriction enzyme amino acid sequence revealed intriguing similarities to an EDTA-resistant nuclease of *Salmonella typhimurium*. Biochemical analyses demonstrated that *BfiI*, like the nuclease of *S. typhimurium*, cleaves DNA in the absence of Mg2+ ions and hydrolyzes an artificial substrate bis(p-nitrophenyl) phosphate. However, unlike the nonspecific *S. typhimurium* nuclease, *BfiI* restriction enzyme cleaves DNA specifically. We propose that the DNA-binding specificity of *BfiI* stems from the C-terminal part of the protein. The catalytic N-terminal subdomain of *BfiI* radically differs from that of type II restriction enzymes and is presumably similar to the EDTA-resistant non-specific nuclease of *S. typhimurium*; therefore, *BfiI* did not require metal ions for catalysis. We suggest that *BfiI* represents a novel subclass of type IIs restriction enzymes that differs from the archetypal *FokI* endonuclease by the fold of its cleavage domain, the domain location, and reaction mechanism.

Type IIs restriction enzymes recognize short non-palindromic DNA sequences and, in the presence of Mg2+ ions, cleave both DNA strands a short distance outside the recognition sequence (1). Currently, our knowledge of the structure and mechanisms of catalysis used by type IIs restriction enzymes is limited to the *FokI* restriction enzyme that recognizes asymmetric nucleotide sequence 5’-GGATG and cleaves both DNA strands 9/13 nucleotides away from the recognition sequence (2). According to proteolytic cleavage and deletion analysis, *FokI* contains two functional domains, one responsible for DNA recognition (N-terminal domain) and the other for cleavage (C-terminal domain). Interestingly, the structural architecture of the *FokI* cleavage domain displays a striking similarity to the monomer of *BamHI* (6), demonstrating that both enzymes share similar catalytic machinery despite the fact that they interact with nucleic acids differently. Protein sequence comparisons suggest that the *StsI* restriction enzyme, which recognizes the same nucleotide sequence as *FokI* but cleaves DNA 10/14 nucleotides away, possesses a similar modular organization (7, 8). However, we still lack evidence to indicate if other type IIs restriction enzymes share a similar structural architecture.

The *BfiI*, isolated from *Bacillus firmus* S8120 strain, is a member of the type IIs restriction enzymes. The enzyme recognizes non-palindromic nucleotide sequence 5’-ACTGGG and cleaves complementary DNA strands 5 and 4 nucleotides beyond the recognition sequence (9). In order to gain an insight into the structural organization and mechanisms of DNA recognition and catalysis employed by the type IIs restriction enzymes, we focused on the structural, biochemical, and mechanichal characterization of the *BfiI* restriction enzyme. Here we report the cloning and sequence analysis of *BfiI* R-M system and a biochemical characterization of the *BfiI* restriction endonuclease. We suggest that *BfiI* uses a novel catalytic domain to perform DNA cleavage that radically differs from the one employed by the archetypal *FokI* endonuclease and other type II restriction enzymes.

EXPERIMENTAL PROCEDURES

Plasmids and Strains—The bacterial strain *Bacillus firmus* S8120 was the source of genomic DNA. The *Escherichia coli* strain ER2267 (e14, mcrA) endA1 supE44 thi-1 Δ(uvrC-mrr)14:IS10 Δ(argF-lac)1U169 recA1F* proA* B* lacI* Δ(lacZ-M15 zff) mini-Tn10 (KmR) was obtained from New England Biolabs and used as a host in cloning procedures. Both strains were grown in Luria broth medium at 37 °C. Ampicillin and kanamycin were used at 60 μg/ml when necessary.

DNA Cloning and Construction of the Genomic Library—Isolation of genomic DNA from *Bacillus firmus* cells pretreated with lysozyme was carried out as described in Ref. 10. Plasmids were prepared by the alkaline-lysis procedure (11) and purified as described (12). DNA manipulations were in accordance with standard procedures (13). Transformations of *E. coli* were carried out using the CaCl2-heat shock method (13) or electrottransformation using a Gene Pulser (Bio-Rad). DNA in ligation mixture was precipitated with ethanol to remove salts and dissolved in H2O before electrottransformation. The gene library of *Bacillus firmus* S8120 was constructed by partially digesting genomic DNA with *Bsp*143I and ligating the fragments into *BamHI*-cleaved and

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The abbreviations used are: R-M, restriction-modification; bp, base pair(s); bis-pNPP, bis(p-nitrophenyl) phosphate; BSA, bovine serum albumin; kb, kilobase pair(s); nt, nucleotide(s); ORF, open reading frame; L1 and L2, linear DNA forms 1 and 2.
**BfiI Restriction Enzyme**

**DNA Sequencing and Analysis of Predicted Amino Acid Sequences** — DNA sequencing was performed in both directions from a series of nested deletions generated by Bal31 nuclease. The Cycle Reader™ DNA sequencing kit from Fermentas was employed, and \\(^{[3]}\overline{[P]}\)dATP was used as end-label to PCR322 sequencing primers with T4 polynucleotide kinase. Sequence data were compiled and analyzed with the MicroGenie sequence analysis software program (Beckman Instruments). Pairwise sequence comparison was performed by FASTA (15). The sequences were searched for putative homologs with PSI-BLAST algorithm (16) using the deduced amino acid sequence of BfiI restriction enzyme as a query.

**Enzymes and Oligonucleotides** — All enzymes, primers, and kits were obtained from Fermentas. The oligodeoxyribonucleotides used in this study were synthesized at Fermentas. The double-stranded 30-bp oligonucleotide containing the BfiI recognition sequence was obtained by annealing two complementary oligonucleotides: 5′-AGC GTA GCA CTT GGG TGA TCA ACT GTG CTG-3′ and 5′-CAG CAC AGT TCA GCC GCG CAG TGC TAC GCT-3′. Control experiments demonstrated that BfiI cleaved such a duplex. The double-stranded 30-bp oligonucleotide lacking the BfiI recognition sequence was obtained by annealing two complementary oligonucleotides: 5′-AAT GAC TCA TCT GGG TGA TCA ACT GTG CTG-3′ and 5′-CAG CAC AGT TCA GCC GCG CAG TGC TAC GCT-3′. All other chemicals used in this study were of the highest quality available.

**Identification of Methylated Cytosines by Sodium Bisulfite Treatment** — The analysis of methylation patterns was performed as described in Ref. 17. In brief, plasmids pUC-BgiM1, pUC-BmI2, and pUC-BgiM1M2 were methylated in vitro by expressing corresponding methylase genes present in cis. Plasmids were linearized with AluI/44I restriction endonuclease, ethanol-precipitated, and dissolved in H₂O. Treatment of DNA samples with sodium bisulfite converts unmodified cytosine residues to uracil (17). Therefore, all unmodified cytosines appear in the thymine lane after strand-specific polymerase chain reaction amplification, whereas modified cytosines remain in the cytosine lane. The bisulfite treatment of DNA was performed as described previously (17), with the exception that an additional step of the thermal DNA denaturation (95°C, 3 min) was introduced into the protocol at the end of each hour of DNA treatment. The modified upper strand DNA was amplified using strand-specific primers 5′-GTG TAG ATA ATT ATG TCA ATT GAG GG-3′ and 5′-CAT TTT CCA ATT ATA AAT GCT TTA ATT TAA-3′, whereas the lower strand was amplified using primers 5′-TTCT CAT TCA ATT ACA TAA CTC CCC-3′ and 5′-ATG ATG ATG ATT TTA AAA GTT TTG TTA TGT GG-3′, respectively. The amplified DNA fragments corresponded to the pUC19 sequences 1702–2300 nt (upper strand) and 1672–2291 nt (lower strand) and included a unique BfiI target located at nt 1744. Polymerase chain reaction fragments were inserted into the Smal-digested and dephosphorylated pUC19, and recombinant plasmids were selected and sequenced.

**Purification of BfiI Restriction Enzyme** — The BfiI protein was purified as described in Ref. 9. In brief, the cells of *B. firmus* SS120 were collected at log phase, disrupted by sonication, and BfiI protein purified by chromatography on phosphocellulose, DEAE-cellulose, heparin-Sepharose, and hydroxyapatite columns. The purified BfiI protein was stored at −20°C in a buffer containing 10 mM Tris-CH₃COOH (pH 7.5), 0.1 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 50% glycerol. The BfiI protein exhibited a single band on the SDS-polyacrylamide gel. Protein concentration was determined by A₂₈₀ using an extinction coefficient calculated from the amino acid composition (18) and is expressed in terms of monomer.

**Plasmid DNA Cleavage by BfiI** — Supercopied plasmid pUC19 was purified twice by ultra centrifugation through a cesium chloride gradient in the presence of ethidium bromide. The plasmid preparation contained >90% supercoiled DNA. Cleavage experiments were performed at 25°C in a reaction buffer consisting of 30 mM Tris-CH₃COOH (pH 8.0) and 100 mM KCl. Reaction mixtures typically contained 2.3 nm supercoiled pUC19, 0.1 mg/ml BSA, and 0–10 mM Mg(CH₃COO)₂ or 0–10 mM CaCl₂ or 0–5 mM MnCl₂. In all cases, the total ionic strength was constant at 130 mM by varying KCH₃COO concentration. Reactions were initiated by adding BfiI to a mixture of the other reaction components. Aliquots were removed at fixed time intervals and mixed with 1/3 volume of loading dye solution containing 400 mM Tris-CH₃COOH, pH 7.2, 0.2% SDS. The samples were heated at 65°C for 10 min and separated by electrophoresis through agarose. Supercopied, open-circular, and linear DNA forms (L1 and L2) were resolved by electrophoresis in the agarose gel, and their amounts were evaluated by densitometric analysis of ethidium bromide-stained gels (19). Nearly identical results were obtained by quantching the samples with equal volume of 1 M HCl, followed by immediate neutralization of samples, DNA precipitation with 2-propanol, and electrophoresis through agarose.

**Bis(p-nitrophenyl) Phosphate Cleavage by BfiI** — Bis(p-nitrophenyl) phosphate was obtained from Sigma. Bis-pNPP cleavage experiments were performed at 25°C in the 30 mM Tris-HCl (pH 7.2–8.5) or 30 mM MES-KOH (pH 6.0–6.8) reaction buffers containing 100 mM KCl·COO. In the pH range 5.0–5.75, the reaction buffer consisted of 30 mM Tris-CH₃COOH, 0.3% SDS. The samples were heated at 65°C for 10 min and separated by electrophoresis through agarose. Purification of BfiI Restriction Enzyme — The BfiI protein was purified from *Bacillus firmus* SS120 by partially digesting genomic DNA with Bsp143I and ligating the fragments into BamHI-cleaved and dephosphorylated pBR322. After the second round of BfiI digestion of plasmid population from partial Bsp143I library (∼300,000 clones) and re-amination of surviving plasmids into *E. coli* cells, several thousand transformants were obtained and individual plasmids were isolated from randomly picked transformants. Screening of 18 transformants revealed 17 BfiI-resistant recombinant plasmids that formed six different groups after restriction mapping. Analysis of the crude cell extracts prepared from representatives selected from each group revealed that three of them possessed both restriction and methylation phenotypes. The absence of restriction endonuclease activity in cells carrying pBfi-KE or pBfi-KM alongside the unaltered modification phenotype suggests that MunI and Eco721 cleavage sites are situated within the regulatory or structural part of the BfiIIR gene. Four plas-
The results of the deletion mapping, indicating that genes and ORFs were identified within the sequenced regions corresponding to the heterologous Bacillus firmus DNA. The open box shows the sequenced DNA fragment. Block arrows denote the location and orientation of BfiI R-M genes bfiIMC1, bfiIMC2, and bfiIR, respectively. Location of the gene for β-lactamase bla, and origin of replication ori of pBR322 are shown as thin black lines. Plasmids pBfi-K, pBfi-KM, pBfi-KE, pBfi-N, pBfi-KN, and pBfi-KB were obtained by deletions of Kmβ2I, Kmβ2I-MunI, Kmβ2I-Eco732I, NdeI, Kmβ2I-NcoI, or Kmβ2I-Bpu1102I fragments from pBfiRM14, respectively. Excision of the NheI-KspAI, Eco32I, NheI-Bpu1102I, or NheI-NcoI fragments from pBfi-KM yielded pBfi-KM-NK, pBfi-KM-E, pBfi-KM-NB, and pBfi-KM-N, respectively. R(+) and R(−) indicates subclones exhibiting and lacking restriction endonuclease phenotype. Methyltransferase protection against digestion by BfiI restriction enzyme is indicated as follows: M(+) = full protection, M(+/-) = partial protection, M(−) = no protection.

Identification of Methylated Bases—Deletion mapping and protein sequence analysis of BfiI methylases suggested that modification of the recognition site of BfiI is accomplished by two independent N4-methylcytosine methylases each of them modifying bases on the opposite strands of the recognition site. To determine the positions of methylation, we applied the sodium bisulfite modification technique adopted recently for mapping N4-methylcytosine residues in DNA (17). To establish the methylation pattern of BfiI methylases, three different recombinant plasmids were constructed by cloning bfiIMC1 and bfiIMC2 genes separately or in combination in a pUC19 vector that has a BfiI target located at nt 1744 and DNA in vivo methylation was accomplished by expressing methylase genes. Plasmid DNA was purified, treated with sodium bisulfite, and analyzed as described under “Experimental Procedures.” As shown in Table I, a single cytosine residue in each DNA strand of the BfiI target survived the bisulfite attack in the case of plasmid containing both genes of BfiI methylases. In comparison, only the unique C of the ACTGGG strand or the second C of the CCCAGT strand were resistant to bisulfite in plasmids, carrying separate genes bfiIMC2 and bfiIMC1, respectively. None of the cytosines survived the bisulfite treatment in the case of non-methylated pUC19 DNA (data not shown). Methylcytosine residues were displayed with frequencies ranging from 16% to 50%, inconsistent with the values reported previously for N4-methylcytosine residues (17). Collectively, based on the presence of conserved motifs characteristic of N4-methylcytosine methylases and the sequencing of bisulfite-treated methylated DNAs, we propose that M.BfiI(C1) modifies a second C base within the strand CCCAGT, whereas the M.BfiI(C2) methylates the unique C of the ACTGGG strand yielding N4-methylcytosines.

Nucleotide and Protein Sequence Analysis of BfiI Restriction Enzyme—The BfiIR gene is located on the DNA strand complementary to that bearing the genes for cognate methylases. Two possible translation start codons TTG at nt 4092 and ATG at nt 4029 and the termination codon TAA at nt 3018 were identified. Despite the presence of putative Shine-Dalgarno sequences upstream of both initiation codons (TAAGGGGG is located 7 bp upstream of TTG, and AAGGA is 9 bp upstream of ATG), we assigned the initiation codon to the TTG. This assumption relies on the experimental observation that cloning and expression of an amplified DNA fragment starting at the TTG but not at ATG codon yielded an active BfiI enzyme (data not shown). The ORF starting at TTG encoded a protein of 358 amino acid residues with a calculated mass of 40 kDa that was close to the value of 39 kDa estimated by SDS-polyacrylamide gel electrophoresis (data not shown). The predicted protein sequence of the bfiR gene was aligned to the protein sequences of other N4-methylcytosine methylases belonging to the S12 class revealed similar levels of homologies.
in the data bases using PSI-BLAST server at NCBI. The analysis failed to reveal significant homologies above the threshold level; however, we noticed that the N-terminal part of the BfiI exhibited marginal similarities to the EDTA-resistant nuclease from Salmonella typhimurium (Fig. 2). The similarities between BfiI and nuclease of S. typhimurium were below statistical significance and could be treated only as guidelines for further experiments. Therefore, seeking for the supportive evidence, we performed biochemical characterization of BfiI restriction enzyme.

**Plasmid DNA Cleavage by BfiI Restriction Enzyme—**Mg$^{2+}$ ions are a necessary cofactor for DNA cleavage by both type II and type IIs restriction enzymes. Strikingly, preliminary data indicated that Mg$^{2+}$ ions are not required for phage λ DNA cleavage by BfiI. In order to study the metal ions requirement for BfiI catalysis and quantitative evaluation of reaction rates of DNA cleavage in the presence and absence of metal ions, we have chosen supercoiled plasmid pUC19 that contains two recognition sites of BfiI, located at 364 and 1744 nucleotides from the origin of replication, respectively. In this case, cleavage of pUC19 by BfiI at the end of the reaction should give two linear DNA fragments of 1.4 and 1.3 kb. However, if either site is cut first in just one strand, an open-circle DNA form will appear prior to linear DNA (Fig. 3A). The plasmid DNA cleavage by restriction enzymes under single-turnover or steady state conditions quite often provides rate constants for different reaction steps (19). Therefore, pUC19 cleavage by BfiI was performed both under single-turnover and multiple-turnover conditions.

Cleavage of supercoiled pUC19 by BfiI in the absence of metal ions under single-turnover reaction conditions at saturating enzyme concentrations (2.3 nM pUC19, 10 nM BfiI) is presented in Fig. 3B. The concentration of supercoiled DNA declines exponentially, and only a small amount of open circular DNA accumulates during the reaction. The predominant reaction product is linear DNA cleaved at a single site (L1), that is subsequently converted to the final reaction product: linear DNA cleaved at both sites (L2). The accumulation of large amounts of L1 under single-turnover reaction conditions suggests that cleavage rates of the two BfiI sites differ significantly, most likely due to differences in flanking nucleotide sequences. Restriction mapping of the intermediate reaction product L1 indicated that pUC19 was predominantly cleaved by BfiI at the recognition site located at nt 1744. A single exponential was fitted to the data of the supercoiled substrate cleavage. The optimal fit yielded a 0.052 ± 0.005 s$^{-1}$ value for the first order reaction rate constant (Fig. 3B). Addition of EDTA (1 mM) or divalent metal ions (1–10 mM Mg$^{2+}$, 1–10 mM Ca$^{2+}$, 1–5 mM Mn$^{2+}$) to the reaction mixture did not affect the rate of plasmid DNA cleavage by BfiI. The values of the first order reaction rate constant remained close to 0.052 s$^{-1}$, and the reaction patterns did not differ significantly from that presented in Fig. 3B (data not shown).

The cleavage of supercoiled pUC19 DNA by BfiI in the presence and absence of metal ions has also been studied under multiple-turnover conditions with limiting enzyme (≤1 nM) and an excess of substrate (2.3 nM). Under these conditions the enzyme must perform several catalytic cycles in order to achieve complete cleavage of substrate DNA. Analysis of the time course of DNA hydrolysis by BfiI in the absence of divalent metal ions revealed a steep decline in concentration of supercoiled DNA over the first 10 min of the reaction followed by a very slow linear steady-state phase (Fig. 3C). Noteworthy, the amount of the supercoiled substrate cleaved during the burst phase equaled half the amount of the BfiI monomer used.

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**TABLE I**

| Bisulfite-treated DNA | Relevant genotype | Upper strand (ACGGGG) | Bottom strand (CCAGGT) | Construction of plasmids used$^b$ |
|----------------------|------------------|-----------------------|------------------------|----------------------------------|
| pUC-BfiIM1M2         | $bfiIMC^1$ $bfiIMC^2$ | 6                      | 4                      | Subcloning of KspAI-Acc651 fragment |
| pUC-BfiIM1           | $bfiIMC^1$       | 6                      | 5                      | Subcloning of KspAI-NcoI fragment |
| pUC-BfiIM2           | $bfiIMC^2$       | 6                      | 5                      | Subcloning of Bpu1102I-Acc651 fragment |

$^a$ Position of the displayed cytosine is underlined.

$^b$ DNA fragments were excised from pBfiIRM14 using indicated restriction enzymes, blunt-ended with T4 DNA polymerase, and inserted into Smal-digested pUC19. Plasmids with BfiI modification gene(s) under the control of P$_{lac}$ were selected.

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*J. Vítkute and V. Petrusyte, unpublished data.*
in the presence of Mg$^{2+}$ ions. The reaction mixture contained 100 mM KCH$_3$COO, 30 mM Tris-CH$_3$COOH, pH 8.0, 2.3 mM pUC19, 10 mM BfiI, and 0.1 mg/ml BSA at 25 °C. Changes of concentrations of supercoiled (A), open circular (D), and linear DNA with single double-strand break (○), and linear DNA with two double-strand breaks (△) are shown. The solid line represents the optimal fit of a single exponential to the time course of the supercoiled pUC19 form cleavage. The best fit yielded 0.052 ± 0.005 s$^{-1}$ for the first order reaction rate constant. B, multiple-turnover cleavage of the supercoiled form of pUC19 by BfiI in the absence of Mg$^{2+}$ ions. The reaction mixture contained 100 mM KCH$_3$COO, 30 mM Tris-CH$_3$COOH, pH 8.0, 2.3 mM pUC19, 10 mM BfiI, and 0.1 mg/ml BSA at 25 °C. A linear regression analysis applied to the data of the supercoiled pUC19 cleavage in the time interval 10–70 min (solid line) yielded $k_{cat}$ value of 0.008 ± 0.001 min$^{-1}$. C, multiple-turnover cleavage of the supercoiled pUC19 by BfiI in the presence of Mg$^{2+}$ ions. The reaction mixture was as in B, except that it contained 10 mM Mg(CH$_3$COO)$_2$, and 70 mM KCH$_3$COO to keep the ionic strength of the buffer equal to that of buffer C. The experimentally determined $k_{cat}$ value equals to 0.066 ± 0.006 min$^{-1}$.  

The ability to cleave bis-pNPP is the intrinsic property of BfiI rather than of the contaminating phosphodiesterase activity. Both the wild type BfiI and the enzyme purified to apparent homogeneity from the E. coli strain containing a cloned BfiI gene, possessed similar catalytic properties. In order to change the purification strategy of BfiI, a recombinant BfiI version containing a hexahistidine tag at the N terminus of the protein was prepared and purified by Ni$^{2+}$-column affinity chromatography. This one-step purification procedure yielded a nearly homogeneous His-tagged BfiI version that retained the ability to cleave both bis-pNPP and DNA.

The pH Dependence of Bis(p-nitrophenyl) Phosphate Hydrolysis Rate by BfiI—The studies of the pH dependence values of the rates of enzymatic reactions sometimes allow determining the $pK_a$ values of ionizable groups at the active site of enzymes responsible for the catalytic activity. Therefore, we have studied the pH dependence of the second order reaction rate of bis-pNPP hydrolysis by BfiI (Fig. 5). The pH dependence of the
rate of bis-pNPP hydrolysis by BfiI exhibited a characteristic, bell-shaped curve with an optimum at pH 5.5–6.0. The slopes of the acidic and alkaline limbs in the log(k) versus pH plot, however, differed (Fig. 5). The alkaline limb gives a slope −1, while the slope of acidic limb is less than +0.5. The fit of the equation $k = k_{\text{max}} [H^+] / ([H^+] + K_a)$ to the experimental data at the alkaline limb (pH 5.5–8.5) yielded the optimal apparent pK_a value of 6.4 ± 0.1 for a catalytically important base at the BfiI active site.

**Inhibition of Bis(p-nitrophenyl) Phosphate Hydrolysis by DNA**—In order to check if the hydrolysis of artificial substrate bis-pNPP and DNA proceeds at the same active site of BfiI, we have studied the effect of DNA on the cleavage rates of bis-pNPP. The single turnover studies of pUC19 cleavage by BfiI indicated that recognition sites on the DNA became saturated by BfiI even at the minor excess of protein, suggesting tight binding (see, above). Thus, if the reaction of the bis-pNPP hydrolysis and DNA cleavage occurs at the same active site of BfiI, DNA might act as an inhibitor of artificial substrate cleavage. Therefore, reaction rates of bis-pNPP hydrolysis by BfiI were studied in the presence of different amounts of synthetic 30-mer oligonucleotides containing and lacking the recognition sequence of BfiI (Fig. 6). The data presented in Fig. 6 demonstrate that increasing concentrations of specific oligonucleotide effectively inhibited the rate of bis-pNPP hydrolysis by BfiI. Interestingly, 30-mer nonspecific oligonucleotide at the same concentrations had only a minor effect on the cleavage rates of the bis-pNPP by BfiI. If we assume that the enzyme bound to the DNA is incapable of artificial substrate cleavage, the reaction rate of bis-pNPP hydrolysis should be proportional to the free enzyme concentration (the concentration of BfiI-bis-pNPP complex can be neglected since $K_m$ value for bis-pNPP is significantly higher than bis-pNPP concentrations used in our experiments). An equation describing the dependence of free enzyme concentration on the total DNA concentration according to a simple equilibrium $E + DNA \leftrightarrow E \cdot DNA$ was fitted to the data for the specific oligonucleotide, presented in Fig. 6 (enzyme concentration was expressed in terms of dimer) to yield a $K_D$ value of 30 nM for the BfiI-specific oligonucleotide complex.

**DISCUSSION**

The restriction-modification system of the *Bacillus firmus* SS120 strain comprises two methyltransferases and a single restriction enzyme and is a typical type IIs system. Each methylase recognizes and methylates bases on the opposite strands of the recognition sequence making the modified DNA resistant to the restriction enzyme cleavage. Mg^{2+} ions are a necessary cofactor for DNA hydrolysis by type II and type IIs restriction enzymes (25). Biochemical experiments strikingly revealed that Mg^{2+} ions are not required for the DNA cleavage by BfiI, raising the question of how catalysis is achieved.

Based mostly on the structural and biochemical studies of FokI endonuclease, the type IIs restriction enzymes are thought to comprise two modules connected by a flexible linker (3–5). In the case of FokI, the N-terminal subdomain is responsible for the DNA binding and the C-terminal for the cleavage.

**Fig. 6. Bis(p-nitrophenyl) phosphate hydrolysis by BfiI in the presence of specific and nonspecific oligonucleotide.** Reaction mixtures contained 100 mM KCH_3COO, 30 mM Tris-CH_3COOH, pH 7.2, 5 mM bis(p-nitrophenyl) phosphate, 400 mM BfiI, 0–500 nM oligonucleotide duplex either containing (□) or lacking (▼) BfiI recognition site at 25 °C. The reaction course was monitored spectrophotometrically at 405 nm, and initial velocities were calculated by linear regression analysis. All displayed results are the mean values of at least three experiments ± one standard deviation. An equation describing the dependence of free enzyme concentration on the total oligonucleotide concentration was fit to the experimental data for specific oligonucleotide (solid line). The obtained $K_D$ of the enzyme-oligonucleotide complex equals 30 nM.

Sequence alignment between Nuc and BfiI indicates (Fig. 2) that all residues found at the active site of Nuc (including active site His) are conserved in BfiI restriction enzyme suggesting a similar organization of the active sites. It is interesting to note that secondary structure predictions for the N-terminal domain of BfiI were very similar to the secondary structure elements of Nuc, suggesting fold similarities (Fig. 2). Thus, it was tempting to suggest that N-terminal domain of

**Fig. 5. pH dependence of bis(p-nitrophenyl) phosphate hydrolysis by BfiI restriction enzyme.** The reaction mixtures typically contained 2–5 mM bis(p-nitrophenyl)phosphate, 400 nM BfiI in the reaction buffer with appropriate pH value. p-Nitrophenolate release was monitored at 405 nm. Initial reaction rates were obtained by linear regression. The solid line represents the optimal fit of the equation $k = k_{\text{max}} [H^+] / ([H^+] + K_a)$ to the experimental data in the pH interval between 5.5 and 8.5. The best fit parameters: $k_{\text{max}} = 5.3 \pm 0.2 \text{ s}^{-1} \text{ M}^{-1}$ and pK_a = 6.4 ± 0.1.

**Note:** The restriction-modification system of the *Bacillus firmus* SS120 strain comprises two methyltransferases and a single restriction enzyme and is a typical type IIs system. Each methylase recognizes and methylates bases on the opposite strands of the recognition sequence making the modified DNA resistant to the restriction enzyme cleavage. Mg^{2+} ions are a necessary cofactor for DNA hydrolysis by type II and type IIs restriction enzymes (25). Biochemical experiments strikingly revealed that Mg^{2+} ions are not required for the DNA cleavage by BfiI, raising the question of how catalysis is achieved.

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Sequence alignment between Nuc and BfiI indicates (Fig. 2) that all residues found at the active site of Nuc (including active site His) are conserved in BfiI restriction enzyme suggesting a similar organization of the active sites. It is interesting to note that secondary structure predictions for the N-terminal domain of BfiI were very similar to the secondary structure elements of Nuc, suggesting fold similarities (Fig. 2). Thus, it was tempting to suggest that N-terminal domain of
BfiI is similar to Nuc. The similarities presented in Fig. 2, however, are below the statistically significant level and should be treated with caution. Therefore, we sought other evidence in support of the hypothesis that BfiI possess a Nuc-like catalytic domain.

Unlike most nuclease, Nuc nuclease cleaves DNA in the absence of metal ions (23, 26, 27). Restriction enzymes studied to date absolutely require Mg$^{2+}$ ions for phosphodiester bond cleavage. Preliminary observations using phage λ DNA and quantitative studies of pUC19 cleavage by BfiI (Fig. 3) indicate that metal ions are unnecessary for the phosphodiester bond cleavage by BfiI and suggest mechanistic similarity to Nuc nuclease. The single turnover experiments with pUC19 and BfiI yielded the first-order rate constant of 0.052 s$^{-1}$ that presumably corresponds to the rate of the chemical step (phosphodiester bond cleavage) and is independent of the metal ion. The value of the rate constant is more than 10-fold lower than values of the rate constants of the chemical step reported for the Mg$^{2+}$-dependent restriction enzymes EcoRI (32), EcoRV (33), and MunI (19). The experiments with pUC19 cleavage under multiple turnover conditions (Fig. 3) revealed, however, that a step other than the chemical step, limits the overall reaction rate of pUC19 cleavage by BfiI. Indeed, the $k_{cat}$ for the cleavage of the closed supercoiled pUC19 form by BfiI in the absence of the metal ion was approximately 400-fold lower than the rate constant of the chemical step. It is possible that, under multiple turnover conditions, dissociation of the enzyme-product complex limits the overall reaction rate. Interestingly, metal ions (Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$) at 5–10 mM concentrations increased the $k_{cat}$ value approximately 10-fold. A similar effect has been reported for vaccinia virus topoisomerase (34). This enzyme did not require metal ions for the DNA cleavage; however, it exhibits metal dependence of product release rate.

Moreover, like the Nuc enzyme, BfiI exhibited the ability to hydrolize the artificial substrate bis-pNPP and metal ions were not required for catalysis. Control experiments revealed that typical type II restriction enzymes like MunI and Cfr10I or type II enzymes FokI did not catalyze hydrolysis of bis-pNPP either in the presence or absence of Mg ions. The reaction rate of the bis-pNPP cleavage by BfiI was much slower than the rate of DNA cleavage. The second order reaction rate constant ($k_{cat}/K_m$) for the bis-pNPP cleavage by BfiI was equal to the 4.2 ± 0.1 M$^{-1}$ s$^{-1}$ (pH 6.0, 25 °C). Noteworthy, the value (4.2 M$^{-1}$ s$^{-1}$) of the second order rate constant for bis-pNTP cleavage by BfiI was close to the $k_{cat}/K_m$ value (10 M$^{-1}$ s$^{-1}$, 30 °C) reported for Nuc cleavage of bis-pNPP (23).

The highest rate of bis-pNPP hydrolysis both by the BfiI restriction enzyme and the Nuc nuclease was observed at pH 5.5–6.0. The alkaline limb of pH dependence of bis-pNPP hydrolysis by BfiI is consistent with the ionization of a base with an apparent pK$_a$ value of 6.4. This value is close to the pK$_a$ value of His residue and supports the assumption that such a residue is located at the active site of BfiI. The pH dependence of bis-pNPP hydrolysis by Nuc has not been reported; however, the coincidence of the optimal pH values for bis-pNPP hydrolysis by BfiI and Nuc suggests similar pH dependence for artificial substrate hydrolysis by Nuc. In contrast to the artificial substrate, BfiI cleaved plasmid DNA both at pH 6.0 (data not shown) and pH 8.0 (Fig. 3B). The ability to hydrolyze DNA at pH 7.5 has also been reported for the Nuc nuclease. The differences in the pH dependence values for hydrolysis of small artificial substrates and DNA by BfiI might be attributed to the perturbation of the pK$_a$ values of active site residues in the enzyme-DNA complex. If we assume that both protonated and unprotonated BfiI forms are able to bind bis-pNPP, the pK$_a$ value, determined from the pH dependence of the $k_{cat}/K_m$ ratio corresponds to the ionization of catalytically important residue at the active site of the free enzyme (24). The pK$_a$ value of the same residue in the enzyme-DNA complex may be shifted significantly. Indeed, such effects were reported for the barnase-catalyzed hydrolysis of RNA and dinucleotides (35). The optimum pH for RNA hydrolysis of barnase was 8.5 and exceeded that of GpA transesterification by 3.5 units. Alternatively, the decrease of the $k_{cat}/K_m$ ratio with the increase of pH in the case of bis-pNPP hydrolysis by BfiI might be explained by decreased binding (increased $K_m$) of the low molecular weight substrate while DNA binding might be less sensitive to the pH change.

Collectively, our data indicate that BfiI exhibits most of the enzymatic properties characteristic for the Nuc nuclease. However, unlike the nonspecific Nuc nuclease, BfiI restriction enzyme cleaves phosphodiester bonds in DNA site-specifically (Fig. 3, B–D). Both the specific DNA cleavage and bis-pNPP hydrolysis proceeds at the same active site of BfiI. Oligonucleotide containing the recognition sequence of BfiI effectively inhibited hydrolysis of bis-pNPP (Fig. 6) at pH 7.0. In contrast, a nonspecific oligonucleotide lacking the recognition sequence of BfiI had only a marginal effect on the rate of bis-pNPP hydrolysis. These experiments indicate that, unlike Nuc, BfiI effectively discriminates between specific and nonspecific DNA. Since sequence comparisons reveal similarities of N-terminal part of BfiI protein to the Nuc nuclease, we propose that DNA-binding specificity of BfiI stems from the C-terminal part of the protein. It is possible that, as in FokI (5), the nucleolytic domain of BfiI is sequestered by the DNA-binding domain. Only upon BfiI binding to its recognition sequence does the cleavage domain swing over to the DNA cleavage site and the enzyme become activated. The possible cross-talking interactions between the DNA binding and cleavage domains of BfiI obviously require further studies.

CONCLUSIONS

The experimental evidence presented here indicates that, in contrast to other restriction enzymes that require metal ions for catalysis, BfiI cleaves DNA specifically in the absence of metal ions. We suggest that, like to other type II enzymes, BfiI is composed of two subdomains that perform separate cleavage and DNA-recognition functions. The catalytic N-terminal subdomain of BfiI is presumably similar to that of nonspecific nuclease Nuc that cleaves DNA in the absence of metal ions. The C-terminal part of the BfiI presumably performs the DNA-binding function. It is tempting to speculate that BfiI evolved by fusion of the catalytic Nuc-like domain to the DNA-binding domain. The archetypal type II restriction enzyme FokI, in contrast to BfiI requires Mg$^{2+}$ ions for DNA cleavage, its cleavage domain is located at the C-terminal part of the protein and is similar to the monomer of BamHI. Therefore, we suggest that BfiI represents a novel subclass of type II restriction enzymes that differ from the archetypal FokI by the fold of the cleavage domain and by the location of the active site and reaction mechanism. Thus, type II restriction enzymes probably form a structurally and mechanistically diverse class. The existence of several different evolutionary lineages of type II restriction enzymes is probable. It will be interesting to see if the Nuc-like fold has been adopted by other restriction enzymes.

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