An alternative approach to study the enzymatic specificities of the CfrBI restriction–modification system

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Restriction–modification systems (RMS) are the main gene-engineering tools and a suitable model to study the molecular mechanisms of catalysis and DNA–protein interactions. Research into the catalytic properties of these enzymes, determination of hydrolysis and DNA-methylation sites remain topical. In our previous work we have cloned and sequenced the CfrBI restriction–modification system (strain *Citrobacter freundii*), which recognizes the nucleotide sequence 5′-CCWWGG-3′.

In this article we describe the cloning of the methyltransferase and restriction endonuclease genes (gene encoding CfrBI DNA methyltransferase (*cfrBIM*) and gene encoding CfrBI restriction endonuclease (*cfrBIR*)) separately to obtain strains overproducing the enzymes of this system. His$_6$-CfrBI, which had been purified to homogeneity, was used to establish the DNA-hydrolysis point in its recognition site. CfrBI was shown to cleave DNA after just the first 5′C within the recognition site and then to generate 4-nt 3′ cohesive ends (5′-C/CWWGG-3′).

To map the site of methylation by M.CfrBI, we exploited the fact that the *Cfr*BI site partially overlaps with the recognition sites of the well-documented enzymes KpnI and ApaI. The M.CfrBI- induced hemimethylation of the internal C residue of the ApaI recognition sequence (GGGC^{N4m}CC) was observed to block cleavage by ApaI. In contrast, KpnI was able to digest its M.CfrBI-hemimethylated site (GGTA^{N4m}CC).

KpnI was used to restrict a fragment of DNA harbouring the CfrBI and KpnI sites, in which the CfrBI site was methylated *in vitro* by His$_6$-M.CfrBI using [³H]-SAM.

The subsequent separation of hydrolysis products by electrophoresis and the enumeration of incorporated [H3]-methyl groups in each of the fragments made it possible to determine that external cytosine undergoes modification in the recognition site.

1. Introduction

The restriction-modification system (RMS) enzymes DNA methyltransferases and endonucleases constitute a unique class of DNA binding proteins, which recognize the same sequence but catalyze totally different types of enzymatic reactions [1, 2].

DNA methyltransferases (MTases) catalyze the transfer from AdoMet to certain N and C atoms in the nucleotides. Site-specific DNA modifications in bacteria usually lead to the formation of three kinds of products: N6-methyladenosine (^{N6m}A), 5-methylcytosine (^{5m}C) and N4-methylcytosine (^{N4m}C). ^{N4m}C modification was first discovered and documented at the laboratory of Dr. A. Janulaitis [3]. N4-methylcytosine has only been found in Prokaryota and Archaea [4].

Our previous work has found that the genes of the CfrBI RMS are localized on the multicopy plasmid pZE8 [5]. The CfrBI RMS recognizing the sequence 5′-CCWWGG-3′ has been previously cloned and sequenced [5, 6]. It also has been shown that CfrBI is an endonuclease with "relaxed" specificity and the true isoschizomer of the StyI prototype [5]. M.CfrBI belongs to the class of ^{N4m}C MTases.

Attempts to transform some commonly used *E. coli* strains by plasmids carrying an intact gene encoding CfrBI DNA methyltransferase (*cfrBIM)* were unsuccessful, apparently due to the McrBC$^+$ phenotype of these strains [5]. Since the McrBC system recognizes 5′R^{me}C [7, 8, 9], the restriction by the *mcrBC*$^+$ strains suggests that M.CfrBI methylates the external 5′-cytosine [5].

The CfrBI RMS is the first system for which the transcription regulation mechanism depending on the methylated base in the promoter region is described in detail [10, 11]; still, it has not been established exactly which of the cytosines is methylated in the site. In what could be a breakthrough, this paper describes the determination of the enzyme

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specificities of this system through the use of purified proteins. Production of these enzymes in purified form was achieved through the cloning and overexpression of the His$_6$-tagged M.CfrBI and CfrBI in *E. coli,* which contributed to their efficient purification.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains used in this study were *Escherichia coli* K802 [12] and M15 [REP4] (Qiagen, Hilden, Germany). The vector plasmid pQE30 was used to obtain strains producing CfrBI RMS enzymes. pUC128 [13] and pUC19 [14] were used to construct plasmids pCFR, pCK, pCA for analysis of enzyme specificity. Plasmids pBGM5 and pXB4 have been described earlier [6, 10]. The plasmid pBGM5 carries the total nucleotide sequence of the CfrBI R-M system. The plasmid pXB4 carrying the M.*CfrBI* gene and the chloramphenicol acetyltransferase gene (*cat*) were constructed from plasmid pBGM5. The plasmid pXB4 was used to develop the CfrBI producer strain.

E. coli strains were grown in the Luria–Bertani (LB) medium at a temperature of 37 °C.

2.2. DNA manipulation and sequence analysis

The isolation of plasmid DNA, restriction analysis, gene cloning and purification of the DNA fragments were all performed in accordance with [15, 16]. The resulting recombinant DNA was analysed through restriction mapping and the cloned DNA sequences were tested as described in [17].

2.3. Oligonucleotides

P1: 5′-TTC<u>GGATCC</u>ACAGTTAAAAACAATTAAAC-3′
P2: 5′-AAT<u>CTGCAG</u>TTAATTTATGATTTTTAAATC-3′
P3: 5′-CGC<u>GGATCC</u>AATTTCAAGGATAAAAATTGTTTCCC-3′
P4: 5′-GGG<u>AAGCTT</u>TTATAATTTAATGTATTTTTCC-3′
P5: 5′-AGT<u>CTAGA</u>CCATGGGCCCCCCCT-3′
P6: 5′-AGT<u>CTAGA</u>CCATGGTACCGGGCCCCCCCT-3′
P7: 5′-AGCGGATAACAATTTCACACAGGA-3′

2.4. Construction of recombinant plasmids for overexpression of cfrBIM and cfrBIR genes

The plasmid pBGM5, carrying the total nucleotide sequence of the CfrBI R-M system, was used as a template in polymerase chain reaction (PCR) to clone *cfrBIM* and gene encoding CfrBI restriction endonuclease (*cfrBIR*).

The "reverse" 29-mer primer (P1, see Section 2.3) used to clone the *cfrBIM* gene contains a BamHI site (underlined) immediately after the ATG start codon; the "forward" 30-mer primer (P2, see Section 2.3) contains a PstI site (underlined) just after the termination codon (TAA) of *cfrBIM*. The *cfrBIM* gene was amplified as an ATG-lacking 1127-bp fragment. This PCR fragment was cloned into a BamHI-PstI-digested pQE 30 expression vector. The plasmid with a functionally active *cfrBIM* gene was named pMet17.

The "forward" 35-mer primer (P3, see Section 2.3) used to clone *cfrBIR* contains a BamHI site (underlined) just after the ATG start codon; the "reverse" 31-mer primer (P4, see Section 2.3) contains a HindIII site (underlined) just after the termination codon (TAA) of *cfrBIR*. The *cfrBIR* gene was amplified as a 1064-bp ATG-lacking fragment. This PCR fragment was cloned into a BamHI-HindIII-digested pQE 30 expression vector. The plasmid with a functionally active *cfrBIR* gene was named pRes10.

All PCR experiments were performed using a Gene Amp PCR System (Perkin Elmer). The PCR operating parameters were: 30 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s).

2.5. Expression and purification of recombinant M.CfrBI and R.CfrBI proteins

The *E. coli* K802 [pREP4] strain was transformed with pMet17 in the case of M.CfrBI or with [pRes10, pXB4] in the case of CfrBI. Expression was induced through the addition of IPTG to a final concentration of 2 mM. Growing cells were harvested at various intervals and the expression of recombinant M.CfrBI or CfrBI was monitored by SDS-PAGE; the assay of the functional activity was also carried out.

The one-step purification of M.CfrBI and CfrBI, nearly to homogeneity, was performed under native conditions by affinity chromatography on nickel ions chelated by nitrilotriacetic acid (Ni^{2+}-NTA) resin. The washing and elution procedure for His$_6$-tagged proteins was optimized for the preparation of M.CfrBI and CfrBI. All protein fractions were checked by SDS gel electrophoresis and assayed for M.CfrBI or CfrBI activity. Two M.CfrBI fractions obtained within the range of 200–250 mM imidazole contained the maximum amount of MTase and displayed the maximum enzymatic activity. Four protein fractions eluted at 180–240 mM imidazole concentrations contained the maximal amount of restriction endonuclease (ENase) and exhibited an excellent restriction activity. These fractions were collected, pooled, dialyzed and stored at –20 °C. The molecular weights of His$_6$-tagged MTase and ENase were determined by 12% SDS gel electrophoresis using the corresponding molecular mass standards.

2.6. R.CfrBI endonuclease activity assay

In the phage plating test, all CfrBI RMS clones were found to restrict φ80 *vir* phage. For this reason, we used the test to select clones with a *cfrBIR* gene on the basis of phage resistance.

The endonuclease activity *in vitro* was detected by incubating the protein fractions with phage φ80 *vir* DNA in a 20 µl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM MgCl$_2$, 1 mM DDT for 1 h at 37 °C, with subsequent electrophoresis on 1% agarose.

2.7. M.CfrBI methyltransferase activity assay

M.CfrBI activity was tested by incubation of MTase with bacteriophage φ80 *vir* DNA. A typical assay was carried out in a 50 µl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM EDTA, 5 mM 2-mercaptoethanol, 80 µM S-adenosyl-L-methionine, 1 µg λ DNA and 2.5 units of His$_6$-M.CfrBI. The reaction mixture was incubated at 37 °C for 1 h. After methylation, DNA was extracted with phenol/chloroform and precipitated with ethanol. The pellet was re-dissolved in a 20-µl reaction buffer and incubated for 1 h at 37 °C with CfrBI. The resulting digested products were analysed by 1% agarose gel electrophoresis; methylation was tested by the absence of digestion.

A quantitative analysis of M.CfrBI activity was based on the incorporation of tritiated methyl groups into substrate DNA. The methylation reactions were carried out as above, except that the reaction mixtures contained labelled 10 µCi [^{3}H] SAM (85 Ci/mM, Amersham) and a PvuII DNA fragment instead of φ80 *vir* DNA. After incubation, the DNA fragment was digested with corresponding ENase. The digested products were loaded onto 2.5% low-melting agarose gel, excised and purified. Then these samples were adsorbed onto 2,5 mm Whatman DE81 paper circles, dipped in 95% ethanol, dried and counted in a liquid scintillator cocktail [18].

2.8. Identification of CfrBI cleavage position

The cleavage position of CfrBI was determined by the primer extension method. For template preparation, a 358-bp EcoRI-KpnI DNA fragment containing a single CfrBI recognition site was excised from pBGM5 and cloned into M13tg130, which yielded the plasmid pCFR. With this plasmid as a template, M13/pUC universal primer (#1201, NEB) and [α-^{32}P] ATP were extended with T7 DNA polymerase (Sequenase 2.0, USB Biochemicals) under standard conditions. After phenol extraction

the DNA was subjected to CfrBI digestion. Addition of large fragment of *E. coli* DNA polymerase I (Klenow) enzyme to one part of the digestion mixture was known to lead to the extension of the reaction product in the case of canonical protruding ends. To localize the CfrBI cleavage site, products of the sequencing reaction [17] with M13/pUC universal primer and pCFR as a template, CfrBI digestion and Klenow fill-in products of the extension reaction were run on the same sequencing gel (7% PAGE).

2.9. Construction of recombinant plasmids with unique overlapping sites for two ENases

The plasmid pUC128 was used as a template in PCR synthesis of DNA fragments with overlapping sites for CfrBI and ApaI as well as CfrBI and KpnI. For the recombinant plasmid carrying CfrBI and ApaI sites, a 23-mer primer (P5, see Section 2.3) with the XbaI site (underlined in Section 2.3) and an M13/pUC reverse sequencing (-48) 24-mer primer

(#1233, NEB) (P7, see Section 2.3) were used. For the recombinant plasmid carrying CfrBI and KpnI sites, the primers used were a 29-mer (P6, see Section 2.3) with the XbaI site (underlined) and M13/pUC reverse sequencing (#1233, NEB) (P7, see Section 2.3). The amplified DNA fragments were digested by EcoRI + XbaI, loaded on 6% polyacrilamide gel, excised and purified. These DNA fragments 48 bp and 52 bp in length were subcloned into XbaI-EcoRI-digested pUC19. The resulting recombinant plasmid with CfrBI and ApaI overlapping sites was named pCA and the plasmid with CfrBI and KpnI sites, pCK.

3. Results and discussion

3.1. Overexpression of M.CfrBI and CfrBI

For overproduction of M.CfrBI, we constructed the plasmid pMet17 (see Section 2.4). The φ80 vir phage DNA from pMet17-harboring cells
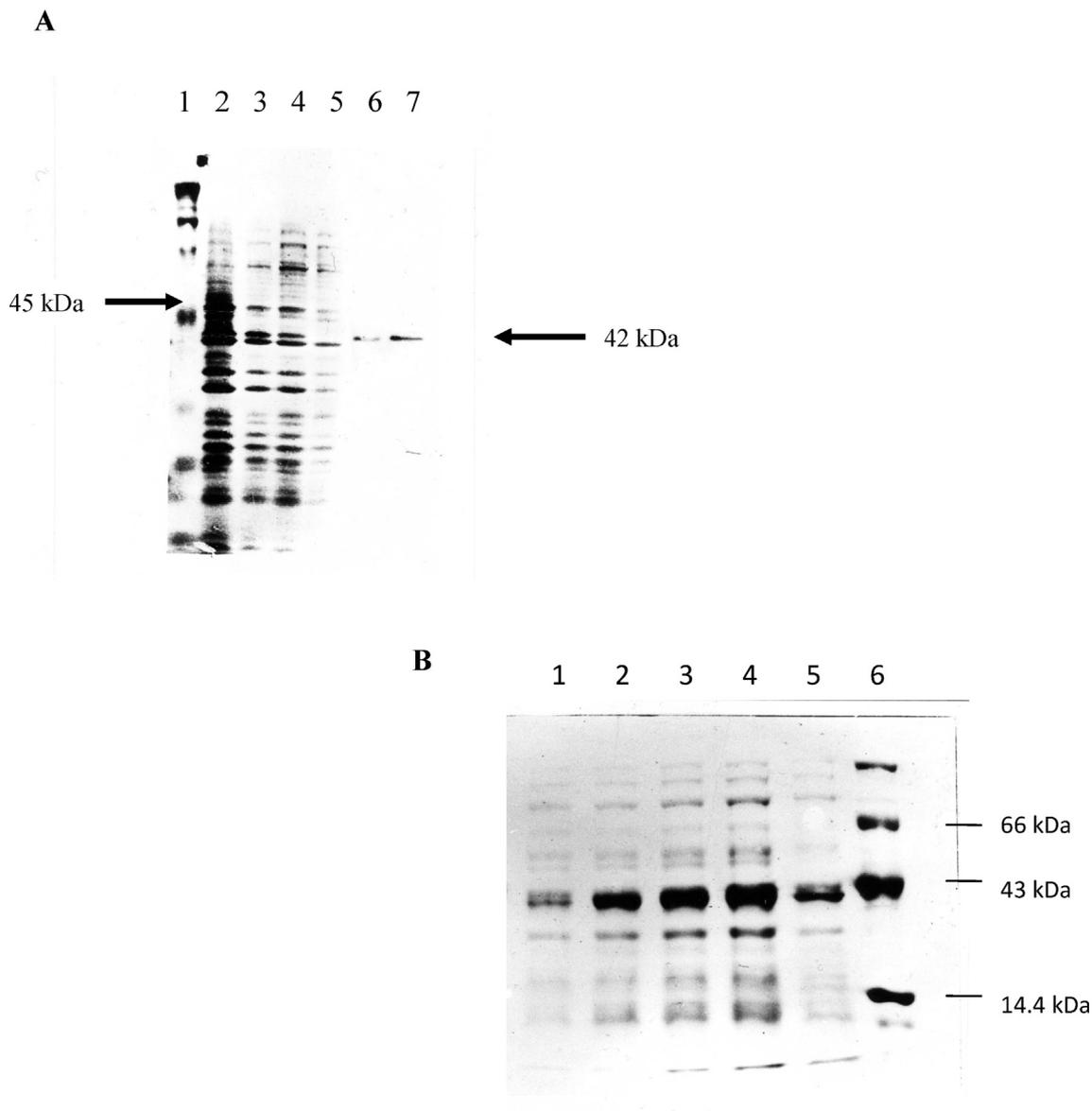
Fig. 1. The time course of the expression of recombinant proteins of CfrBI system. A. M.CfrBI in *E.coli* K 802 [pRep4] cells under IPTG induction. 12% SDS polyacrylamide gel analysis. Lanes: 1 - protein markers; 2, 3, 4, 5 - overproduction of recombinant M.CfrBI under IPTG induced conditions at different time intervals (3 h, 2 h, 1 h); lanes 6 and 7- M.CfrBI was purified under native conditions by affinity chromatography on Ni^{2+}-NTA resin (0.1 μg and 0.5 μg protein, respectively). The arrow points to the *cfrBIM* gene product of approximately 42 kDa. B. CfrBI in *E.coli* K 802 [pXB4, pRep4] cells under IPTG induction. 12% SDS polyacrylamide gel analysis. Lanes: 1, 2, 3, 4 - overproduction of recombinant CfrBI under IPTG induced conditions at different time intervals (1 h, 3 h, 4 h, 5 h); 5- CfrBI was purified under native conditions by affinity chromatography on Ni^{2+}-NTA resin (5 μg protein); 6 - protein markers.

were CfrBI-resistant, indicating that the modification gene was functional in *E. coli*. Under IPTG induction, analysis of the protein profiles from the pellet and supernatant fractions of lysed *E. coli* K802 cells [pMet17, pREP4] showed that His$_6$-tagged M.CfrBI occurred mostly in the latter fraction. Thus, under native conditions we could purify M.CfrBI nearly to homogeneity in one step. Fig. 1A shows M.CfrBI production in *E. coli* K802 [pREP4] cells carrying the recombinant plasmid pMet17 under IPTG induction (2 mM). Expression of MTase was monitored by SDS-PAGE analysis of protein samples taken at various intervals during the cell growth.

Upon 4 h induction with IPTG, M.CfrBI constituted about 1–2% of the cellular protein. The relative molecular weight of M.CfrBI was about 42 kDa, which agrees with the sequencing data. M.CfrBI was characterized by a protein concentration of 1 mg/ml and activity of 5–10 units/µl.

The effect of Na$^+$ and K$^+$ cations on His$_6$-M.CfrBI activity was investigated in various pH buffers using a protection assay with φ80 *vir* phage DNA. In the presence of K$^+$, the methylase activity strongly increased, whereas at Na$^+$ concentrations above 75 mM it drastically decreased. The optimal reaction buffer was shown to contain 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM EDTA, 5 mM 2-mercaptoethanol, 80 µM SAM. Purified His$_6$-tagged M.CfrBI was further used to localize a methylated base within the CfrBI recognition site.

In order to get the overproduction of CfrBI, we constructed the plasmid pRes10 (see Section 2.4). Propagation of the φ80 *vir* phage in *E. coli* K802 [pRes10, pXB4, pREP4] strain was restricted to 1–2 orders of magnitude as compared to the control strain with the plasmids pXB4 and pREP4. The *cfrBIR* endonuclease gene was expressed by adding 2MM IPTG (Fig. 1B). Expression of ENase was monitored by SDS-PAGE analysis made at various intervals during the cell growth. A 5 h IPTG induction provided an end result at the equivalent of 40% CfrBI in proportion to the total supernatant. The relative molecular weight of CfrBI was approximately 40 kDa, which is consistent with the predicted molecular weight based on the sequencing data.

The effects of several factors on His$_6$-R.CfrBI activity were then studied. The optimal conditions for its activity were found to be within the 7.8–8.0 pH range and at a temperature of 37 °C. In the presence of Mn^{2+} and Co^{2+}, the digestion was far less efficient. Thus, the optimal reaction buffer contained 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT. The functional purity of R.CfrBI was confirmed by the restriction–ligation–restriction test and its suitability for localization of the CfrBI cleavage site.

3.2. Localization of CfrBI endonuclease cleavage site

The cleavage site of CfrBI was determined using the primer extension method with pCFR as a template and M13/pUC universal primer. Within the plasmid pCFR, the CfrBI site was positioned conveniently for determination of the CfrBI cleavage site. It was shown that CfrBI endonuclease cleaved DNA after the first 5′-cytosine within the recognition sequence, generating 4-nt 3′ cohesive ends (Fig. 2). These results demonstrate that CfrBI endonuclease is an isoschizomer of StyI [19].

3.3. Localization of a methylated base within the CfrBI recognition site

In order to determine which C is the target for the action of M.CfrBI in its recognition sequence, we used an approach based on the overlapping between the MTase recognition site and the cutting sites of type-II ENase [20]. We modified the approach using the PCR technique to construct a convenient sequence of DNA.

Two recombinant plasmids with unique overlapping sites were constructed. pCK had a recognition sequence for CfrBI and KpnI (Fig. 3A, 1), and pCA, for CfrBI and ApaI (Fig. 3A, 2). Methylation of the CfrBI site in these overlapping sequences resulted in ^{N4m}C-hemimethylation of the ApaI and KpnI sites.

Site-specific DNA methylation is known to block the cleavage of cognate restriction endonuclease. Moreover, ENases are sometimes
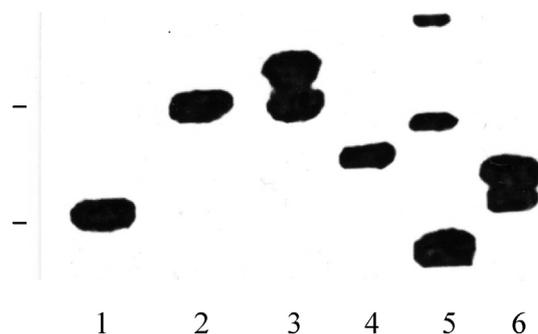

Fig. 2. Identification of the CfrBI cleavage position. The DNA cleavage product comigrates with the band corresponding to C in the DNA strand (lane 1). Addition of the Klenow enzyme yields a band, which is 4 nt longer, comigrating with 3′ flanking G-residue (lane 2). The size ladder was obtained by using M13/pUC universal primer and pCFR as a template (lanes 3, 4, 5, 6). The positions of the cleavage point and G residue, corresponding to the Klenow fill-in product, are marked by arrows.
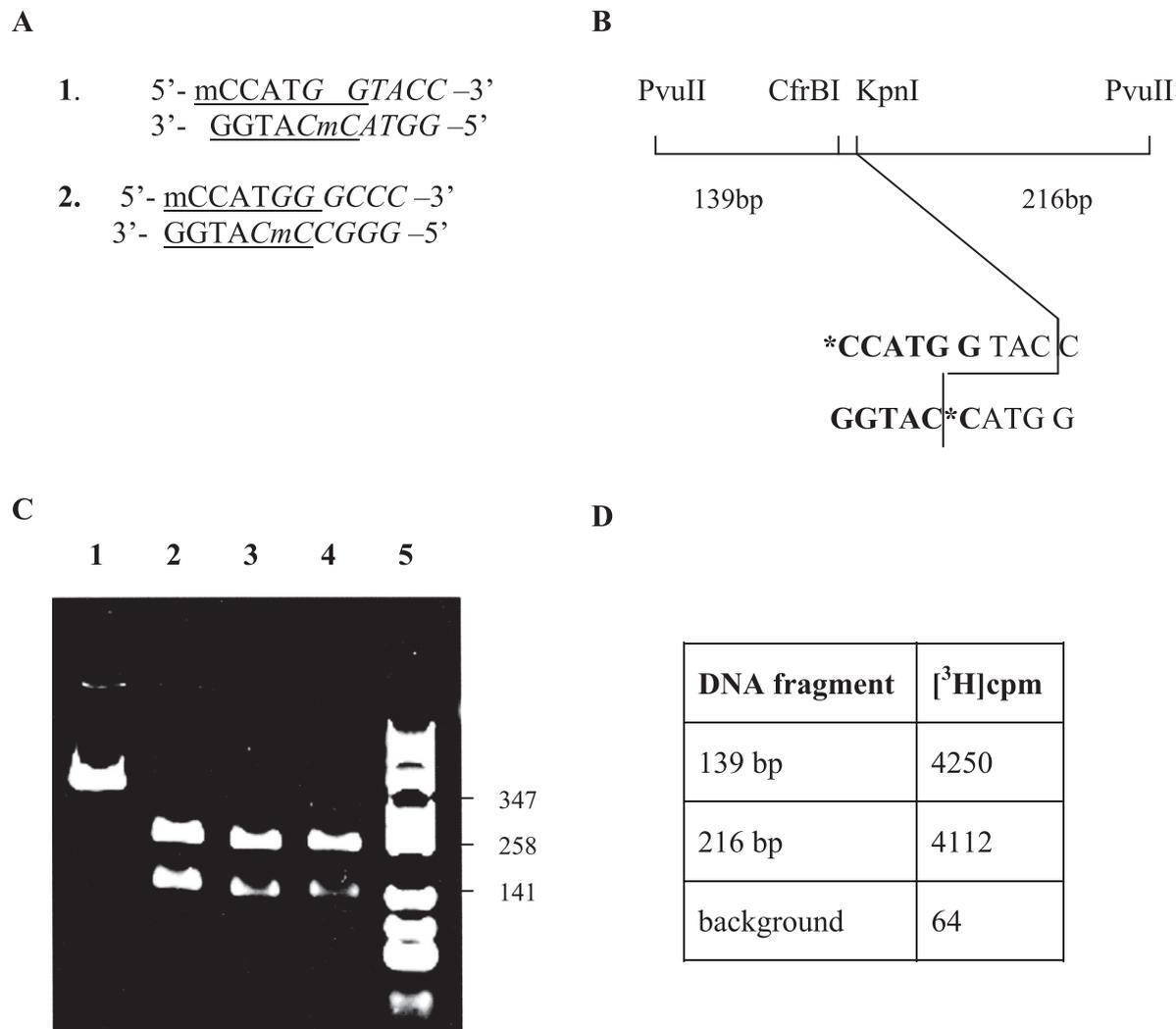
sensitive to "non-canonical" methylation of their recognition sites [21]. To date, the sensitivities of KpnI and ApaI to ^{N4m}C methylation have not been fully investigated. However, it has been reported [22, 23, 24, 25] that ApaI is sensitive to ^{5m}C-methylation of external or internal C within the recognition sequence (GGG^{5m}CCC or GGGCC^{5m}C), but, to our knowledge, the sensitivity to ^{N4m}C-methylation has not been the subject of a specific study. Although restriction by KpnI is not inhibited by ^{5m}C-methylation of external or internal C residues (GGTA^{5m}CC or GGTAC^{5m}C), it is blocked by ^{N4m}C-methylation of internal or external C within the recognition sequence (GGTA^{N4m}CC or GGTAC^{N4m}C) [26].

Methylation *in vivo* of the CfrBI site by M.CfrBI occurred when cells carrying pCK or pCA were grown in the presence of the compatible plasmid pXB4. The plasmid pXB4 carried the native *cfrBIM* gene and provided constitutive synthesis of M.CfrBI [10]. The total plasmid pCA + pXB4 and pCK + pXB4 DNAs were digested by appropriate ENases (Fig. 4). KpnI could digest hemimethylated DNA of the KpnI site (Fig. 4, lane 7). In contrast, ApaI did not cleave its hemimethylated site (data not shown). It should be noted that a hemimethylated GGTA^{N4m}CC target sequence cut by KpnI is slower than its unmodified analogue. The above results of *in vivo* methylation allowed taking a 355-bp PvuII-PvuII DNA fragment from pCK as a model of the methylated CfrBI recognition site for *in vitro* experiments. This DNA fragment (Fig. 3A) with unique overlapping CfrBI and KpnI recognition sites was excised and purified.

The CfrBI site was methylated by M.CfrBI *in vitro* through introduction of labelled methyl groups with the use of a SAM [3H] methyl donor. Cleavage of the DNA PvuII fragment with KpnI led to the formation of two fragments 139 bp and 216 bp in length (Fig. 3B). The 139-bp DNA fragment contained three C residues each of which being potential targets for M.CfrBI. The 216-bp DNA fragment had a single C that could be methylated by M.CfrBI. Therefore, when external C bases underwent methylation, the joined [3H] methyl groups of the longer and shorter fragments should be at a ratio of 1:1. Methylation of the internal C residues meant the joining of all [3H] methyl groups only to the 139-bp DNA fragment. As it follows from Fig. 3C and D, both DNA fragments had an approximately equal content of [3H] methyl groups. This allows the conclusion to be reached that the external C residues of the CfrBI recognition site are methylated by M. CfrBI.

During the determination of M.CfrBI we were also able to conclude that M.CfrBI-induced hemimethylation of the internal C residues of the ApaI recognition sequence (GGGC^{N4m}CC) blocked ApaI cleavage (data not shown). In contrast, KpnI could digest its M.CfrBI-hemimethylated site (GGTA^{N4m}CC) (Fig. 3C).

Thus we have demonstrated in this paper that CfrBI endonuclease cleaves DNA after the first 5′-cytosine within the recognition sequence, generating 4-nt 3′ cohesive ends. The external cytosine of the M.CfrBI recognition sequence 5′-^{N4m}CCWWGG-3′was established to be the target

A

1.   5'- mCCAT*G   GTACC* –3'
      3'-   *GGTAC*mCATGG –5'

2.   5'- mCCAT*GG GCCC* –3'
      3'-   *GGTAC*mCCGGG –5'

B

PvuII   CfrBI KpnI   PvuII

139bp   216bp

***CCATG G** TAC C*

GGTAC ***C**ATG G*

C

1   2   3   4   5

347
258
141

D

DNA fragment	[^{3}H]cpm
139 bp	4250
216 bp	4112
background	64

Fig. 3. Localization of the C bases methylated by M.CfrBI at the CfrBI site. A. DNA sequence with unique overlapping sites for: 1. CfrBI and KpnI in pCK; 2. CfrBI and ApaI in pCA. CfrBI site is underlined. KpnI and ApaI sites are in italics. The small *m* shows C in a sequence of the CfrBI site that is the target for the action of M.CfrBI. B. Map of the 355-bp PvuII fragment. The fragment contains the sequence with overlapping CfrBI and KpnI cutting sites. The CfrBI site is bolded. Subsequent cleavage of the PvuII fragment by KpnI separates specific segments of the CfrBI recognition sequence, as shown in the enlarged sequence, and leads to the formation of DNA fragments 139 bp and 216 bp in length. The connected vertical lines represent the staggered cut by KpnI. C. Electrophoregram of cleaved products that were run in 2.5% agarose gel. Lanes: 1- PvuII fragment is methylated by M.CfrBI and digested by CfrBI; 2 - nonmethylated PvuII fragment is digested by CfrBI; 3 - nonmethylated PvuII fragment is digested by KpnI; 4 - methylated PvuII fragment is digested by KpnI; 5 - R.Sau3A digested pUC19. DNA fragments in lane 4 were individually cut out from 2.5% low-melting agarose gel and DNA samples were counted in a liquid scintillator cocktail. The labeled methyl-group content of the fragments is shown in cpm in Table D. The deduced M.CfrBI methylation cytosines are indicated by asterisks in the enlarged sequence on panel B.

for methylation by M.CfrBI.

The identification and determination of DNA methylation and the parallel description of methylated nucleotides underwent multiple stages of study over an extensive period of time. During the first stage, it was based primarily on such methods as TLC (followed by high performance liquid chromatography and mass spectrometry). This approach allowed a detailed analysis to be carried out on the quantitative methylation of the genome as a whole, but without the determination of exact nucleotide sequences.

The use of restriction endonucleases in limiting hydrolysis to enzymes sensitive to DNA methylation provided genomic analysis, but only in certain and limited areas of genomic DNA. For methylation of the C5-cytosine, the situation underwent a dramatic change with the development of the method of bisulfite conversion, which, combined with the related development of a technology to determine the primary structure of DNA, provided a genome-wide analysis of 5-methylcytosine to a resolution of one nucleotide [27, 28]. Furthermore, since 2008, this technology has allowed researchers to create the first full-genome maps of

plant and mouse DNA methylation [29, 30, 31].

In connection with the development of the methodology for sequencing Single Molecules in Real Time (SMRT), it only became possible to determine the positions of any type of nucleotide methylation in complete genomes in 2010 [32]. The latter led to a new view of the role of DNA methylation on the bacteria present in defense mechanisms, with relation to cell division, gene expression and DNA repair [33]. Liu with coauthors [34] used single-wall carbon nanotubes to selectively detect modified 5-hydroxymethylcytosine in single-stranded DNA, which can be used to screen specific genomic DNA. The proposed strategy can be extended to many other useful applications in the field of chemistry and biology [35]. Despite the rapid development of the technologies described above, the exact determination of nucleotides and the nature of methylation remain an ongoing issue. It is not always necessary in every case to employ the latest technology; this is especially true when it comes to a specific RMS or DNA modification within a particular fragment. The approach we used to determine a modified base in the CfrBI site is a confirmation of this.

Fig. 4. Resistance of the recombinant plasmids pXB4 and pCK to the action of CfrBI and KpnI. pXB4 contains a unique CfrBI site. The plasmid DNAs were prepared from *E.coli* K 802 cells. These DNAs were treated with 10 units of respective ENase in a 20 µl reaction mixture at 37 °C for 1 h. The cleavage products were analyzed by 1% agarose gel electrophoresis. Lanes: 1 - pXB4; 2 - pXB4/KpnI; 3 - pXB4/CfrBI; 4 - pCK/KpnI; 5 - pCK/CfrBI; 6 - pCK; 7 - [pXB4 + pCK] cut with KpnI; 8 - [pXB4 + pCK] cut with CfrBI.

Declarations

Author contribution statement

Marina V. Zakharova, Irina V. Beletskaya, Elena M. Ibryashkina: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Alexander S. Solonin: Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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