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The Second Methyltransferase of the BstF5I Restriction–Modification System Is Homologous to the C-Terminal Domains of FokI and StsI Methylases

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The bstF5IM-2 gene for the second DNA methyltransferase of Bacillus stearothermophilus F5 (M.BstF5I-2) was cloned and sequenced. On the chromosome, the gene is located downstream of the gene for M.BstF5I-1 and is oriented similarly. The protein encoded has conserved regions specific for adenine methylases of subclass D12. M.BstF5I-2 is homologous to the C-terminal domains of M.FokI and M.StsI and recognizes the same sites. M.BstF5I-1 modifies the DNA upper strand and M.BstF5I-2 modifies the other strand in the recognition site.

INTRODUCTION

Site-specific DNA methyltransferases (methylases, M.) are common in prokaryotes. Together with restriction endonucleases, they form a restriction–modification system which protects bacterial cells from foreign DNA. Methylases transfer a methyl group from S-adenosylmethionine to a nucleotide of the recognition sequence, to produce N6-methyladenine, C5-methylcytosine, or N4-methylcytosine and are accordingly classed into three groups. As revealed by comparison, enzymes of the same group share several conserved motifs. Thus ten motifs have been found in adenine methylases [1, 2]. Based on their composition and arrangement, these enzymes are divided into three subgroups—D₁₂, D₂₁, and N₁₂ [3] — which also appreciably differ in spatial structure.

Most adenine methylases recognize palindromic DNA sequences. One such enzyme is sufficient for cell protection, as it modifies both DNA strands in the recognition site. When nonpalindromic sequences are recognized, a restriction–modification system includes two methylases, each modifying only one DNA strand in the recognition site. Homologous systems FokI [4] and StsI [5] are exceptions: they each include one methylase which consists of two domains each having a complete set of D₁₂ conserved motifs [6]. The domains are of 300–350 amino acid residues, thus being comparable in length with other DNA methylases. DNA fragments encoding the domains M.FokIN and M.FokIC of the FokI methylase have been cloned. They are expressed to produce two functional methylases, each modifying only one strand in its recognition site [7]. Hence the FokI methylase gene can be assumed to result from fusion

of two methylase genes [8].

We previously found *Bacillus stearothermophilus* strain F5 producing a restriction endonuclease which, like nucleases FokI and StsI, recognized 5'-GGATG-3'. However, the cleavage site was 2/0 with BstF5I, 9/13 with FokI, and 10/14 with StsI [9]. We also cloned the gene for M.BstF5I-1 and showed that this enzyme is a D₂₁ methylase nonhomologous to any domain of M.FokI and M.StsI, which belong to the D₁₂ subgroup [10]. M.BstF5I-1 methylated an adenine on the upper strand in the recognition site, thus being similar to the N domains of M.FokI and M.StsI.

The objectives of this work were to clone the gene and to analyze the primary structure of M.BstF5I-2, another methylase of the BstF5I restriction–modification system.

EXPERIMENTAL

To obtain a genome library, *B. stearothermophilus* F5 DNA was partly digested with AluI. The fragments were ligated into the SmaI site of pMTL22. The resulting DNA was used to transform *Escherichia coli* RR1. Plasmid pool was isolated and treated with BstF5I. Transformation of *E. coli* RR1 with the hydrolysis products yielded two clones with plasmids resistant to BstF5I. Restriction mapping showed that the plasmids were identical and contained an insert of about 1200 nt. The plasmid was designated pF521. The insert was sequenced by the Maxam–Gilbert method.

Analysis of homology and alignment of nucleotide and amino acid sequences were performed with the FASTA and ALIGN programs of the FASTA 2.0 package [11]; multiple alignment was done with the Clustal W 1.7 program [12]. The spatial structure of M.DpnM (available at <http://www.rcsb.org/pdb/>, PDB Id: 2DPM) was viewed and analyzed with the Quick-PDB 1.1 JAVA applet (I. Shindyalov, P. Bourne).

Gene engineering manipulations followed the published protocols [13].

The DNA preparations, enzymes, and oligonucleotides were from SibEnzyme (Novosibirsk).

RESULTS AND DISCUSSION

Nucleotide and Amino Acid Sequences of M.BstF5I-2

We previously cloned the Ksp22I fragment of *B. stearothermophilus* F5 DNA containing the M.BstF5I-1 gene [10]. The AluI fragment cloned in this work had a 119-nt overlap with the Ksp22I fragment. The total chromosome region sequenced was 3363 nt in size (Fig. 1). Nucleotide sequence analysis revealed an open reading frame (ORF) at position 2197–3081 and the Shine–Dalgarno signal sequence (Fig. 2). The absence of BstF5I restriction sites indicates that the ORF codes for a site-specific DNA methyltransferase.

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Fig. 1. Location of the genes for BstF5I methylases in the sequenced fragment of the bacterial chromosome.

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Fig. 2. Structure of the spacer between *bstF5IM-1* and *bstF5IM-2*. The start codon and the Shine–Dalgarno sequence (SD) of *bstF5IM-2* are in bold.

The M.BstF5I-2 gene (*bstF5IM-2*) is downstream of *bstF5IM-1* (Fig. 1). The genes are oriented similarly and separated by a 204-nt spacer. The spacer contains a tandem repeat (units of 33 and 34 nt; of these, 31 are identical), an inverted repeat (8-nt units), and a 32-nt AT-rich sequence (Fig. 2). The role of these elements in transcription and translation of the genes the operon for the BstF5I restriction–modification system is still unclear. A combination of the inverted repeat and the AT-rich sequence resembles Rho-independent terminators but has less GC in the repeat; this element may act as a transcription attenuator.

The homology between *bstF5IM-2* and the genes for the C domain of M.FokI and M.StsI was 61.6 and 59.6%, respectively. M.BstF5I-2 had all conserved motifs characteristic of D₁₂ methylases. Its amino acid sequence had 54.0% identity and 79.8% similarity with M.FokIC, and 49.7% identity and 80.7% similarity with M.StsIC (Fig. 3). On the strength of these data, we assumed that M.BstF5I-2 modifies an adenine in the lower strand of the recognition site.

Collation of the Amino Acid Sequences of DNA Methylases with the Spatial Structure of M.DpnM

The first DNA-methylase spatial structure has recently been published for D₁₂ DpnM adenine methylase recognizing 5'-GATC-3' [1]. The DpnM molecule is C-shaped, with the ends embracing doublestranded DNA. Its two domains are linked together by two α -helical bridges. The large domain forms a cavity for methylase cofactor S-adenosylmethionine and an active center.

As shown with amino acid sequence alignment, M.BstF5I-2, its isoschizomers M.FokIC and M.StsIC, and M.DpnM are similar in size and in the relative position of all conserved motifs (Fig. 3). This suggests similarity in the spatial arrangement of structural elements, i.e., in the tertiary structure of the

entire molecule. The secondary structure elements (α -helices and β -sheets) and motifs conserved among methylases of this type are indicated in Fig. 3. Structural elements of all methylases are termed as accepted for M.DpnM.

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Fig. 3. Aligned nucleotide acid sequences of M.BstF5I-2, M.FokIC, and M.StsIC correlated with the M.DpnM sequence and secondary structure elements (shadowed bars, α -helices; shadowed arrows, β -sheets). The motifs conserved among DNA methyltransferases (single lines, designations as in [1]) and the putative TRD (double line) are indicated above the sequences. The M.DpnM residues interacting with the cofactor and the substrate adenine are in bold. The M.DpnM residues with unknown spatial arrangement are in small letters. Amino acid residues (*) identical, (:) functionally similar, and (.) functionally compatible in the first three and all methylases are indicated in lines 4 and 6, respectively.

The four enzymes do not have homologous regions other than the known conserved motifs and the target recognition domain (TRD), as reported earlier. A higher homology is characteristic of regions forming the core of the large domain (with the cavity for the cofactor) and the active center (α A, β 1, α B, β 2, β 4, α I, β 5, and β 7).

In addition, the enzymes must have elements determining site-specific interaction with DNA. One of these elements is TRD located in the small domain between α F and α G and forming a loop protruding outward. Presumably, site-specific DNA binding also requires two flexible loops enriched in serine, threonine, arginine, and lysine residues and located between β 4 and α I and between β 6 and β 7 in the large domain [1]. We compared the sequences of these loops in the four enzymes. The three isoschizomers modify the adenine in the 5'-CATCC-3' site. Most probably, their regions involved in site-specific interaction with DNA are homologous but differ from those of M.DpnM. This was observed for amino acid residues 198–208 (a loop between β 4 and α I) of M.BstF5I-2: this region was nearly identical to the corresponding regions of M.FokIC and M.StsIC but showed no homology to that of M.DpnM. Though less clearly, the same was also seen with TRD and region 276–281 (a loop between β 6 and β 7). Surprisingly, region 8–13 proved highly homologous in the three isoschizomers. All these regions are not classed with the standard conserved motifs, and their similarity in amino acid sequence and arrangement may implicate them in sitespecific interaction with DNA. As *B. stearothermophilus* F5 is thermophilic, its enzymes are thermostable, suggesting certain structural features of M.BstF5I-2. In amino acid composition, M.BstF5I-2 is characterized by a higher cysteine content: its molecule includes six cysteine residues, whereas M.FokIC has three and M.StsIC has none. Correlation with the M.DpnM spatial structure showed that four residues (Cys-42, Cys-45, Cys-58, and Cys-190) of M.BstF5I-2 are in regions located close together and forming the cavity for S-adenosylmethionine. Hence the enzyme thermostability can be attributed to cysteine bridging.

Amino Acid Sequence of Putative DNA-Binding Regions in Methylases of the M.DpnM Group

We compared the regions possibly involved in interacting with DNA (see above) in methylases of the M.DpnM group (Fig. 4). Recent data were also considered, and the recognition sites of M.FokIC and M.StsIC erroneously indicated in [1, 2] were corrected. The highest homology was characteristic of TRD (Fig. 4a). Possibly, this region interacts with 5'-(G/C)AT-3' common for all recognition sites. Substrate binding is due to the interaction of positively charged amino acid residues of TRD and negatively charged phosphate groups of the DNA backbone [1].

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Fig. 4. Amino acid sequences of putative DNA-binding regions in methylases of the M.DpnM group, subclass D₁₂. The recognition sites are indicated on the right, the bases modified are in capital letters. The residues conserved among most enzymes are in bold. Conserved regions and sequence homology are indicated as in Fig. 3.

Sequences of the N-terminal region of DNA methylases are shown in Fig. 4b. The N-terminal peptide with YIKSPLNY, which is homologous in the three FokI isoschizomers, is known to vary in CATCC-recognizing enzymes. As shown in experiments on subcloning the M.FokIC gene, a shortened protein (amino acid residues 369–647) has no enzymic activity, whereas a larger protein (335–647) methylates DNA [7]. M.BstF5I-2 has only 12 residues more than the homologous inactive protein but possesses the enzymic activity. Possibly, this fragment (in particular, YIKSP identical in the three isoschizomers) is important for the DNA-modifying activity, though it has not been included in the conserved motif X [1, 2]. According to the spatial model, the corresponding region of M.DpnM plays a structural role, rather than being involved in DNA binding or catalysis. However, DNA binding can change the conformation of individual protein regions, and we think that the above element participates in the interaction of M.BstF5I-2, M.FokIC, and M.StsIC with the substrate. Possibly, the element enters the neighboring DNA groove, which facilitates sequence recognition.

We analyzed the amino acid composition of methylase regions corresponding to the M.DpnM loops located between β_4 and α_1 and between β_6 and β_7 (Fig. 4c,d). Structural analysis has failed to establish the spatial arrangement of atoms in the amino acid residues of these loops, suggesting their flexibility. Indeed, the loops mostly consist of residues with a high local flexibility (G, D, S, P, N, T, K A). The amino acid composition of these regions varies with recognition sequence of enzymes (excluding T4 M.T4Dam which differs from other methylases recognizing 5'-GATC-3'). On the strength of the primary structure data, we assumed that the regions shown in Figs. 4b–4d are involved in recognizing the nucleotides flanking the central 5'-AT-3' site. The two *B. stearothermophilus* methylases together modify both DNA strands at 5'-GGATG-3' sites, which is sufficient for the function of the restriction–

modification system. M.BstF5I-1 and M.BstF5I-2 belong to the subgroups D₂₁ and D₁₂, respectively. As assumed previously, BstF5I restriction nuclease is evolutionarily related to nucleases of other *B. stearothermophilus* strains, rather than of the FokI or StsI restriction–modification systems [9]. M.BstF5I-1 is not homologous to M.FokI and M.StsI, which favors this assumption. However, the high homology of M.BstF5I-2 to M.FokI and M.StsI poses again the problem of the origin and relations of the BstF5I restriction–modification system with the FokI and StsI systems. How did the two different methylases arise in evolution? Did M.BstF5I-2 originate from M.FokIC or independently? Further studies of the M.BstF5I-2 enzymic activity will elucidate these problems.

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