

A novel site-specific endonuclease Glul recognizes methylated DNA sequence 5'-G(5mC)^NG(5mC)-3'/ 3'-(5mC)GN^(5mC)G

Category: [New enzymes](#)

Hits: 10366

V.A. Chernukhin, E.V. Chmuzh, Yu.E. Tomilova, T.N. Nayakshina, D.A. Gonchar, V.S. Dedkov, S.Kh. Degtyarev

Translated from "Ovchinnikov bulletin of biotechnology and physical and chemical biology" V.3, No 2, pp 13-17, 2007

A novel site-specific endonuclease Glul from the bacterial strain GL24 has been isolated and characterized. The enzyme recognizes methylated DNA sequence 5'-G(5mC)^NG(5mC)-3'/3'-(5mC)GN^(5mC)G-5' and cleaves it as it is shown by arrow. Due to its ability to cleave only modified DNA Glul may be useful for genetic engineering experiments as well as for determination of DNA methylation status in eucaryotes.

INTRODUCTION

Only four site-specific endonucleases are currently known to cleave only methylated DNA, do not require cofactors besides Mg^{2+} ions and, therefore, can be considered to be IIM site-specific endonucleases [1]. These are restriction endonuclease DpnI, which recognizes and cleaves the nucleotide sequence 5'-G(6mA)↓TC-3' [2], and site-specific endonucleases BslI, Glal and BslI described in our works. The latter ones recognize and cleave DNA sequences 5'-G(5mC)↓NGC-3' [3], 5'-G(5mC)↓G(5mC)-3'/3'-(5mC)G↓(5mC)G-5' [4], [5] and 5'-G(5mC)N↓GC-3' [6], respectively.

The present paper describes a new representative of this group, enzyme Glul, which is an isoschizomer of the site-specific endonuclease BslI [3] but requires more methylated cytosines in the recognition site for its activity to appear. Enzyme Glul recognizes the DNA sequence 5'-G(5mC)↓NG(5mC)-3'/3'-(5mC)GN↓(5mC)G-5' and cleaves it as indicated by arrows producing 5'-protruding ends.

EXPERIMENTAL CONDITIONS

The producer strain growth

The strain was grown in the fermenter at the temperature 30°C in 20 L of nutrient medium containing 1% Trypton (Organotechnie, France), 0.5% yeast extract (Organotechnie, France), 0.5% NaCl and 0.05% MgCl₂, at pH 7.5 with aeration at 10 L/min and stirring at 200 rev/min. When the culture reached the late logarithmic growth stage, the cells were collected by centrifugation and 100 g of biomass was obtained and kept at -20°C.

Enzyme isolation

All procedures of enzyme isolation were performed at the 4°C. 100g of frozen cells were suspended in 250 ml of Buffer A (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 0.01 % Triton X-100) containing 0.05 M NaCl, 0.1 % Triton X-100, 0.1 mg/ml of lysozyme and 1 mM phenyl methyl sulphonyl fluoride (PMSF). The cells were disrupted by ultrasonic disintegrator Soniprep 150 (MSE, England) 10 times 1 min each at 1 min intervals to cool the suspension.

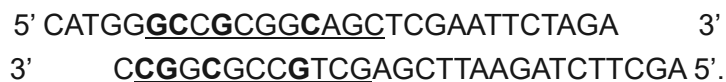
The crude lysate was clarified by centrifugation for 30 min at 12,000g on a J-2-21 centrifuge (Beckman, USA). Enzyme preparation was obtained by chromatographic purification of the extract on the following sorbents: 100 ml of phosphocellulose P-11 (Whatman, England), 15 ml hydroxyapatite (Bio-Rad, USA), 4 ml of heparin-sepharose (Bio-Rad, USA), and 10 ml hydroxyapatite (Bio-Rad, USA).

The supernatant was initially applied to a P-11 phosphocellulose column pre-equilibrated with Buffer A containing 0.05 M NaCl. The column was washed with 200 ml of Buffer A with 0.05 M NaCl, then with 1,000 ml of a linear gradient of the NaCl concentration (0.2M-0.95M) in Buffer A, 10 ml-fractions were collected. Endonuclease-containing fractions were combined and applied to a hydroxyapatite column pre-equilibrated with Buffer B (5 mM KH₂PO₄, pH 7.2, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 0.01 % Triton X-100) containing 0.02 M NaCl. The column was washed with 30 ml of Buffer B containing 0.02 M NaCl, then the protein was eluted by a linear gradient of the KH₂PO₄ concentration (0.005M-0.2M) in 400 ml of Buffer B, and 10 ml-fractions were collected. Active fractions were combined, dialyzed against 20 volumes of Buffer A with 0.02 M NaCl and applied to a heparin-sepharose column equilibrated with Buffer A containing 0.2 M NaCl. The column was washed with 8 ml of Buffer A containing 0.2 M NaCl. The enzyme was eluted by a linear gradient of NaCl concentration (0.2M–0.9M) in 120 ml of Buffer A, and 3 ml-fractions were collected. Fractions containing the enzymatic activity were combined and applied to a hydroxyapatite column pre-equilibrated with Buffer B containing 0.02 M NaCl. The column was washed with 20 ml of Buffer B containing 0.02 M NaCl. The adsorbed material was eluted by the linear gradient of concentration KH₂PO₄ (0.005M-0.17M) in 320 ml of Buffer B, and 6.4 ml-fractions were collected. Active fractions were combined and dialyzed against a concentrating buffer (50% glycerol, 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 0.01 % Triton X-100, 0.1 M NaCl). The enzyme preparation was stored at -20°C.

Enzyme activity determination

The DNA of plasmid pFsp4HI3, a derivative of plasmid pFsp4HI2, was used as a substrate to determine the activity of Glul [7]. Plasmid pFsp4HI2 carries the gene of Fsp4HI DNA-methyltransferase from *Flavobacterium* sp. 4H, which modifies the first cytosine base in the nucleotide sequence 5'-GCNGC-3'

[7]. Thus, plasmid pFsp4HI2 contains methylated sites 5'-G(5mC)NGC-3'. Plasmid pFsp4HI3 was obtained by inserting the following synthetic oligonucleotide duplex into plasmid pFsp4HI2 at the sites HindIII and Bsp19I:



The obtained plasmid pFsp4HI3 differs from pFsp4HI2 in a four-nucleotide substitution (the corresponding nucleotides are shown in bold type within the structure of the duplex). This substitution results in the formation of the nucleotide sequence 5'-GCCGCGGCAGC-3' (this sequence is underlined within the structure of the duplex), which represents three overlapping sites 5'-GCNGC-3'. Since the plasmid includes the the M.Fsp4HI DNA-methyltransferase gene modifying all of these three sites, as a result, the nucleotide sequence 5'-G(5mC)GG(5mC)-3'/3'-(5mC)GC(5mC)G-5' containing 4 methylated cytosine residues and presenting a fully methylated recognition site appears in the plasmid.

As it was established separately, GluI displays the maximum of activity in a buffer of the following composition: 10 mM Tris-HCl, pH 9.0, 7.5 mM MgCl₂, 75 mM NaCl, 1 mM β-mercaptoethanol. This buffer was used in all the experiments on DNA cleavage by enzyme GluI.

The activity of the site-specific endonuclease GluI in chromatographic profiles was determined by incubating aliquots of fractions with the DNA of plasmid pFsp4HI3 linearized with restriction endonuclease BglII. 2 μl-aliquots from the corresponding chromatographic fractions were added to 10 μl of the reaction mixture containing linearized plasmid DNA in the reaction buffer. The reaction mixture was incubated for 30 min at 37°C. The reaction products were separated in 1% agarose gel.

The minimal amount of the enzyme required for complete cleavage of 1 μg of the DNA of plasmid pFsp4HI3 hydrolyzed by restriction endonuclease BglII for 1 hour at the temperature of 37° C in 50 μl of the reaction mixture was considered to be an activity unit (a.u.).

Determination of the recognition site and positions of DNA hydrolysis

Different DNAs were hydrolyzed to determine the specificity of GluI. Plasmid and phage DNAs as well as synthetic oligonucleotide DNA duplexes containing or non-containing methylated bases were used as substrates in the enzyme specificity determination.

The positions of DNA hydrolysis by the site-specific endonuclease GluI were determined by comparing the lengths of the fragments produced in the cleavage of [³²P]-labeled synthetic oligonucleotide duplexes by endonucleases GluI and BslI. The products of partial cleavage of the same duplexes by endonuclease III from *E.coli* (ExoIII) were used as fragment length markers.

DNA cleavage was performed under optimal conditions (37°C, reaction buffer – 10 mM TrisHCl, pH 9.0, 7.5 mM MgCl₂, 75 mM NaCl, 1 mM β-mercaptoethanol) for 1 hour. The cleavage products of plasmid and phage DNAs were separated by gel-electrophoresis in 1.2% agarose. DNA fragments formed as a result of the cleavage of oligonucleotide duplexes were separated by electrophoresis in denaturing 20% polyacrylamide gel (PAAG) with 7 M urea.

RESULTS AND DISCUSSION

The new enzyme Glul was isolated from an unidentified bacteria Glacial ice bacterium GL24. Glacial ice bacterium GL24 is characterized as follows:

The cells are Gram-positive, aerobic, irregular, small rods of 0.5-(1-1.5) μm , single and forming clusters, immobile and non-sporiferous. On Luria-Betrani (LB) medium, they form light-brown, small semi-transparent (1-2 mm), rounded colonies with smooth edges. The growth temperature is from 10 to 30°C. The cells produce catalase. Oxidase is not detected.

The site-specific endonuclease Glul produced by Glacial ice bacterium GL24 was isolated from cell extract by the consecutive chromatographies on sorbents as described in "Experimental conditions".

The enzyme yield was 200 a.u./g of raw biomass, and the concentration was 1,000 a.u./ml.

Glul endonuclease does not hydrolyze standard viral and plasmid DNAs used to screen and to determine the activity of restriction endonucleases such as DNAs of phages λ (dam+/dcm+ and dam-/dcm-), T7, adenovirus-2, and plasmids pUC19 and pBR322. Plasmids containing the genes of some bacterial methylases modifying cytosine bases in the DNA were also tested in DNA cleavage experiments: M.Fsp4HI methylates DNA with the formation of the sequence 5'-G(5mC)NGC-3'[7], M.HspAI modifies DNA with the formation of the sequence 5'-G(5mC)GC-3' [5], M.FauIA and M.FauIB methylate DNA with the formation of the sequences 5'-C(5mC)CGC-3' and 5'-G(5mC)GGG-3', respectively [8]. Also, no DNA hydrolysis is observed during incubation of plasmids carrying the genes of these methylases with Glul preparation. Enzyme Glul cleaves only the DNA of plasmid pFsp4HI3 obtained as described in "Experimental conditions". Due to having the methylase gene of Fsp4HI, this plasmid contains methylated sequences 5'-G(5mC)NGC-3' as well as a single fully methylated site 5'-G(5mC)NG(5mC)-3'/3'-(5mC)GN(5mC)G-5'.

Figure 1 shows the results of the linearized plasmids pFsp4HI2 and pFsp4HI3 hydrolysis by the site-specific endonuclease Glul. As it is seen from the Figure, plasmid pFsp4HI2 is fully cleaved by enzyme BslI for which the sequence 5'-G(5mC)NGC-3' is a recognition site [3]. Enzyme Glul does not cleave plasmid pFsp4HI2, however, it hydrolyzes the DNA of plasmid pFsp4HI3. The double digestions of the plasmid pFsp4HI3 by the site-specific endonuclease Glul and restriction endonucleases PciI, DseDI and BglII was performed to determine the DNA cleavage sites (Fig. 1). The comparison of the lengths of DNA fragments obtained at double digestions shows that DNA cleavage by endonuclease Glul occurs at location of the site 5'-G(5mC)GG(5mC)-3'/3'-(5mC)GC(5mC)G-5'.

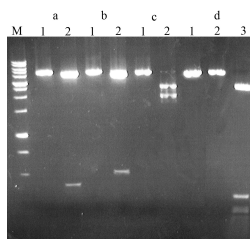


Fig. 1. Cleavage of plasmid DNA by endonucleases Glul (run 2) and BslI (run 3). a - plasmid pFsp4HI3 linearized with PciI, b - plasmid pFsp4HI3 linearized with DseDI, c - plasmid pFsp4HI3 linearized with BglII, d - plasmid pFsp4HI2 linearized with BglII, Run 1- the corresponding plasmid without hydrolysis. M – molecular weight marker of 1 Kb (SybEnzyme) with the following DNA fragment lengths (kb): 10; 8; 6; 5; 4; 3; 2.5; 2; 1.5; 1; 0.75; 0.5; 0.25.

Experiments on the cleavage of synthetic oligonucleotide duplexes containing the sequence 5'-GCNGC-3' with methylated or non-methylated cytosine bases (the sites of 5'-GCNGC-3' are underlined) were performed to confirm the above data and to determine DNA hydrolysis site:

NN01: 5' GCTTGTACTTTAGCGGCATTGATTCTCACCACG 3'
 NN02: 5' CGTGGTGAGAATCAATGCCGCTAAAGTACAAGC 3'
 NN1: 5' GCTTGTACTTTAG(5mC)GGCATTGATTCTCACCACG 3'
 NN2: 5' CGTGGTGAGAATCAATG(5mC)CGCTAAAGTACAAGC 3'
 DD1: 5' GCTTGTACTTTAG(5mC)GG(5mC)ATTGATTCTCACCACG 3'
 DD2: 5' CGTGGTGAGAATCAATG(5mC)CG(5mC)TAAAGTACAAGC 3'
 4mT: 5' GGGAAAAG(5mC)TG(5mC)AAAAGAGGAAAGGG 3'
 4mA: 5' CCCTTTCCTCTTTTG(5mC)AG(5mC)TTTTCCC 3'

As it is seen from Fig.2, endonuclease GluI cleaves duplexes DD1*/DD2 and 4mT*/4mA containing the fully methylated sequence 5'-G(5mC)NG(5mC)-3' but doesn't cleave duplex NN01*/NN02, which contains the same non-methylated sequence, as well as duplex NN1*/NN2, which contains the methylated sequence 5'-G(5mC)NGC-3'/3'-CGN(5mC)G-5'. The obtained data shows that the site-specific endonuclease GluI recognizes and cleaves only fully methylated sequence 5'-G(5mC)NG(5mC)-3'/3'-(5mC)GN(5mC)G-5'.

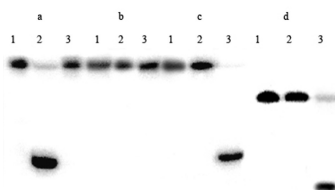


Fig. 2. Cleavage of oligonucleotide duplexes NN01*/NN02 (a), NN1*/NN2 (b), DD1*/DD2 (c) and 4mT*/4mA (d) with endonucleases Fsp4HI (run 2) and GluI (run 3). Run 1 – the corresponding duplex without hydrolysis. Labeled oligonucleotides are shown with the sign *.

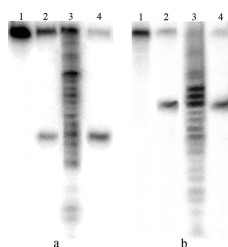
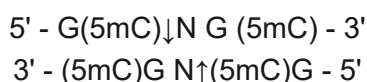


Fig. 3. The determination of DNA cleavage position by endonuclease GluI. a – duplex DD1*/DD2, b – duplex DD2*/DD1. Runs: 1 – duplex without hydrolysis, 2 – hydrolysis by endonuclease GluI, 3 –

hydrolysis by endonuclease III, 4 – hydrolysis by endonuclease Bsl. Labeled oligonucleotides are shown with the sign *.

Figure 3 shows the results of oligonucleotide duplex DD1/DD2 hydrolysis by endonucleases Glul and Bsl. The comparison of the lengths of the fragments producing in the cleavage of this substrate indicates that Glul cleaves the methylated duplex with four C5-methylcytosines similar to Bsl. Therefore, Glul cleaves the recognition site as shown by arrows:



Thus, in the present work we described the new site-specific endonuclease Glul belonging to a rare group of enzymes, which recognize and cleave only methylated DNA. The nucleotide sequence recognized by the new enzyme and the DNA hydrolysis site coincide with those for enzyme Bsl, however, unlike Bsl, Glul is inactive on substrates with two methyl groups and cleaves DNA containing a fully methylated recognition site.

Hydrolysis of fully methylated DNA is not an exclusive property of the new enzyme. It was shown earlier that endonuclease Glal, which also belongs to the group of IIM site-specific endonucleases, displays the maximum of activity on the fully methylated recognition sequence 5'-G(5mC)↓G(5mC)-3'/3'-(5mC)G↓(5mC)G-5' [5].

A possible role of enzymes, which recognize and cleave only methylated DNA, in the protection of bacteria against infection by phages having methylated DNA was discussed previously [4]. In particular, a number of bacteriophages are known to contain the genes of site-specific DNA-methyltransferases, which results in the methylation of phage DNAs [9]. However, a preferable cleavage of excessively methylated DNA by Glal and Glul endonucleases indicates that these enzymes may have some other functions in a bacterial cell.

We believe that Glul can be used for detection and analysis of methylated sites in eucaryote e DNA, which usually contain a considerable number of 5-methylcytosine bases. DNA methylation is known to play an important role in the regulation of cellular processes and needs to be investigated [10].

ABBREVIATIONS USED

PMSF – phenyl methyl sulphonyl fluoride
BSA - bovine serum albumin
ExoIII - exonuclease III from *E.coli*
PAAG – polyacrylamide gel
a.u. - activity unit

SibEnzyme Ltd.: Patent RU 2322492 C1

REFERENCES

1. Roberts R.J., Belfort M., Bestor, T., Bhagwat A.S., Bickle T.A. et al. // Nucleic Acids Res. – 2003. – V. 31. – P. 1805-1812.
2. Lacks S. and Greenberg B.J. // Biol. Chem. - 1975. - V. 250. - P. 4060-4066.
3. Chmuzh E.V., Kashirina Yu.G., Tomilova Yu.E., Mezentseva N.V., Dedkov V.S., Gonchar D.A., Abdurashitov M.A., Degtryarev S.Kh. New endonuclease restriction Bis I from *Bacillus subtilis* T30 recognizes methylated sequence DNA 5'-G(5mC)[^]NGC-3' // Biotechnology. - 2005. - 3. - P.22-26 (in Russian). [[english online version](#)].
4. Chernukhin V.A, Nayakshina T.N., Abdurashitov M.A., Tomilova Yu.E., Mezentseva N.V., Dedkov V.S., Mikhnenkova N.A., Gonchar D.A., Degtryarev S.Kh. New restriction endonuclease Glal recognizes methylated sequence 5'-G(5mC)[^]GC-3' // Biotechnology. - 2006. - 4. - P. 26-30 (in Russian). [[english online version](#)]
5. Tomilova J.E., Chernukhin V.A., Degtyarev S.Kh Dependence of site-specific endonuclease Glal activity on quantity and location of methylcytosines in the recognition sequence 5'-GCGC-3'. // Bulletin of biotechnology and physico-chemical biology V.2, No 1, pp 30-39 (2006) (Russian) [[english online version](#)]
6. Chernukhin V.A., Tomilova J.E., Chmuzh E.V., Sokolova O.O, Dedkov V.S, Degtyarev S.Kh // Biotechnology. – [[english online version](#)]
7. Chmuzh E.V., Kashirina Yu.G., Tomilova Yu.E., Chernukhin V.A., Okhapkina S. S., Gonchar D.A., Dedkov V.S., Abdurashitov M.A., Degtryarev S.Kh. . // Molecular Biology (Russian). – 2007. – V. 41. – N 1. – P. 1-9.
8. Chernukhin V.A., Kashirina Yu.G., Sukhanova K.S., Abdurashitov M.A., Gonchar D.A., and Degtyarev S. Kh. Isolation and exploration of biochemical propeties of DNA Methyltransferase FaulA modifying non-palindromic sequence 5'-CCCGC-3'// Biochemistry (Moscow), Vol. 70, No 6, (2005), 829-837. (In Russian)
9. Lange C., Noyer-Weidner M., Trautner T.A., Weiner M., Zahler S.A. // Gene. - 1991. - V. 100. - P. 213-218.
10. Costello J.F. and Plass C. // J. Med. Genet. - 2001. - V. 38. - P. 285-303.