

## A novel restriction endonuclease Glal recognizes methylated sequence 5'-G(5mC)<sup>↓</sup>GC-3'

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*A novel restriction endonuclease Glal from the bacterial strain GL29 has been isolated and characterized. The enzyme recognizes methylated DNA sequence 5'-G(5mC)<sup>↓</sup>GC-3' and cleaves it as indicated by arrow. Due to its ability to cleave only modified DNA Glal may find a practical application in genetic engineering experiments as well as in determination of DNA methylation status*

### INTRODUCTION

As a rule type II restriction - modification (RM) system includes two enzymes - restriction endonuclease (RE) and DNA methyltransferase (M., MTase). Type II RE recognizes a short DNA sequence (usually 4-8 bp) and cuts it within or nearby this recognition site. Bacterial MTase methylates the recognition sequence protecting host DNA from cleavage by a cognate restriction enzyme whereas a foreign unmethylated DNA is cleaved by RE.

However, there are several restriction endonucleases presenting in a bacterial cell without cognate DNA methyltransferases. These enzymes belong to so called IIM subtype and cleave methylated recognition sequence only. At present time there are two known restriction enzymes of this type. DpnI restriction enzyme recognizes and cleaves 5'-G(6mA)<sup>↑</sup>TC-3' [1] and BspI restriction enzymes cleaves 5'-G(5mC)<sup>↑</sup>NGC-3' [2].

In this article we describe a new restriction enzyme Glal which recognizes nucleotide sequence 5'-G(5mC)<sup>↑</sup>GC-3' and requires only Mg<sup>2+</sup> ions as a cofactor. Likewise DpnI and BspI, Glal belongs to subtype IIM restriction endonucleases [3].

### MATERIALS AND METHODS

## Strain growth

The strain was grown at 28°C in 20 l fermenter (LKB, Sweden) with aeration (10 l/min). Nutritious medium included 1% tryptone (Organotechnie, France), 0.5% yeast extract (Organotechnie, France), 0.5% NaCl and 0.05% MgCl<sub>2</sub> (pH 7.5). At a late logarithmic stage of growth bacterial cells were precipitated by centrifugation. The precipitate of wet cells was stored at -20°C.

## Purification of Glal

The enzyme purification was performed based on the column chromatography technique. 100 g of biomass were suspended in 300 ml of buffer A (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM β-mercaptoethanol) containing 0.1 M NaCl, 0.3 mg/ml lysozyme, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% triton X100. Cells were disrupted by ultrasonic treatment. The cell debris was removed by centrifugation at 12000 rpm for 30 min.

Supernatant was loaded onto 100 ml phosphocellulose P-11 ("Whatman", England) column pre-equilibrated with buffer A containing 0.1 M NaCl. The column was washed with 200 ml of buffer A containing 0.1 M NaCl. The gradient 0.1-0.65 M NaCl in buffer A (1000 ml) was applied for enzyme's elution and 10 ml fractions were collected. The elution fractions with Glal activity were pooled and dialysed against 20 volumes of buffer A. Dialysed fractions were loaded onto 15 ml heparine-sepharose ("Bio-Rad", USA) column pre-equilibrated with buffer A containing 0.05 M NaCl. The column was washed with 30 ml buffer A containing 0.05 M NaCl. Elution was performed using 0.05-0.45 M NaCl gradient in buffer A (300 ml) and 5 ml fractions were collected. The fractions with Glal activity were pooled together and were loaded onto 4 ml hydroxyapatite column pre-equilibrated with buffer containing 0.01 M K<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 0.05 M NaCl. The column was washed with 10 ml equilibrating buffer. The enzyme was eluted with 0.01-0.4 M K<sub>2</sub>HPO<sub>4</sub> gradient (100 ml) containing 0.05M NaCl, 0.1 mM EDTA, 7 mM β-mercaptoethanol. 2.5 ml fractions were collected, analyzed and those of them with the most enzymatic activity were pooled and dialysed against 20 volumes of storage buffer (50% glycerol, 10 mM Tris-HCl, pH 7.55, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 0.2 M NaCl). Purified preparation of Glal was stored at -20°C.

## Enzyme assay

A plasmid pHspAI was used as a DNA substrate for detection of Glal enzymatic activity. This plasmid has been obtained by introduction of HspAI DNA fragment carrying gene of HspAI DNA methyltransferase into pUC19 plasmid. The activity of Glal was measured in 50 µl of a reaction mixture containing pHspAI DNA. Reaction mixture included 1 µg of pHspAI plasmid DNA, 33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 1mM DTT. After incubation for one hour at 37°C the reaction was terminated by addition of 10 µl stop solution. One unit of Glal activity is determined as the amount of enzyme required to complete cleavage of 1 µg pHspAI plasmid DNA for 1 hour at 37°C in a 50 µl of a reaction mixture.

The DNA fragments were resolved by electrophoresis in 1% and 2% agarose gels.

## Determination of the recognition sequence and the cleavage position

The recognition sequence was determined based on the comparison of pHspAI digestion pattern by Glal with theoretically predicted cleavage pattern for RE recognizing 5'-GCGC-3'. The recognition sequence was confirmed by Glal cleavage of synthetic oligonucleotide duplexes.

To get the complete pHspAI DNA digestion the preparation of Glal was concentrated by Vivaspin 500 concentrator (Sartorius). High concentration Glal preparation was added to 20 µl of reaction mixture with 1 µg pHspAI plasmid DNA and incubated for 1 hour at 37°C.

The cleavage position was determined by digestion of [<sup>32</sup>P]-labeled oligonucleotides containing the modified recognition sequence 5'-GCGC-3'. Oligonucleotide fragments separation was performed by electrophoresis in 20% PAAG (Tris-borate buffer with 7M urea).

## RESULTS AND DISCUSSION

Growth of bacterial strain GL29 in a nutritious medium allows us to harvest approx. 5 g/l of cells. Glal was isolated from frozen cells by several chromatographic steps as described in "Materials and methods". The yield of Glal final preparation was 1.8 ml with concentration 100 u/ml.

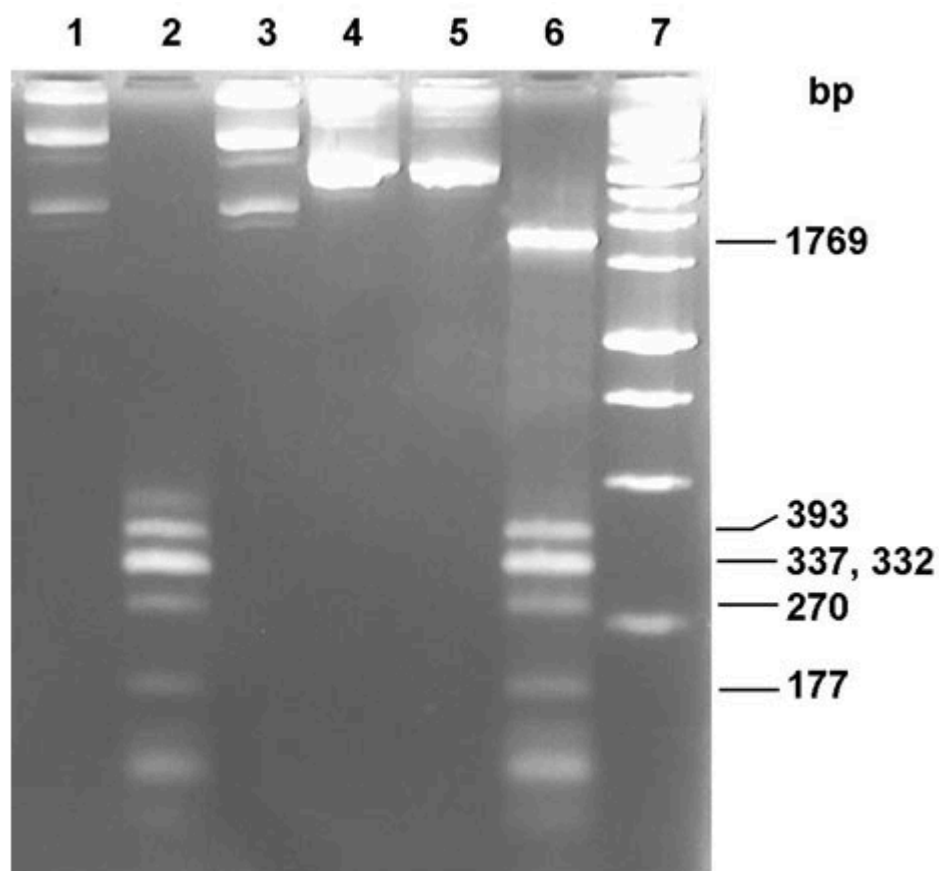
Glal doesn't cleave any standard substrates including lambda (dam<sup>+</sup>, dcm<sup>+</sup> and dam<sup>-</sup>, dcm<sup>-</sup>) and T7 phage DNAs, Adenovirus-2 DNA, pUC19 and pBR322 plasmid DNAs. We have also tested several other plasmids as possible substrates for Glal which carry genes of some DNA methyltransferases modifying the cytosine residues in definite DNA sequences: M.Fsp4HI (recognition sequence 5'-G(5mC)NGC-3'), M.FaulA and M.FaulB (recognition sequences CCCGC and GCGGG, respectively). We have not observed cleavage of these DNAs. Glal cleaves a plasmid pHspAI, which carries the gene of HspAI DNA methyltransferase (recognition sequence 5'-GCGC-3'). This plasmid was obtained by ligation of 1.4 kb EcoRI fragment of Haemophilus species A1 DNA and pUC19 vector linearized by R.EcoRI. The insertion of pHspAI comprized a gene of HspAI MTase.

A primary amino acid structure of M.HspAI deduced from the nucleotide sequence of M.HspAI gene shows a very high homology with M.HinP1I - 99% identity (Fig. 1). Since M.HinP1I modifies C5-position of inner cytosine in 5'-GCGC-3' sequence [4] we can conclude that M.HspAI methylates this site at the same position and the modified sequences 5'-G(5mC)GC-3' are present in the pHspAI DNA.

|           |                                                                        |     |
|-----------|------------------------------------------------------------------------|-----|
|           | 1                                                                      | 50  |
| M. HinP1I | MEKLKFIDFCAGIGGGRLGLELNGMECIAHSEVDLNP AKTYE IFFNDSRN                   |     |
| M. HspAI  | MEKL <u>R</u> FIDFCAGIGGGRLGLE <u>F</u> NGMECIAHSEVDLNP AKTYE IFFNDSRN |     |
|           | 51                                                                     | 100 |
| M. HinP1I | LGDLTQLAPKSLPDFDLMIAGFPCQTF SIIGKRDGFLDDR GQIIYYLSNI                   |     |
| M. HspAI  | LGDLTQLAPKSLPDFDLMIAGFPCQTF SIIGKRDGFLD <u>N</u> R GQIIYYLSNI          |     |
|           | 101                                                                    | 150 |
| M. HinP1I | LKVKKVPFFILENVKGLVNHNQGETLKSILEILE GCNYDVYYKVLDSQFY                    |     |
| M. HspAI  | LKVKKVPFFILENVKGLVNHNQGETLKSILEILE <u>S</u> CNYDVYYKVLDSQFY            |     |
|           | 151                                                                    | 200 |
| M. HinP1I | GTPQMRERIYFVGIRKDIHHSYVFPEPKSNNGNELSNYLIGDGKYKFLI                      |     |
| M. HspAI  | GTPQMRERIYFVGIRKDIHHSYVFPEPKSN <u>N</u> ENELSNYLIGDGKYKFLI             |     |
|           | 201                                                                    | 250 |
| M. HinP1I | NKTFENYINNKYNKDRISLDQIYEKNYQVIDWRQSDIRIYENKIPTLR TG                    |     |
| M. HspAI  | NKTFENYINNKYNKDRISLDQIYEKNYQVIDWRQSDIRIYENKIPTLR TG                    |     |
|           | 251                                                                    | 300 |
| M. HinP1I | RHGILYVKDNQLYKLSGYEALLQGFPKEIAQKAIDYKLPDSKILSQAGN                      |     |
| M. HspAI  | RHGILYVKDNQLYKLSGYEALLQGFPKEIAQKAIDYKLPDSKILSQAGN                      |     |
|           | 301                                                                    | 322 |
| M. HinP1I | AMTVNVIQ EIAEKLLISINKGK                                                |     |
| M. HspAI  | AMTVNVIQ <u>K</u> IAEKLLISINKGK                                        |     |

**Fig. 1.**Alignment of HspAI and HinPI DNA methyltransferases primary structures. Identity is about 99%. Identical residues are given in bold.

The DNA fragments pattern produced by Glal cleavage of pHspAI DNA corresponds to the predicted one for pHspAI cleavage by RE cutting a recognition sequence 5'-GCGC-3' (Fig 2). These data indicate that the enzyme Glal recognizes and cleaves nucleotide sequence 5'-G(5mC)GC-3'.



**Fig. 2.** Glal and HspAI cleavage of plasmid DNAs.

Lanes: 1 - pUC19 plasmid DNA, 2 - pUC19 plasmid DNA incubated with HspAI, 3 - pUC19 plasmid DNA incubated with Glal, 4 - pHspAI plasmid DNA, 5 - pHspAI plasmid DNA incubated with HspAI, 6 - pHspAI plasmid DNA incubated with Glal, 7 - DNA fragment size marker 1Kb (SibEnzyme). Theoretical lengths of pHspAI fragments produced by cleavage at 5'-GCGC-3' sites are shown at right. Electrophoresis has been run in 2% agarose gel

For final verification of Glal recognition site and its cleavage position determination we have used synthetic oligonucleotide duplexes containing or not containing modified cytosine residues (5'-GCGC-3' sites are underlined):

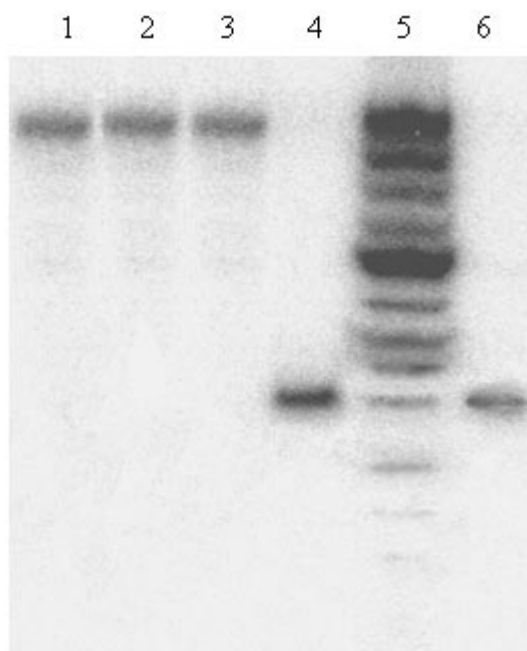
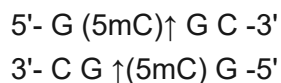
- 1: 5'-GTGCATGG(5mC)GCCTATGCG-3'
- 2: 5'-CGCATAGG(5mC)GCCATGCAC-3'
- 3: 5'-GTGCATGGGCGCCTATGCG-3'
- 4: 5'-CGCATAGGGCGCCATGCAC-3'

The results of these oligonucleotide duplexes cleavage are shown on Fig. 3. As expected C5-modified duplex 1/2 is cut with Glal, whereas unmethylated duplex 3/4 isn't cut.

Comparison of oligonucleotides cleavage by Glal and PspN4I (recognition sequence 5'-GGN<sup>N</sup>NCC-3')

clearly indicates that Glal and PspN4I cleave DNA in the same way.

Hence, Glal recognizes and cleaves the following sequence as indicated by the arrows:



**Fig. 3.** The cleavage of oligonucleotide duplexes with Glal and PspN4I restriction endonucleases. Labeled chains in duplexes are marked with \*. Lanes: 1 - duplex 3\*/4, 2 - duplex 3\*/4 incubated with Glal, 3 - duplex 1\*/2, 4 - duplex 1\*/2 incubated with Glal, 5 - duplex 1\*/2 incubated with Exonuclease III (marker of DNA fragment length), 6 - duplex 1\*/2 incubated with PspN4I

A detailed study of Glal ability to cleave sites with different methylation patterns will be presented in other publications.

So, in this work we present a novel RE Glal belonging to a rare group of restriction enzymes which recognize a methylated nucleotide sequence only. The known members of this group are listed in Table I. A comparison of presented data shows that the recognition sequences of all three enzymes share an obvious similarity: i) the second nucleotide in recognition sites is modified and ii) the cleavage occurs after methylated base. The recognition sequences of BslI and Glal differ only by one indefinite nucleotide (N) located in the middle of the site. Besides, Glal and DpnI cut recognition sequences producing blunt ends whereas Glal and DpnI analogues (recognizing the same but unmethylated site) cut DNA producing sticky ends.

| Restriction enzyme | Recognition site with cleavage position | Similar REs which recognize unmethylated sites |
|--------------------|-----------------------------------------|------------------------------------------------|
| DpnI               | G(6mA)↑TC                               | MboI - ↑GATC, ChaI - GATC↑, BstKTI - GAT↑C     |
| BisI               | G(5mC)↑NGC                              | Fnu4HI - GC↑NGC, BthCI - GCNG↑C                |
| GlaI               | G(5mC)↑GC                               | HhaI - GCG↑C, HlnPII - G↑CGC                   |

**Table I.** Recognition sites of known members of type IIM restriction enzymes

The similarity of DpnI, BisI and GlaI recognition sites may reflect the common evolutionary origin of known type IIM restriction enzymes.

As it was mentioned earlier [2] a role of restriction enzymes specific for methylated DNA may be considered as a protection of bacterial cell from penetration of modified bacteriophage's DNA. It's well known that some bacteriophages contain genes of DNA-methyltransferases and these enzymes activity result in phage DNA methylation [5].

GlaI may be useful for determination of eukaryotic DNA methylation status because its recognition site contains methylated CG sequence specific for eukaryotic genomes.

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**SibEnzyme Ltd.: Patent RU 2287012 C1**

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