

A new methyl-directed site-specific endonuclease Krol recognizes and cuts DNA sequence 5'-G[↑]C(5mC)GGC-3'

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We have discovered and purified a new methyl-directed site-specific DNA endonuclease Krol from bacterial strain Kocuria rosea 307. Krol recognizes and cuts DNA sequence 5'-G[↑]C(5mC)GGC-3'/3'-CGG(5mC)C[↓]G-5' and doesn't cleave unmethylated DNA. A new enzyme cleaves both strands of the recognition sequence if one or two central cytosines are methylated. Krol is a first methyl-directed site-specific DNA endonuclease that recognizes the non-degenerate hexanucleotide sequence. Due to its ability to cleave only modified DNA Krol may find a practical application in genetic engineering experiments as well as in determination of DNA methylation status.

INTRODUCTION

Type II restriction endonucleases are the most known and well studied enzymes among all site-specific DNA endonucleases. As a rule the restriction endonuclease (RE) and corresponding DNA-methyltransferase form restriction-modification system which protects bacterial cell from penetration of a foreign DNA. REase cleaves a foreign DNA at a short recognition site, whereas cognate MTase modifies the same sequence in host DNA protecting it against RE digestion.

Methyl-directed site-specific endonucleases (MD endonucleases) hydrolyze only methylated DNA and their biochemical properties are similar to the restriction endonucleases ones. At first the enzymes that recognize and cut DNA sequence 5'-G(m6A)TC-3' were discovered [1]. MD endonucleases which cleave the sites with 5-methylcytosines were described quite recently [2-5]. These enzymes recognize definite DNA sequences with 5-methylcytosines, require only Mg²⁺ ions as a cofactor, hydrolyze DNA in the same way as restriction endonucleases [6]. MD endonuclease Glal recognizes and cuts DNA sequence 5'-Pu(5mC)[↑]GPy-3'/3'-PyG[↓](5mC)Pu-5' [2], [6]. Site-specific endonucleases BlnI [3], BlnI [4] and Glul [5] cleave the sequence 5'-GCNGC-3' with fully or partially methylated cytosines in the recognition site.

The present work describes a new methyl-directed site-specific endonuclease Krol recognizing DNA sequence 5'-G $\uparrow$ CCGGC-3'/3'-CGGCC $\downarrow$ G-5' with one or two 5mC in central dinucleotide 5'-CG-3'/3'-GC-5'.

MATERIALS AND METHODS

The producer strain growth. The strain was grown in a fermenter 1601-013 (LKB, Sweden) at 30°C in 10 L of nutrient medium containing 1% Tryptone (Organotechnie, France), 0.5% yeast extract (Organotechnie, France), 0.5% NaCl, and 0.05% MgCl₂, pH 7.5, with aeration at 10 L/min and stirring at 200 rev/min. At a late logarithmic stage of growth bacterial cells were precipitated by centrifugation. Obtained pellet of cells (100 g) was stored at -20°C.

Enzyme isolation. All procedures of enzyme isolation were performed at 4°C. Twenty grams of frozen cells were suspended in 80 ml of Buffer A (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM β -mercaptoethanol) containing 0.05 M NaCl, 0.3 mg/ml of lysozyme and 0.1 mM phenyl-methyl-sulphonyl fluoride (PMSF) and mixed for 1 h. The cells were disrupted by ultrasonic disintegrator Soniprep 150 (MSE, England). The crude lysate was clarified by centrifugation for 30 min at 15,000g on J2-21 centrifuge (Beckman, USA).

The enzyme preparation was obtained by chromatographic purification of the supernatant on the following resins: 40 ml of phosphocellulose P-11 (Whatman, England), 4 ml of hydroxyapatite (Bio-Rad, USA), and 4 ml of heparin-sepharose (Bio-Rad, USA).

The supernatant was initially loaded to a phosphocellulose P-11 column pre-equilibrated with Buffer A containing 0.05 M NaCl. The column was washed with 80 ml of Buffer A with 0.05 M NaCl. The enzyme was eluted with 400 ml of a linear gradient of NaCl concentration (0.05 M-1.0 M) in Buffer A, 10 ml-fractions were collected. Fractions with endonuclease activity 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). The column was washed with 10 ml of Buffer B, then the protein was eluted with 160 ml of linear gradient of KH₂PO₄ concentration (0.01 M-0.2 M) in Buffer B, and 4 ml-fractions were collected. Active fractions were combined and applied to a heparin-sepharose column equilibrated with Buffer A. The column was washed with 8 ml of Buffer A. The enzyme was eluted with 120 ml of linear gradient of NaCl concentration (0.05 M-0.5 M) in Buffer A and 3 ml-fractions were collected. Active fractions were combined and dialyzed against a concentrating buffer (50% glycerol, 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM β -mercaptoethanol, 0.05 M NaCl). The enzyme preparation was stored at -20°C.

Plasmid construction. The plasmid pMHpall-1 has been obtained by introduction of PCR fragment carrying a gene of M.Hpall DNA methyltransferase from *Haemophilus parainfluenzae* into pMTL22 plasmid at the restriction sites PstI and XbaI. PCR fragment was amplified using following primers:

M.Hpall_up (PstI): 5'-agagatacagactgcagtttatgaaagatgtg-3'

M.Hpall_low (XbaI): 5'-gccaataatctagagccggcgtaaggctcactccac-3'

An obtained pMHpall-1 plasmid DNA carries three 5'-GCCGGC-3' sites.

The plasmid pMMspl-1 has been obtained by introduction of PCR fragment carrying a gene of M.Mspl

DNA methyltransferase from *Moraxella species* into pMTL22 plasmid at the restriction sites BamHI and PstI. PCR fragment was amplified using following primers:

M.MspI_up (BamHI): 5'-agatttggatcccaaatgaaacctgaaatattg-3'

M.MspI_low (PstI): 5'-gtcggactgcaggccgctaactgaatttcgtatatga-3'

An obtained pMMspI-1 plasmid DNA carries two 5'-GCCGGC-3' sites.

Hydrolysis of plasmid and phage DNA with endonuclease Krol. The reaction was conducted in 20 µl of the reaction mixture containing "Krol buffer" (1x) of a following composition: 10 mM Tris HCl, pH 8.5 (at 25°C), 20 mM NaCl, 3 mM MgCl₂, 1mM DTT) and 0.5 µg of plasmid or phage DNA at the temperature of 37°C for 2 hours. The reaction products were separated in 1% agarose gel in TAE buffer.

Determination of a DNA hydrolysis position. The position of DNA hydrolysis by methyl-directed site-specific endonuclease Krol was determined by comparison of DNA fragments lengths after cleavage of [³²P]-labeled synthetic oligonucleotide duplexes with Krol and MroNI endonucleases. DNA cleavage was performed in 20 µl of the reaction mixture containing "Krol buffer" (1x) at the temperature of 37°C for 1 hour. A partial cleavage product of one of these duplexes by Exonuclease III from E.coli (ExoIII) was used as a fragment length marker. The products of hydrolysis were separated by electrophoresis in 20% PAAG with 7 M urea in TBE buffer.

Oligodeoxyribonucleotides were synthesized at SibEnzyme Ltd. (Russia). The duplexes from a following oligonucleotides were used as a substrates for Krol endonuclease:

17 5'-CCATAGGATGCCTCTATGGCCGGCCACGGACCGTATC-3'

18 5'-GATACGGTCCGTGGCCGGCCATAGAGGCATCCTATGG-3'

19 5'-CCAAGGATGCCTCTATGGC(5mC)GGCCACGGACCGTATC-3'

20 5'-GATACGGTCCGTGGC(5mC)GGCCATAGAGGCATCCTATGG-3'

21 5'-GCTAGTTGGAAGGCCGCGAAGAATGATG-3'

22 5'-CATCATTCTTCGCCGGCCTTCCAACACTAGC-3'

23 5'-GCTAGTTGGAAGGC(5mC)GGCGAAGAATGATG-3'

24 5'-CATCATTCTTCGC(5mC)GGCCTTCCAACACTAGC-3'

RESULTS AND DISCUSSION

Strain-producer description. The strain-producer of Krol endonuclease was isolated from a soil sample. The bacterial cells are spherical with a diameter of 1-2 µm and don't form spores. Cells are gram-positive, catalase-positive and obligate aerobic. They grow at the temperature of 10-40°C and pH from 6 to 9. Based on morphological and biochemical properties [7] as well as the nucleotide sequence of 16S ribosomal RNA gene [8], the strain was identified as *Kocuria rosea* (old name *Micrococcus roseus*).

The enzyme Krol was purified from cell extract by three consecutive chromatographic steps on the resins as described in "Materials and Methods". The enzyme yield was 1.5 ml, and the concentration was 1000 units/ml.

Determination of Krol substrate specificity. Methylated and unmethylated phage λ DNA/AspA2I, DNA of phage T7 and pBR322 were used as a substrates for determination of Krol specificity. MTases M.SssI (recognition sequence 5'-CG-3') and M.CviPI (recognition sequence 5'-GC-3') were used to modify the substrate DNAs. DNA fragments patterns produced by Krol were compared with ones produced by MroNI restriction enzyme (NaeI neoschizomer) that cleaves nonmethylated DNA sequence 5'-G $\uparrow$ CCGGC-3'. Figure 1 presents the results of above mentioned DNAs hydrolysis with Krol and MroNI enzymes.

Figure 1. Digestion of substrate DNAs with Krol and MroNI endonucleases. Electrophoresis in 1% agarose gel. M - 1 Kb molecular weight DNA Ladder (SibEnzyme) 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.

Lanes:

- 1 - λ DNA/AspA2I; 2 - λ DNA/AspA2I + M.SssI
- 3 - λ DNA/AspA2I + M.CviPI; 4 - λ DNA/AspA2I + MroNI
- 5 - λ DNA/AspA2I + M.SssI + MroNI; 6 - λ DNA/AspA2I + Krol
- 7 - λ DNA/AspA2I + M.CviPI + Krol; 8 - λ DNA/AspA2I + M.SssI + Krol
- 9 - pBR322 DNA; 10 - pBR322 DNA + M.SssI
- 11 - pBR322 DNA + Krol; 12 - pBR322 DNA + MroNI
- 13 - pBR322 DNA + M.SssI + Krol

As it is seen from Figure 1 Krol doesn't cut unmodified DNAs (lanes 6 and 11) and λ DNA methylated with M.CviPI (lane 7). But Krol cleaves λ and pBR322 DNAs modified with M.SssI (lanes 8 and 13, respectively) producing the patterns, which are similar to ones of these unmethylated substrates hydrolysis with MroNI (lanes 4 and 12).

Thus, methylation of 5'-GCCGGC-3' site with M.SssI results in formation of 5'-GC(5mC)GGC-3' site and we can conclude that Krol recognizes and cleaves DNA sequence 5'-GC(5mC)GGC-3'/3'-CGG(5mC)CG-5'. At the same time we don't observe Krol hydrolysis of methylated site 5'-G(5mC)CGG(5mC)-3'/3'-(5mC)GGC(5mC)G-5' (lane 7).

We have constructed an additional plasmid substrates to determine Krol endonuclease activity and confirm the recognition sequence. The first plasmid pMHpall-1 carries a gene of M.Hpall MTase. M.Hpall methylates the sequence 5'-CCGG-3' producing 5'-C(5mC)GG-3'/3'-GG(5mC)C-5' site. The obtained pMHpall-1 plasmid has three sites 5'-GC(5mC)GGC-3'/3'-CGG(5mC)CG with internal cytosine residues modified. The second plasmid DNA pMMspl-1 carries a gene of M.Mspl MTase. This MTase modifies the sequence 5'-CCGG-3' producing 5'-(5mC)CGG-3'/3'-GGC(5mC)-5' site. The plasmid pMMpsl-1 encloses two sites 5'-G(5mC)CGGC-3'/3'-CGGC(5mC)G-5' with first modified cytosine residue.

The results of pMHpall-1/Dril and pMMspl-1/Dril hydrolysis with Krol are shown in the Figure 2. As it is seen from this figure Krol cuts pMHpall-1/Dril producing DNA fragments that correspond to a theoretical pattern of this plasmid digestion at sites 5'-GCCGGC-3' (1250, 1215, 1060 and 101 bp). This figure also demonstrates that Krol doesn't cleave pMMspl-1/Dril DNA, although it contains two sequences 5'-G(5mC)CGGC-3'/3'-CGGC(5mC)G-5'.

Figure 2. Digestion of pMHpall-1 and pMMspl-1 plasmid DNA with endonuclease Krol. Electrophoresis in 1% agarose gel. M - 1 Kb molecular weight DNA Ladder.

Lanes:

1 - DNA pMMspl-1/Dril; 2 - DNA pMMspl-1/Dril + Krol
3 - DNA pMHpall-1/Dril; 4 - DNA pMHpall-1/Dril + Krol

An obtained data confirm the previous result that Krol cleaves DNA sequence 5'-GC(5mC)GGC-3'/3'-CGG(5mC)CG-5', however it doesn't cut 5'-G(5mC)CGGC-3'/3'-CGGC(5mC)G-5'. Thus, a presence of central 5-methylcytosines in the recognition site is necessary for Krol activity.

Determination of Krol activity. The plasmid pMHpall-1 is used as a substrate for determination of Krol activity. One unit of Krol activity is defined as the amount of enzyme required to completely digest 1 µg of pMHpall-1/Dril DNA in SE Buffer "Y" at 37°C for one hour in a total reaction volume of 50 µl. Figure 3 represents the result of pMHpall-1/Dril DNA hydrolysis with different quantities of Krol. According to the data given in the Figure 3 the specific activity of Krol is 1000 uints/ml.

Figure 3. Krol endonuclease activity assay. Electrophoresis in 1% agarose gel. M - 1 Kb molecular weight DNA Ladder.

Lanes:

1 - DNA pMHpall-1/Dril; 2 - DNA pMHpall-1/Dril + 0.5 μ l of Krol enzyme preparation

3 - DNA pMHpall-1/Dril + 1 μ l of Krol enzyme preparation; 4 - DNA pMHpall-1/Dril + 2 μ l of Krol enzyme preparation

Determination of Krol endonuclease cleavage position. Digestion of synthetic oligonucleotide duplexes (see "Materials and Methods") has been performed for confirmation of recognition sequence and determination of Krol endonuclease cleavage position.

The duplexes 17/18 and 19/20 have the same primary structure and differ by a presence of 5-methylcytosines in Krol recognition sequence of the last couple. Krol cleavage position has been determined by comparison of DNA fragments lengths produced by MroNI hydrolysis of oligonucleotide duplex 17/18 and fragments produced by Krol digestion of oligonucleotide duplex 19/20. The result of this experiment is shown in the Figure 4.

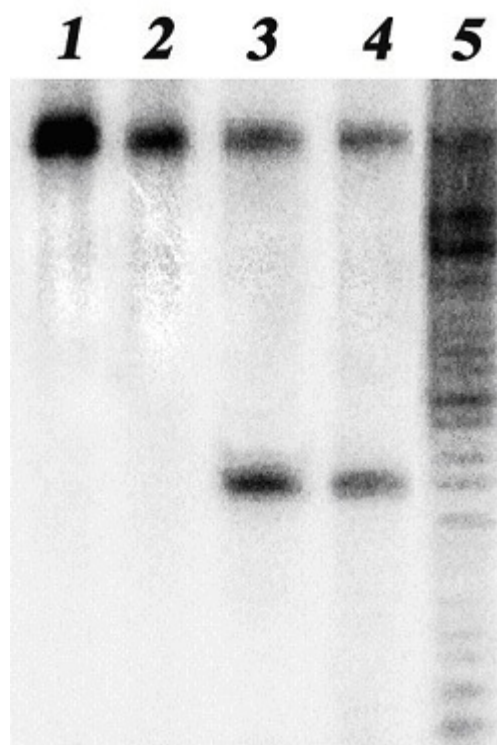


Figure 4. Determination of DNA cleavage position by endonuclease Krol. The products were separated by electrophoresis in 20% PAAG with 7 M urea. Labeled chain is shown with *.

Lanes:

1 – duplex 17*/18; 2 – duplex 19*/20

3 – duplex 17*/18 + MroNI; 4 – duplex 19*/20 + Krol

5 – duplex 19*/20 + ExoIII

As it is seen from Fig. 4 the products of DNA cleavage with both enzymes have an equal length. Thus, MroNI and Krol cleave their recognition sequences (5'-GCCGGC-3' or 5'-GC(5mC)GGC-3', respectively) at the same position before the first cytosine residue producing cohesive 5'-protruding tetranucleotide ends.

Digestion of hemi-methylated substrates with Krol. An ability of Krol and MroNI to hydrolyse hemi-methylated recognition sequence has been studied by hydrolysis of the duplexes formed by oligonucleotides 21, 22, 23 and 24. The results of cleavage of unmethylated, hemi-methylated and fully methylated oligonucleotide duplexes are presented in the Figure 5.

Figure 5. Digestion of oligonucleotide duplexes with MroNI and KroI endonucleases. A symbol of substrate is indicated above, analyzed site is indicated below. C – control single-stranded oligonucleotide. The products were separated by electrophoresis in 20% PAAG with 7 M urea. Labeled chain is shown with *.

The data in the Figure 5 demonstrate that MroNI and KroI don't cut a single strand with the recognition sequence (see 21*, 22* and 23*, 24*, respectively). MroNI restriction endonuclease cleaves both strands of unmodified duplexes only (21*/22 and 22*/21). KroI, in the contrary, cleaves both strands of the DNA duplexes with two 5-methylcytosines (23*/24 and 24*/23). These results correspond to the literature data: as a rule restriction endonucleases don't cleave fully- and hemi-methylated recognition sites [9], whereas MD endonucleases cut recognition site with two and more 5-methylcytosines [6]. Surprisingly, KroI hydrolyses both strands of DNA duplexes with one 5-methylcytosine (21*/24, 22*/23, 23*/22 and 24*/21).

Thus, KroI cleaves both strands of recognition sequences $G\uparrow C(5m)CGGC-3'/3'-CGGCC\downarrow G-5'$ (Fig. 5) and $G\uparrow C(5mC)GGC-3'/3'-CGG(5mC)C\downarrow G-5'$ (Fig. 2, lane 4) but doesn't cut DNA sequences 5'- $G(5mC)CGGC-3'/3'-CGGC(5mC)G-5'$ (Fig. 2, lane 2) and 5'- $G(5mC)CGG(5mC)-3'/3'-(5mC)GGC(5mC)G-5'$ (Fig. 1, lane 7).

KroI is a first methyl-directed site-specific endonuclease that recognizes non-degenerate hexanucleotide sequence with one 5-methylcytosine. MroNI and KroI cut recognition sites 5'-GCCGGC-3' and 5'-GC(5mC)GGC-3', respectively, in the same way, producing cohesive 5'-protruding tetranucleotides ends. Moreover both enzymes are isolated from the same bacterial species *Micrococcus roseus* [10].

A pair of restriction endonuclease and MD-endonuclease, which have similar substrate specificity and isolated from similar species of microorganism, has already been described earlier. These are MD endonuclease DpnI (recognition sequence 5'-G(m6A)TC-3'/3'-CT(m6A)G-5') and restriction endonuclease DpnII (recognition sequence 5'-GATC-3'/3'-CTAG-5'). However, unlike KroI and MroNI enzymes, DpnI and DpnII cleave DNA in a different way: DpnI produces blunt ends but DpnII forms 5'-protruding tetranucleotide ends [11].

Endonuclease KroI may be useful in determination and analysis of methylated DNA regions in epigenetic studies. KroI and MroNI may be used similar to HpaII and MspI applications [12].

Abbreviations used:

5mC – 5-methylcytosine

BSA – bovine serum albumin
ExoIII – exonuclease III from E.coli
M., MTase – DNA-methyltransferase
MD endonuclease – methyl-directed endonuclease
PAAG – polyacrylamide gel
PMSF – phenyl-methyl-sulphonyl fluoride

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