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Isolation and Characterization of Site-Specific DNA-methyltransferases from *Bacillus coagulans* K

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Abstract—Two site-specific DNA methyltransferases, M.*Bco*KIA and M.*Bco*KIB, were isolated from the thermophilic strain *Bacillus coagulans* K. Each of the methylases protects the recognition site 5′-CTCTTC-3′/5′-GAAGAG-3′ from cleavage with the cognate restriction endonuclease *Bco*KI. It is shown that M.*Bco*KIB is an N6-adenine specific methylase and M.*Bco*KIA is an N4-cytosine specific methylase. According to bisulfite mapping, M.*Bco*KIA methylates the first cytosine in the sequence 5′-**C**TCTTC-3′.

Key words: DNA methyltransferase, IIS restriction endonuclease, bisulfite mapping, N4-methylcytosine, N6-methyladenine

Restriction endonuclease R.*Bco*KI was previously isolated by us from the strain *Bacillus coagulans* K [1], and it was shown to be an isoschizomer of type IIS restriction endonuclease *Ksp*632I [2]. The endonuclease recognizes the site 5′-CTCTTC-3′/5′-GAAGAG-3′ and cleaves DNA at a distance of one nucleotide from the site in the top strand and at a distance of four nucleotides from the site in the bottom strand. In orthodox type II systems recognizing palindromic sites, one DNA-methyltransferase is enough to methylate the site, because the same base is methylated in its different strands. In contrast to orthodox systems, type IIS systems contain as a rule two methylases. The requirement for two methylases can be rationalized, first, by the asymmetry of the recognition site and, second, quite often each chain of such a site contains a different type of bases that are to be methylated. Thus, in the top strand of the *Bco*KI site only one of the cytosine residues can be the target of methylation, while in the bottom strand this is one of the adenine residues. Therefore, for methylation in both strands cytosine-specific and adenine-specific methylases are required, though there are methylases capable of the two types of methylation [3].

In this paper it is shown that the strain *Bacillus coagulans* K contains two methylases, M.*Bco*KIA and M.*Bco*KIB, with cytosine- and adenine specificity, respectively, and the position of the methylated cytosine in the site is identified.

MATERIALS AND METHODS

DNAs of phages λ dam⁻dcm⁻, M13tg130, and M13tg131, restriction endonuclease *Bco*KI, *Taq* DNA polymerase, phage T4 DNA ligase, methylases *Ssc*L1I, *Bsp*LU11IIIb, and *Pvu*II were prepared in our laboratory. We also used restriction endonucleases *Bam*HI, *Xba*I, and *Hin*dIII (Fermentas, Lithuania), salmon sperm DNA, calf thymus DNA, S-adenosine-L-methionine (AdoMet) (Sigma, USA), and [methyl-^{3}H]AdoMet (15 Ci/mmol) (Amersham, England). The methylated bases C5-cytosine (5mC) and N6-adenine (N6mA) were a kind gift of M. D. Kirnos.

Oligonucleotides IIS1 (5′-GATCCTGAAG-CGGGTGAAGACGAAGAGACCT-3′), IIS2 (5′-TCAGCTAGAGGTCTCTTCGTCTTCACCCGCT-3′), AC1 (5′-ATAATAATTCTAGATAGGAAATAGTTAT-

Abbreviations: 5mC) C5-methylcytosine; N4mC) N4-methylcytosine; N6mA) N6-methyladenine; AdoMet) S-adenosyl-L-methionine.

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GATT-3′), AC2 (5′-AATCACAAGCTTATAAAACAAC-CAA-3′), B9 (5′-GTTTTCCCAGTCACGAC-3′), and B10 (5′-AACAGCTATGACCATG-3′) were from Syntol (Russia).

Isolation of DNA and DNA cleavage and ligation were performed using standard techniques [4]. DNA was purified with Silica quartz powder as described by us [5]. Electrocompetent cells for transformation were prepared according to Dower et al. [6].

Isolation and purification of methylases. *Bacillus coagulans* K cells were grown in 4 liters of LB medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) with intensive aeration at 48°C during 12 h to reach the late logarithmic phase. The cells were precipitated by 6000*g* centrifugation for 40 min at 4°C; the precipitate was suspended in buffer TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and centrifuged again under the same conditions. Thirty grams of crude biomass was obtained.

To isolate methylases, 300 ml of lysing buffer (20 mM KH_2PO_4, pH 6.8, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 7 mM 2-mercaptoethanol, 1 mM EDTA) was added to the cells thawed at 0°C. The suspension was homogenized thoroughly, 5 ml of freshly prepared lysozyme solution (10 mg/ml) in lysing buffer were added, and the mixture was incubated on ice for 30 min. Then the cells were disrupted with an UZDN-1A ultrasonic disintegrator (Russia) for 23 min with 1 min sonifications and 2 min pauses. The lysate was centrifuged (40 min, 30,000*g*, 4°C). The proteins from the supernatant were precipitated with ammonium sulfate to 75% saturation. The precipitate was pooled by centrifugation at 6000*g* for 20 min, resuspended in 20 ml of buffer A (20 mM KH_2PO_4, pH 6.8, 7 mM 2-mercaptoethanol, 1 mM EDTA), and the solution was dialyzed against buffer A overnight. The dialyzed solution was centrifuged at 6000*g* for 20 min. Twenty milliliters of buffer A containing 50 mM NaCl was added to the supernatant.

The cell lysate was used for further purification of methylases on chromatography columns. All the stages of chromatographic purification were performed at 4°C. Columns of 30-ml volume with heparin-Sepharose (Pharmacia, Sweden) and of 30- and 3-ml volume with phosphocellulose P11 (Whatman, England) were used. The columns were equilibrated with buffer A containing 50 mM NaCl. The rate of the protein loading onto the columns and their following elution was 20 and 5 ml/h, correspondingly, on the 30- and 3-ml phosphocellulose columns and 10 ml/h on the heparin-Sepharose column. The proteins were eluted from the columns using a linear gradient of NaCl in buffer A.

The methylase activity in the fractions was determined by the protection from hydrolysis of the substrate DNA, preliminarily incubated with aliquots from fractions, by restriction endonuclease *Bco*KI.

Analysis of fractions for methylase activity. The reaction was done in 20 μl methylase buffer (50 mM Tris-HCl, pH 8.0, 4 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 mg/ml gelatin) containing 80 μM AdoMet, 0.5 μg phage λ dam^-dcm^- DNA, and 3 μl aliquot from the fractions. The reaction mixture was incubated at 48°C for 1 h. Then 2 units of restriction endonuclease *Bco*KI and $MgCl_2$ to the final concentration of 15 mM were added to the mixture. Then the mixture was incubated again for 1 h at 48°C. The preparations obtained were analyzed in 1% agarose gel.

The DNA-methylase activity of the final preparation of methylases was determined in a similar way. One unit of methylase activity is defined as the amount of methylase required to completely protect 1 μg of phage λ DNA against cleavage by *Bco*KI restriction endonuclease.

Methylation of DNA with [^{3}H]AdoMet. Phage λ DNA (2.5 μg) was methylated in 25 μl of the methylase buffer in the presence of 10 pmol [^{3}H]AdoMet and 4 units of methylase. Salmon sperm DNA (0.3 mg) was methylated in 200 μl of methylase buffer containing 10 pmol [^{3}H]AdoMet and 30 units of methylase. After incubation of the reaction mixtures at 48°C for 4 h, the percentage of the radioactive label incorporated into the DNA was determined. After that 1/5 volume of 10% SDS and 2.5 volumes of buffer TE were added to the samples and the mixtures were incubated at 65°C for 1 h. Then 15 μg of thymus DNA and 1 ml of 10% trichloroacetic acid (TCA) were added to the preparations and the mixture was kept on ice for 40 min. The mixture was filtered through GF/C filter (Whatman, England), the filters with precipitated DNA on them were washed thrice with 5 ml of 5% TCA and twice with 5 ml of 96% ethanol, dried, and placed in flasks with 3 ml of Liquid Scintillation Supersolve X (Koch-Light Ltd., England). Radioactivity of precipitated DNA was measured using LS1801 counter (Beckman, USA). The percentage of the incorporated label was determined as a ratio of the radioactivity of DNA precipitated on the filter to that before the procedure.

Determination of the nature of methylated base by acidic depurinization. The methylated base (adenine or cytosine) was determined according to [7] with a slight modification. After determining the radioactivity incorporated into the DNA, the filters were washed thrice for 5 min in 3 ml of toluene, dried, placed in 2 ml of 0.5 M HCl, and incubated at 60°C for 40 min. Then the filters were washed thrice with 5 ml of 50 mM HCl and twice with 5 ml of 96% ethanol. After drying the radioactivity was measured again. The methylase specificity was determined as the ratio of the radioactivity remaining after the acidic treatment of the preparations to the radioactivity retained in the precipitated DNA.

DNA hydrolysis to bases. DNA was hydrolyzed to bases according to Marshak and Vogel [8]. Salmon sperm DNA (0.3 mg) was methylated in the presence of [^{3}H]AdoMet. The methylated DNA was precipitated with ethanol and dissolved in 20 μl of 58% perchloric acid. A

hermetic tube with the probe was incubated on a boiling water bath for 1 h. Then 80 μl of H_2O and 85 μl of 2 M KOH (up to pH 5-8) were added, and the formed insoluble $KClO_4$ was removed by centrifugation at 12,000*g*. The absence of radioactivity on the filter after precipitation of the hydrolyzed DNA proved the completeness of hydrolysis.

Thin layer chromatography assay. We used plates covered with cellulose MN 300 (Macherey and Nagel, Germany). Fractionation was done in one direction in the liquid phase of the isobutanol–H_2O–NH_4OH system with the ratio of 60 : 10 : 0.1 v/v. After the fractionation, the plates were dried and the spots were localized by absorption at 254 nm. The sorbent in the place corresponding to the spot was scraped off and placed in a tube. H_2O (200 μl) was added to the tube and the mixture was stirred at 60°C for 15 min. After centrifugation, the supernatant was layered on GF/C filters. The filters were dried and the radioactivity was counted.

Localization of methylcytosine using the bisulfite method (conversion). Methylated cytosine was determined by a slightly modified bisulfite method [9].

Methylation was done in a volume of 100 μl methylase buffer containing 80 μM AdoMet, 10 μg of single stranded DNA M13tg130(BcoK), and 25 pmol of oligonucleotide IIS2. Before this, the oligonucleotide was annealed with DNA, for which the mixture was incubated at 60°C for 10 min and then kept at room temperature for 15 min. After that 30 units of methylase M.*Bco*KIA was added and the mixture was incubated for 3 h at 48°C, which is the optimal temperature for the functioning of M.*Bco*KIA. Due to the long length of the oligonucleotide, it remained bound to the single stranded DNA during the reaction. After the methylation reaction, an aliquot containing 0.25 μg DNA was digested by R.*Bco*KI to monitor the efficiency of the reaction. Then the sample was extracted with phenol with subsequent re-precipitation with ethanol of the main part of the methylated sample. To eliminate non-methylated sites in the following reactions, the re-precipitated DNA was incubated at 48°C for 1 h with 10 units of restriction endonuclease *Bco*KI, after which phenol extraction was repeated.

Conversion. Bisulfite solution (2.5 M) was prepared just before the experiment. NaOH (750 μl, 2 M) was added to 2.5 ml of H_2O, 1.9 g of sodium metabisulfite was dissolved in the mixture, and 550 μl of freshly prepared 1 M hydroquinone was added. The mixture was filtered through a 0.45-μm HA filter (Millipore Corp., USA).

Methylated DNA (0.1 μg) was denatured in a volume of 21 μl on a boiling bath for 5 min, rapidly cooled, and centrifuged in Eppendorf (Germany) centrifuge. Then 4 μl of 3 M NaOH was added, and the mixture was incubated at 50°C for 15 min and mixed with 50 μl of hot 2.5% low-melting agarose (SeaAq, USA). Ten-microliter portions of the resulting mixture were introduced into mineral oil (Sigma, USA) layered on the bisulfite solution to obtain DNA-agarose beads. Each bead contained 13 ng of DNA. The tubes were placed on ice for 30 min and then incubated at 50°C for 3.5 h. After the incubation, the beads were washed four times with 1 ml TE for 15 min. Then the beads were incubated twice for 15 min in 500 μl of 0.2 M NaOH and washed thrice with 1 ml of TE for 10 min. The beads were stored in a small amount of TE at 4°C.

Amplification of converted DNA. The DNA contained in agarose beads was used as a template for PCR. Immediately before the reaction, the beads were washed twice with 1 ml of H_2O for 15 min. PCR was done in 100 μl volume (the volume of one added DNA-agarose bead included) containing Taq-buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$, 0.08% Nonidet 40), 100 μM dNTPs, oligonucleotides AC1 and AC2 (20 pmol each), and 2.5 units of *Taq* DNA polymerase. Amplification was performed at 94°C for 3 min followed by 30 cycles each consisting of 30 sec at 94°C, 30 sec at 59°C, and 30 sec at 72°C.

DNA sequencing was done according to Sanger et al. [10] as modified by Promega (USA) [11]. In the reaction, we used ^{32}P-labeled B9 and B10 primers complementary to DNA regions flanking the polylinker of phage M13tg DNA. The reaction products were analyzed in 6% polyacrylamide gel containing 7 M urea at 50°C on a Macrophor instrument (LKB, Sweden).

RESULTS AND DISCUSSION

Isolation of site-specific methylases. The scheme of isolation of methylases is given in Fig. 1. Cell lysate (21 ml) was loaded onto a phosphocellulose column (30 ml), the column was washed with buffer A containing 50 mM NaCl, and the bound proteins were eluted with a NaCl gradient (0.05-1.5 M) in buffer A. Methylase activity was found in fractions 13-14 corresponding to NaCl concentrations from 720 to 780 mM, as well as in fractions 18-19 corresponding to NaCl concentrations from 1.0 to 1.06 M. Endonuclease activity was observed in fractions 16-18. The existence of the two, separated from each other, sets of fractions with methylase activity showed the presence of two methylases (M.*Bco*KIA and M.*Bco*KIB) in the lysate. Fractions of each of the methylases with maximal activity were pooled and used for further purification. Purification of each methylase was done on separate columns but by the same scheme (Fig. 1).

The second resin was heparin-Sepharose. Before loading onto the column, NaCl was removed from the fractions by dialysis overnight against buffer A. Proteins were eluted with a NaCl linear gradient (50-800 mM) in buffer A. Methylase M.*Bco*KIA was eluted from the column in fractions corresponding to NaCl concentrations

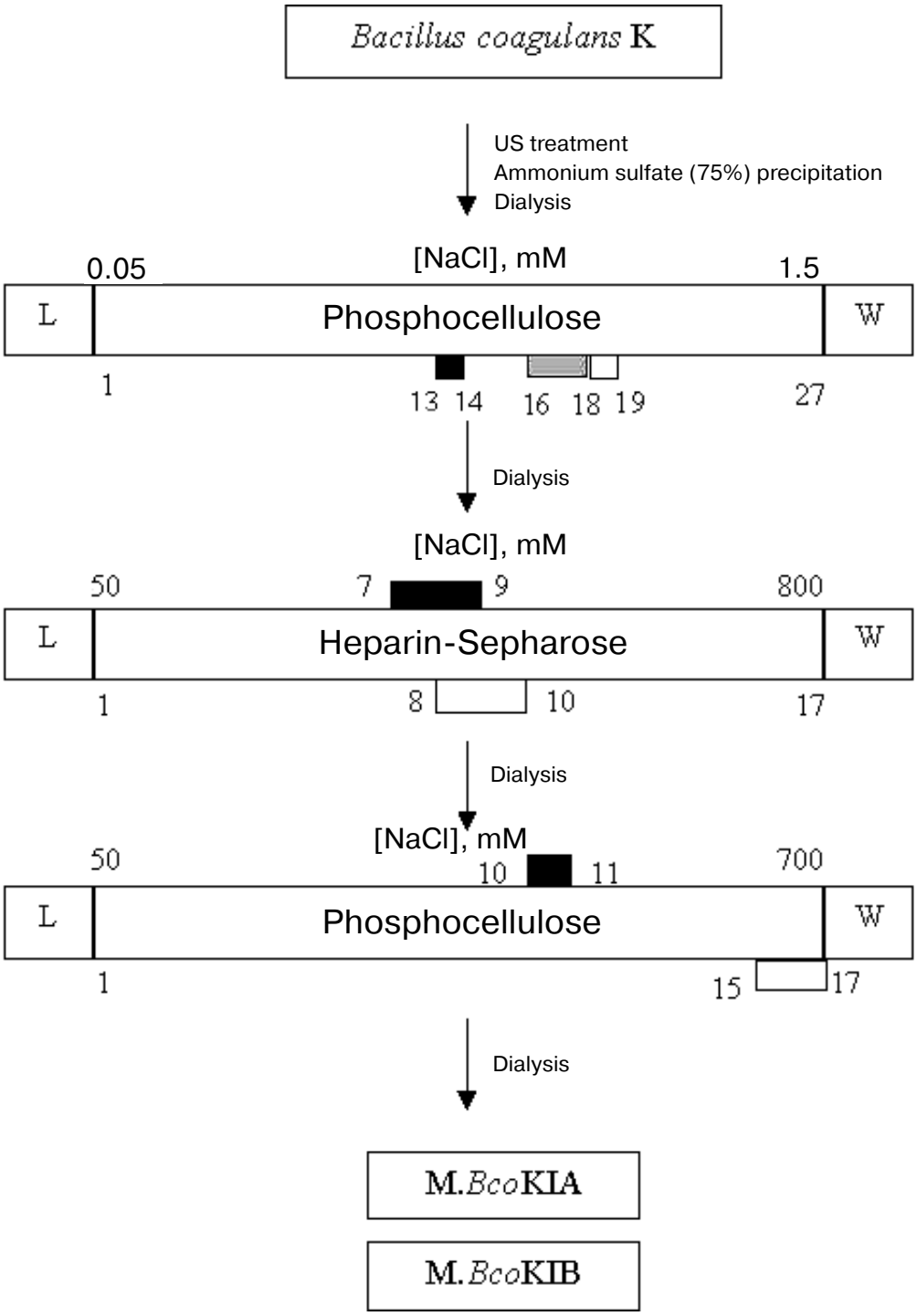

Fig. 1. General scheme of isolation of methylases M.*Bco*KIA and M.*Bco*KIB. The stages of purification are shown by rectangles. Numerals above the rectangles indicate original and final molar concentrations of NaCl. L, the stage of loading and washing the column after it; W, washing the column after the gradient. Fractions are shown that contain the enzyme: M.*Bco*KIA is given in black, M.*Bco*KIB is designated by empty rectangles; the fractions shown in gray contain the restriction endonuclease.

Table 1. Determination of the nature of the methylated base

Methylase	Radioactivity, cpm		*a**, %	Methylated base
	before HCl hydrolysis	after HCl hydrolysis		
M.*Bco*KIA	11266	6119	54	C
M.*Bco*KIB	11998	238	2	A
M.*Bsp*LU11IIIb	13539	6620	49	C
M. *Ssc*L1I	12877	302	2	A

* *a* is radioactivity (%) retained on the filter after depurinization.

from 330 to 420 mM. Methylase M.*Bco*KIB was eluted from the column in fractions corresponding to NaCl concentrations from 380 to 470 mM. However, it is seen that NaCl concentrations at which methylases were eluted overlap partially, which means that each of the methylases contains an admixture of the other methylase.

Taking into account that on the phosphocellulose column the methylases M.*Bco*KIA and M.*Bco*KIB were separated quite well, we used this column (3 ml volume) once again. In this column, M.*Bco*KIA was eluted in fractions with NaCl concentrations from 410 to 450 mM, and M.*Bco*KIB was eluted in fractions with NaCl concentrations from 620 to 700 mM, i.e., in this case the NaCl concentrations did not overlap. Thus, it can be assumed that methylases have no overlapping contamination.

The fractions from the latter column that had maximal activity were pooled, dialyzed against storage buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 200 μg/ml gelatin, 50% glycerol), and used in further work. Optimal conditions for revealing the activity are the same for both methylases: 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM 2-mercaptoethanol, 48°C. Both methylases had the activity of 5 units/μl. The total yield was 2250 units of M.*Bco*KIA and 3000 units of M.*Bco*KIB.

Determination of methylase specificity. To determine the nature of the methylated base (adenine or cytosine), acidic depurinization was used. The method is based on different stability of the glycoside bond between deoxyribose and purine and pyrimidine bases in the acid medium: this bond is less stable in purine bases. In such conditions, purine bases are mostly cleaved off from DNA. The cleaved bases are not retained on the GF/C filter upon filtration of the precipitated DNA. As a result, the ratio of the radioactivity of the DNA retained on the filter before and after the acid treatment can be used to determine which base, adenine of cytosine, has incorporated [methyl-^{3}H].

The specificity was determined on phage λ dam$^-$dcm$^-$ DNA. Adenine-specific methylase M.*Ssc*L1I [12] and cytosine-specific methylase M.*Bsp*LU11IIIb [13] were used as controls. The results given in Table 1 show that for methylase M.*Bco*KIA the portion of the radioactivity retained after acid hydrolysis is 54% of the original radioactivity, which corresponds to that for cytosine-specific methylases. The other methylase, M.*Bco*KIB, is adenine-specific, since 2% of the label is retained after hydrolysis.

Thus, it has been found that M.*Bco*KIA is a cytosine-specific methylase and M.*Bco*KIB is adenine-specific.

Determination of the nature of the methylated base by thin-layer chromatography assay. To determine to what cytosine atom (C5 or N4) M.*Bco*KIA transfers the methyl group, we used the thin-layer chromatography assay. 5mC and N6mA were used as markers of mobility of bases. Due to unavailability of N4mC, we used hydrolyzate of DNA methylated in the presence of [^{3}H]AdoMet with methylase M.*Pvu*II, which is an N4-cytosine specific methylase [14]. The methylated bases 5mC and N6mA were placed on a plate both separately in a 100 μg portion and mixed with 50 μl of hydrolyzed DNA: 5mC + DNA methylated with M.*Bco*KIA; N6mA + DNA methylated with M.*Bco*KIB; 5mC + N6mA + DNA methylated with M.*Pvu*II. The results showed that the mobility (R_f) of the radioactivity spot in the hydrolyzate of DNA methylated with M.*Bco*KIA is compatible with the mobility of the radioactivity spot in the hydrolyzate of DNA methylated with M.*Pvu*II. Consequently, M.*Bco*KIA methylates cytosine in position N4. The mobility of the radioactivity spot in the hydrolyzate of DNA methylated with M.*Bco*KIB is compatible with that of N6mA. This supports the conclusion that M.*Bco*KIB is an N6-adenine-specific methylase (Table 2).

Determination of the position of methylated cytosine. To answer the question what cytosine is methylated within the site, we used bisulfite mapping. It is based on selective interaction of bisulfite with non-methylated cytosines, as a result of which cytosines transfer (convert) to uracils. After replication during PCR upon sequenc-

ing, converted cytosines will be read out as thymines, whereas methylated cytosines are not converted.

As a substrate we used recombinant phage M13tg130(BcoK) DNA the polylinker of which contained an incorporated *Bco*KI site. The recombinant DNA was constructed by inserting of the duplex of the oligonucleotides IIS1 and IIS2 into the *Xba*I and *Bam*HI sites of the polylinker. In choosing a vector for the cloning of the duplex, such an orientation of the duplex within the vector was anticipated that the single stranded phage DNA would contain the top strand of site *Bco*KI (5′-CTCTTC-3′).

Conversion was performed on single stranded DNA M13tg130(BcoK). After the reaction, agarose beads containing converted DNA were used in PCR. PCR was done with primers AC1 and AC2. AC2 is complementary to the chain containing the target cytosine taking into account cytosines that are transferred to uracils during conversion. AC1 is complementary to the DNA chain formed as a result of synthesis on the template of converted DNA from primer AC2. Sites of restriction endonucleases *Xba*I and *Hin*dIII were introduced into primers AC1 and AC2, respectively.

Synthesis during PCR of a DNA fragment of the expected length indicates that the conversion was successful. The fragment of 160 bp synthesized in PCR was purified, cleaved with restriction endonucleases *Xba*I and *Hin*dIII, and cloned by sites of the endonucleases. Vector M13tg131 was taken for cloning because in this case a chain containing the converted sequence of site *Bco*KI during the sequencing of the single stranded DNA would be read out.

The recombinant phage M13tg131(BcoK) DNA was used to transform TG2 *E. coli*. Single stranded DNAs were isolated from the recombinant phages. The presence of the fragment insertion in such DNAs was checked in PCR on the DNAs with the use of oligonucleotides B9 and B10 complementary to DNA regions flanking the polylinker. Then DNAs containing the insertion were sequenced.

Table 2. The results of thin-layer chromatography on cellulose

Bases and hydrolyzate of DNA methylated in the presence of [methyl-^{3}H]AdoMet	R_f	Radioactivity, cpm
N6mA	0.7	—
5mC	0.38	—
M.*Pvu*II (N4mC)	0.5	2737
M.*Bco*KIA	0.47	9707
M.*Bco*KIB	0.65	9639

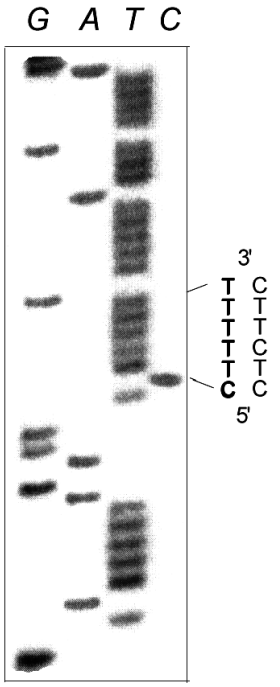

Fig. 2. Determination of the position of cytosine by bisulfite treatment. G, A, T, and C are "sequencing" lanes. The methylated site after conversion is shown in bold type and that before conversion in regular type.

The results of sequencing are given in Fig. 2. It is seen that all but one cytosine residues of the fragment were converted into uracils because they are read out as thymines. The only unconverted and therefore methylated cytosine corresponds to the first cytosine in the sequence 5′-**C**TCTTC-3′ (it is given in bold type). An analogous situation is observed for 5 of the 7 sequenced DNAs.

Thus, it was shown that strain *Bacillus coagulans* K contains two methylases. M.*Bco*KIB is an N6-adenine-specific methylase and modifies one of the adenines in the bottom strand of the recognized site 5′-CTCTTC-3′/5′-GAAGAG-3′. M.*Bco*KIA is an N4-cytosine-specific methylase and modifies the first cytosine in the sequence 5′-**C**TCTTC-3′.

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