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## New Restriction Endonuclease AluBI from *Arthrobacter luteus* B - AluI Isoshizomer, nonsensitive to Presence of 5-methylcytosine in the Recognition Sequence AGCT

Category: [New enzymes](#)

Hits: 11139

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*A new restriction endonuclease AluBI has been discovered and characterized. AluBI is an isoshizomer of well known restriction endonuclease AluI, which recognizes DNA sequence AGCT. Unlike AluI, enzyme AluBI is able to cleave DNA when recognition sequence contains 5-methylcytosines. AluBI also cleaves recognition sequence with two methylated cytosines, one modified at N4 and other at C5 positions. However, new enzyme doesn't cleave DNA with two N4-methylcytosines in the recognition site.*

Bacterial site-specific DNA-endonucleases (restriction endonucleases, RE) and DNA-methyltransferases of the same specificity form so-called restriction-modification systems (RM-system). Nowadays there are more than 250 different recognition sequences of type II restriction endonucleases (prototypes) and more than 3500 characterized enzymes (isoschizomers). Isoschizomers have an identical recognition sequence but may differ in ability to hydrolyze methylated site. There are only a few examples of such isoschizomers pairs (EcoRII - MvaI [6,7], HpaII-MspI [8]), which recognize DNA sequences 5'-CCGG-3' and 5'-CC(A/T)GG-3', respectively, and are used in study of natural DNAs methylation status [1-5,16].

A well known RE AluI from bacterial strain *Arthrobacter luteus* recognizes and cleaves sequence 5'-AGCT-3' [9]. This enzyme has found a wide practical application in biotechnology and molecular biology and has become one of the most popular restriction endonucleases.

AluI RM-system includes DNA-methyltransferase, that methylates recognition sequence 5'-AGCT-3' in position C5 [14]. Such modification prevents DNA from hydrolysis by restriction endonuclease AluI.

In the present work we describe isolation and substrate specificity of a new restriction endonuclease AluBI, which is capable to cleave both non-modified DNA and the recognition sequence 5'-AGCT-3' with 5-methylcytosine(s).

## MATERIALS AND METHODS

Reagents manufactured by Sigma (USA), Serva (Germany), ICN (USA) and Helikon (Russia) were used in the work.

### Strain cultivation and enzyme isolation

Colonies *Arthrobacter luteus* B were grown on LB agar at 30°C and were transferred into the flasks, which contain 300 ml of liquid LB medium. Flasks were cultivated in shaker (New Brunswick Scientific USA) two days at 30°C. The cells were precipitated by centrifugation for 30 minutes at 5000 g in J2-21 centrifuge (Beckman, USA) and were frozen at -20°C.

Enzyme isolation was performed at 4°C. Frozen cells (37 g) were suspended in 100 ml of the buffer A (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM 2-mercaptoethanol), containing 0.05 M NaCl, 0.3 mg/ml lysozyme, 0.1 mM phenylmethylsulfonyl fluoride (PSMF). The suspension was incubated during 1 hour with permanent mixing and then cells were disrupted by ultrasonic treatment as was described earlier. The cell debris was removed by centrifugation at 15000 rpm for 30 min.

Supernatant was applied on column with 45 ml phosphocellulose P-11 («Whatman», England). The column was washed with buffer A containing 0.05 M NaCl. 0.05-0.6 M NaCl gradient in buffer A (500 ml) was used for enzyme elution and 10 ml fractions were collected. The elution fractions with endonuclease activity were pooled and dialyzed against 20 volumes of buffer A.

Dialyzed fractions were applied on 7 ml heparine-sepharose column («Bio-Rad», USA). Elution was performed using 0.05-0.5 M NaCl gradient in buffer A (120 ml) and 3 ml fractions were collected. The fractions with enzyme activity were combined and applied on 4 ml column with hydroxyapatite (BioRad, USA). The enzyme was eluted with K<sub>2</sub>HPO<sub>4</sub> gradient, 2 ml fractions were collected and analyzed. Fractions with a high enzyme's activity were pooled and concentrated by dialysis against 20 volumes of 50% glycerol, 10 mM Tris-HCl, pH 7.55, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 0.05 M NaCl. Purified enzyme was stored at -20°C.

A total of 2.5 ml of enzyme preparation with the specific activity of 5000 u/ml were purified.

Restriction endonucleases AluI (concentration 3000 u/ml), AluB I (concentration 3000 u/ml), T4 polynucleotide kinase and buffer solutions manufactured by SibEnzyme (Russia) were used for experiments.

### Determination of enzymes sensitivity on synthetic oligonucleotide duplexes

Following oligonucleotides, served as substrates for endonucleases Alu I and AluB I, have been synthesized at SibEnzyme Ltd. (Russia).

Alu1: 5'-GGT ATA GGA TGA AGCT TTC GCG GGT TAA GG-3'

Alu2: 5'-CC TTA ACC CGC GAA AGCT TCA TCC TAT TCC-3'

Alu3: 5'-GGT ATA GGA TGA (6mA)GCT TTC GCG GGT TAA GG-3'

Alu4: 5'-CC TTA ACC CGC GAA (6mA)GCT TCA TCC TAT TCC-3'

Alu5: 5'-GGT ATA GGA TGA AG(5mC)I TTC GCG GGT TAA GG-3'

Alu6: 5'-CC TTA ACC CGC GAA AG(5mC)I TCA TCC TAT TCC-3'

Alu7: 5'-GGT ATA GGA TGA AG(4mC)I TTC GCG GGT TAA GG-3'

Alu8: 5'-CC TTA ACC CGC GAA AG(4mC)ITCA TCC TAT TCC-3'

Oligonucleotides with even number are complementary to oligonucleotides with odd number. All oligonucleotide duplexes have identical primary structure and differ from each other by a presence or an absence of the methylated bases in sequence AGCT (sequences AGCT recognized by AluI and AluBI, are underlined).

## Preparation of [<sup>32</sup>P]-labeled oligonucleotide duplexes

One of the chains of oligonucleotide duplex was labeled at 5'-end using T4-polynucleotide kinase and γ-[<sup>32</sup>P]ATP. After oligonucleotide purification, complementary unlabeled oligonucleotide was added, the tube was heated at 95°C for 5 minutes followed by cooling to room temperature on the bench.

## Hydrolysis of oligonucleotide duplexes with endonucleases Alu I and AluB I

The hydrolysis reaction was carried out in optimal conditions (37°C, SE-buffer Y - 33 mM Tris, acetate pH 7.9 (at temperature 25°C), 10 mM Mg (CH<sub>3</sub>COO)<sub>2</sub>, 66mM KCH<sub>3</sub>COO, 1mM DTT) by addition of 1 μl (3 units) enzyme AluI or AluBI in 20 μl the reaction mixture containing the oligonucleotide duplex in concentration of 66,7 nM. Reaction time is 50 minutes. Products of the oligonucleotide cleavage were separated by electrophoresis in denaturing 20% polyacrylamide gel (PAAG) with 7 M urea.

# RESULTS AND DISCUSSION

## The description of strain

The strain *Arthrobacter luteus* B has been isolated from natural sources as described earlier [10]. Taxonomic determination of AluBI strain-producer has been done according to [11]. Strain *Arthrobacter luteus* B was characterized by following features:

## Morphological features

Strain forms white, smooth, brilliant, opaque, convex, round colonies of 4 mm in diameter on Luria-Bertrani medium. Cells are coccus-shaped, single, in pairs or in short chains have 0,8-1,2 microns diameter.

## Physiological and biochemical features

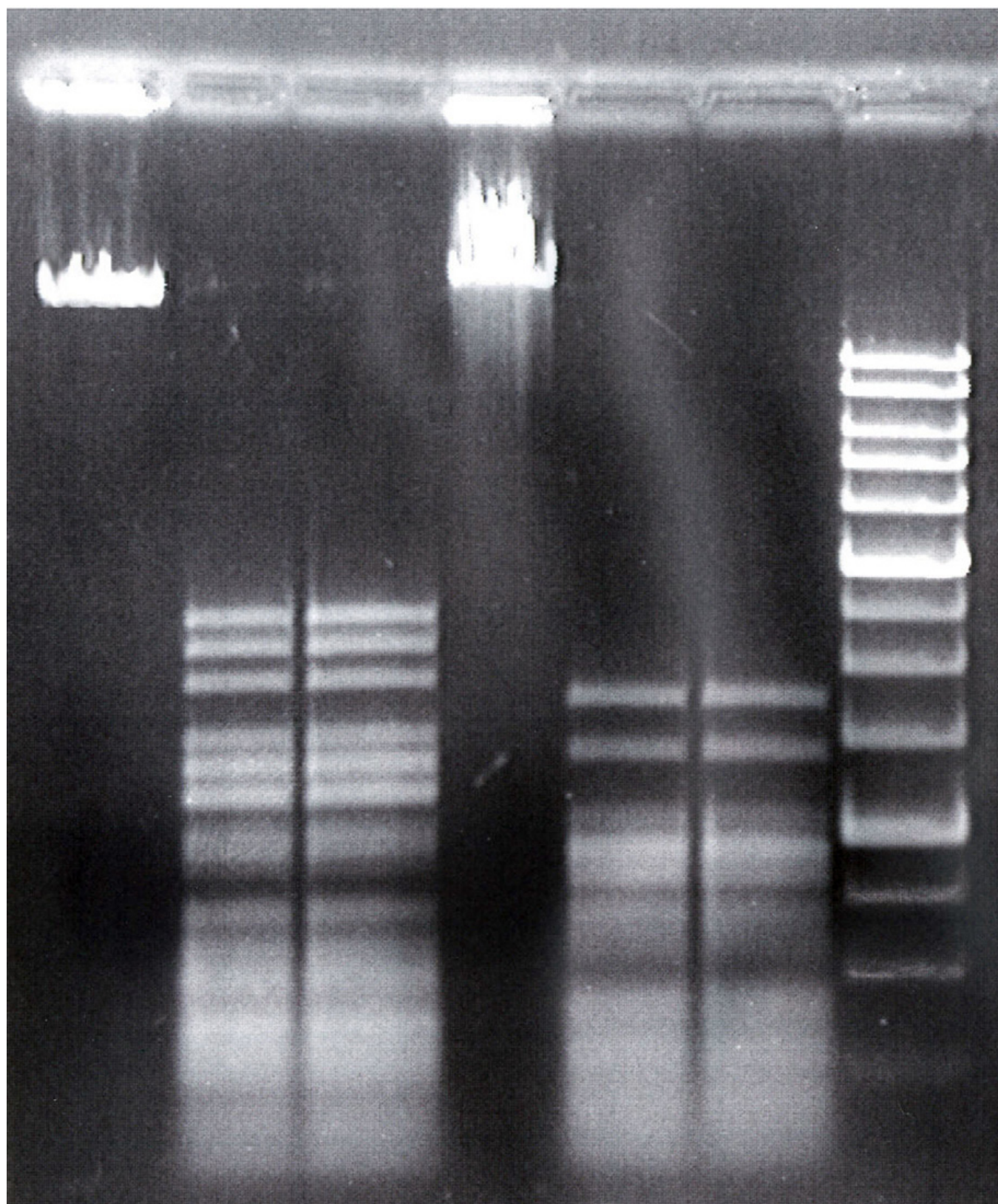
Bacterial cells are Gram-positive, not capable to anaerobic growth, catalase-positive, oxydase-negative. The strain grows at temperature 10-40°C. The guanines and cytosines percentage in a host DNA 73-77 % was established according to [12].

Strain was identified as a bacterial species *Arthrobacter luteus* B. Restriction endonucleases was named AluBI according to the standard nomenclature [13].

## Determination of restriction endonuclease AluBI specificity

Specificity of enzyme was determined by comparison of  $\lambda$  and T7 bacteriophages DNAs digestion patterns (fig. 1).

1 2 3 4 5 6 7



**Fig 1.** Digestion of  $\lambda$  and T7 DNA with AluI and AluBI restriction endonucleases.

Lanes:

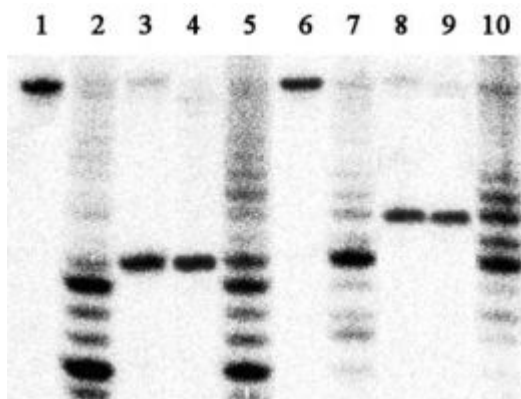
- 1 –  $\lambda$  DNA;
- 2 –  $\lambda$  DNA, RE AluI
- 3 –  $\lambda$  DNA, RE AluBI;
- 4 – T7 DNA;
- 5 – T7 DNA, RE AluI;
- 6 – T7 DNA, RE AluBI;
- 7 – 1 kb DNA ladder

Reaction mixtures containing DNA at concentration 0,02 mg/ml, enzyme in SE-buffer Y (33 mM Tris-acetate, pH 7,9, 10 mM MgCl<sub>2</sub>, 66 mM potassium acetate, 1 mM DTT) was incubated at 37°C for 60 minutes and was separated by electrophoresis in 1 % agarose gel.

As shown on fig. 1  $\lambda$  and T7 DNA hydrolysis with AluI and AluBI produces a similar DNA digestion fragments of identical length have appeared as a result of the bacteriophages DNAs treatment with patterns. So, AluBI recognize and cleave DNA sequence 5'-AGCT-3'.

### Determination of site-specific endonuclease AluBI DNA cleavage positions

Comparison of DNA fragment lengths, which formed during cleavage of oligonucleotide duplexes Alu1\*/Alu2 and Alu2\*/Alu1 (labeled strand is marked by a symbol <\*>) was carried out. These duplexes have non-methylated recognition sequence 5'-AGCT-3'. Products of partial hydrolysis of the same duplexes with exonuclease ExoIII were used as a fragments length marker. Results of oligonucleotide duplexes digestion are presented on fig. 2.



**Fig. 2.** Determination of DNA cleavage positions of restriction endonuclease AluBI.

Lanes:

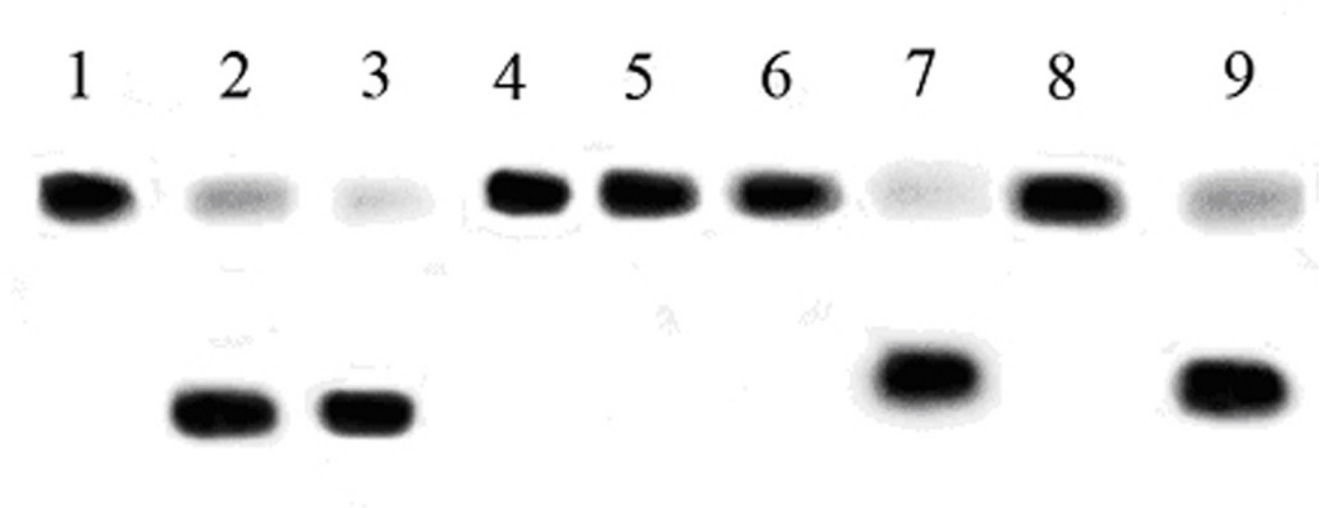
- 1 - duplex Alu1\*/ Alu2
- 2, 5 - duplex Alu1\*/ Alu2, exonuclease III
- 3 - duplex Alu1\*/ Alu2, RE AluI;
- 4 - duplex Alu1\*/ Alu2, RE AluBI;

- 6 - duplex Alu2\*/ Alu1;
- 7, 10 - duplex Alu2\*/ Alu1, exonuclease III
- 8 - duplex Alu2\*/ Alu1, RE AluI;
- 9 - duplex Alu2\*/ Alu1, RE AluBI;

As shown on fig. 2, lengths of the fragments, which are produced by the cleavage of oligonucleotide duplexes Alu1\*/Alu2 and Alu2\*/Alu1 with both enzymes, are identical. Thus, the restriction endonuclease AluI and AluBI cleaves the recognition sequence in the same way, producing blunt ends.

### Determination and comparison of AluI and AluBI methylation sensitivity

Results of methylated and non-methylated synthetic oligonucleotide duplexes digestion are presented on fig. 3-5.



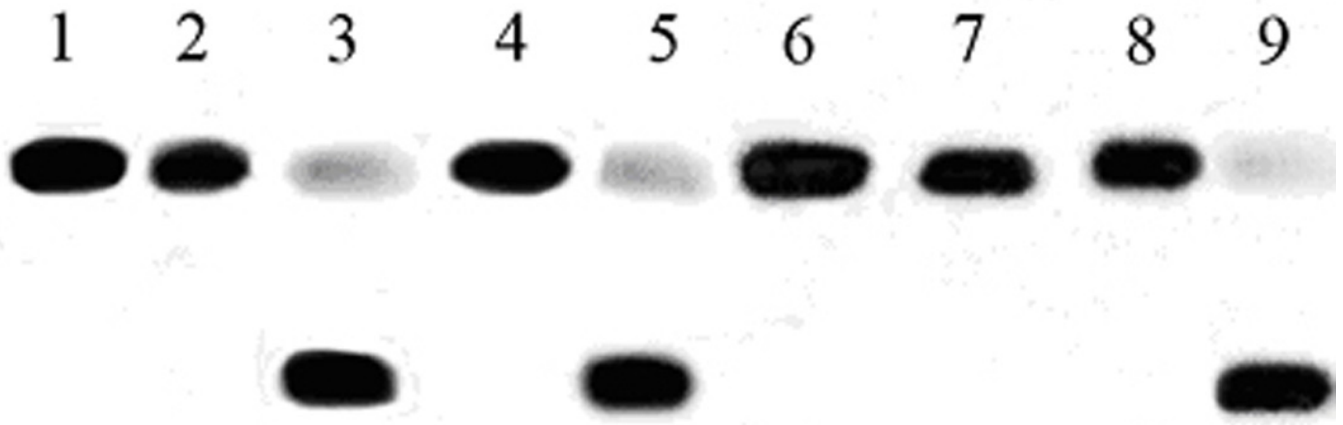
**Fig. 3.** AluI and AluBI activity in cleavage of semi-methylated oligonucleotides

Lanes:

- 1 -duplex Alu1\*/ Alu2;
- 2 - duplex Alu1\*/ Alu2, RE AluI;
- 3 - duplex Alu1\*/ Alu2, RE AluBI;
- 4 - duplex Alu1\*/ Alu4, RE AluI;
- 5 - duplex Alu1\*/ Alu4, RE AluBI;
- 6 - duplex Alu1\*/ Alu6, RE AluI;.
- 7 - duplex Alu1\*/ Alu6, RE AluBI;
- 8 - ä duplex Alu1\*/ Alu8, RE AluI;
- 9 - duplex Alu1\*/ Alu8, RE AluBI;

Results of AluI and AluBI hydrolysis of oligonucleotide duplexes containing recognition sequences without the methylated bases or with only one methylated base (semi-methylated site) are presented on

fig.3. AluI and AluBI hydrolyze the oligonucleotide duplex containing the non-methylated recognition sequence (lanes 2, 3) and do not cleave DNA with methylated adenine (lanes 4, 5). In presence of C4 and C5 methylated cytosine in complementary strand, AluBI hydrolyzes unmodified strand (lanes 7, 9) whereas AluI does not (lanes 6, 8).

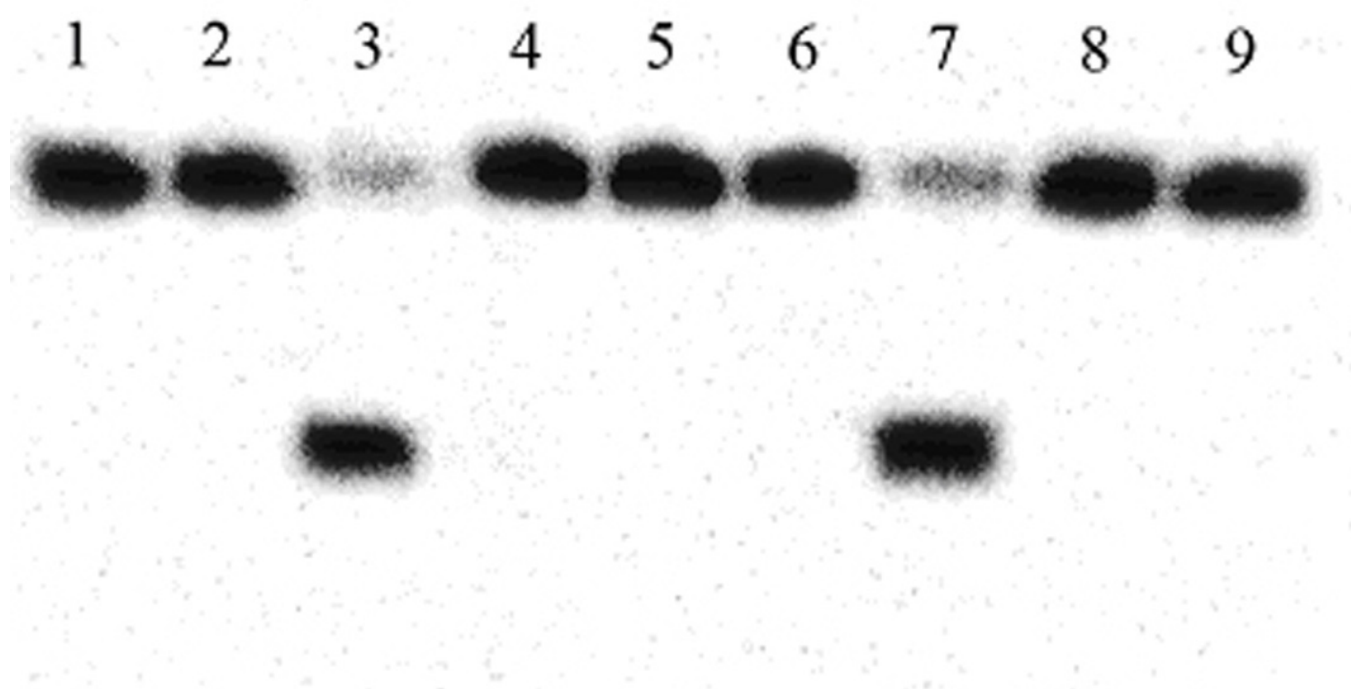


**Fig. 4.** AluI and AluBI activity in cleavage of methylated oligonucleotides

Lanes:

- 1 -duplex Alu5\*/ Alu2;
- 2 - duplex Alu5\*/ Alu2, RE AluI;
- 3 - duplex Alu5\*/ Alu2, RE AluBI;
- 4 - duplex Alu5\*/ Alu6, RE AluI;
- 5 - duplex Alu5\*/ Alu6, RE AluBI;
- 6 - duplex Alu5\*/ Alu4 RE AluBI;
- 7 - duplex Alu5\*/ Alu4, RE AluI;
- 8 - duplex Alu5\*/ Alu8, RE AluI;
- 9 - duplex Alu5\*/Alu8, RE AluBI;

Data on AluI and AluBI hydrolysis of the oligonucleotide duplexes, which contain 5-methylcytosine or N4-methylcytosine in the labeled strand of recognition sequence, are presented on fig.4 and on fig.5, respectively. According to the data on fig. 4 AluBI hydrolyzes methylated strand if complementary strand is unmodified (lane 3) or contains 5-methylcytosine (lane 5); however AluI does not cleaves such methylated DNA (lanes 2, 4). Thus, AluBI, unlike AluI, hydrolyzes both strands of DNA in the presence of one or two 5-methylcytosines in the recognition sequence. However, the strand with 5-methylcytosine is not hydrolyzed with AluI and AluBI if complementary strand contains N6-methyladenine (lanes 6, 7). Finally, AluBI, unlike AluI, cleaves the strand with 5-methylcytosine in the presence of N4-methylcytosine in complementary strand (lanes 8, 9).



**Fig. 5.** AluI and AluBI activity in cleavage of methylated oligonucleotides

Lanes:

- 1 -duplex Alu7\*/ Alu2;
- 2 - duplex Alu7\*/ Alu2, RE AluI;
- 3 - duplex Alu7\*/ Alu2, RE AluBI;
- 4 - duplex Alu7\*/ Alu8, RE AluI;
- 5 - duplex Alu7\*/ Alu8, RE AluBI;
- 6 - duplex Alu7\*/ Alu6 RE AluI;
- 7 - duplex Alu7\*/ Alu6, RE AluBI;
- 8 - duplex Alu7\*/ Alu4, RE AluI;
- 9 - duplex Alu7\*/Alu4, RE AluBI;

As shown on fig.5 AluBI, unlike AluI, hydrolyzes the strand with N4-methylcytosine in recognition sequence if complementary strand is not modified (lanes 2 and 3). However, both AluI and AluBI do not cleave oligonucleotide containing two N4-methylcytosine in the recognition sequence (lane 4, 5). Thus, AluBI, unlike AluI, is able to hydrolyze DNA if the recognition sequence contains only one N4-methylcytosine. The labeled strand with N4-methylcytosine is also hydrolyzed by AluBI, but not AluI if the complementary strand of recognition sequence contains 5-methylcytosine (lanes 6 and 7). At the same time, as one would expect, both enzymes does not cleave oligonucleotide if its structure contains N6-methylcytosine (lanes 8 and 9).

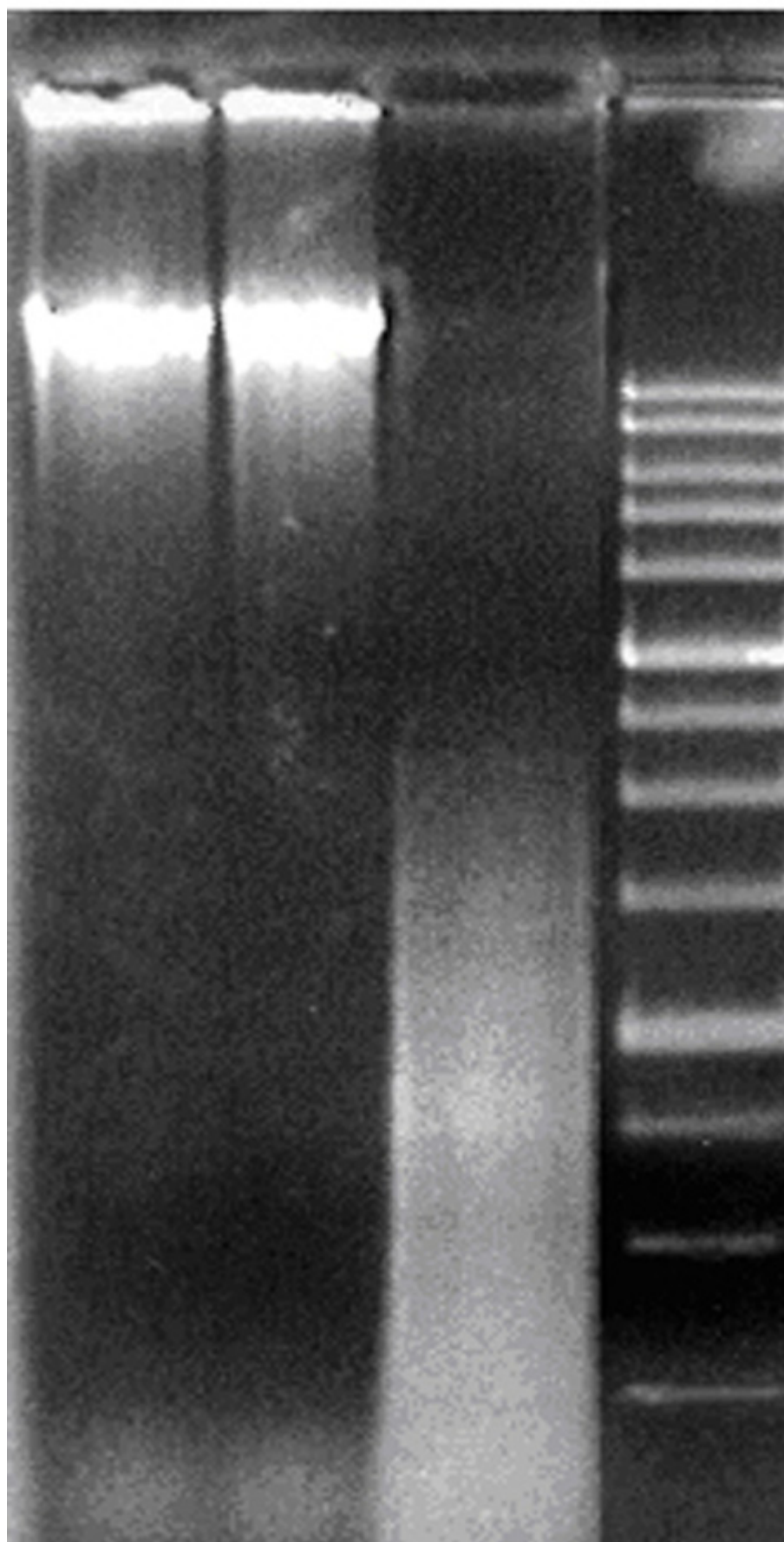
Thus, unlike AluI, restriction endonuclease AluBI is capable to cleave a recognition sequence 5'-AGCT-3' if one or two cytosines in position C5 or one cytosine in position N4 are methylated.

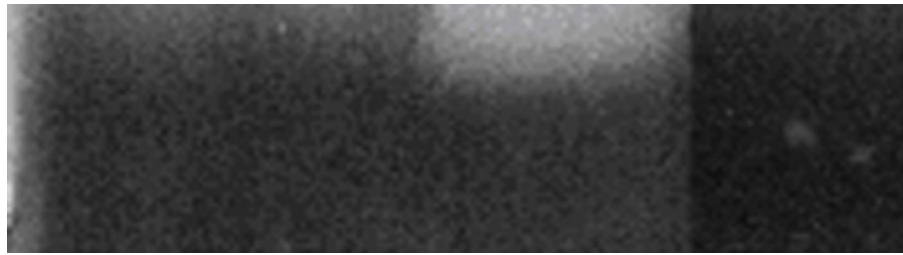
**Application opportunities of restriction endonuclease Alu and AluBI for determination of the methylation status of chromosomal DNAs and for selective**

## hydrolysis of C5-methylated DNAs

Bacterial DNA of strain *Arthrobacter luteus*, which is a producer of AluI restriction endonucleases, contains 5-methylcytosine in a sequence 5'-AGCT-3' [14]. On fig.6 we have presented data on the cleavage of *Arthrobacter luteus* DNA with AluI and AluBI. According to data provided on fig.6 AluI doesn't cleave DNA, whereas AluBI cut this chromosomal DNA very well. Because of various sensitivity of enzymes AluI and AluBI to C5-methylation of the recognition sequence, this restriction endonucleases can be used for determination of the modified bases in sequence 5'-AGCT-3' in chromosomal DNAs.

**1 2 3 4**





**Fig. 6.** Digestion of *Arthrobacter luteus* chromosomal DNA with restriction endonucleases AluI and AluBI

Lanes:

- 1 - *Arthrobacter luteus* chromosomal DNA;
- 2 - *Arthrobacter luteus* chromosomal DNA, RE AluI;
- 3 - *Arthrobacter luteus* chromosomal DNA, RE AluBI;
- 4 - 1 kb DNA ladder

AluI and AluBI may be used for determination of DNA methylation status for eukaryotic genomes. The presence of 5-methylcytosine in DNA, as a result of CNG and CT methylation, is shown for some eukaryotes [15,16]. In this case, due to partial or full overlapping these methylation sites with sequence 5'-AGCT-3', AluBI is able to hydrolyze the modified recognition sequence whereas AluI is not.

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