

A simple technique for detection of restriction endonucleases in bacterial colonies

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A simple technique is proposed for detection of bacterial restriction endonucleases. Analysis is performed directly in the cells from colonies cultivated on Petri dishes. The cells are collected by a microbiological loop into microtubes and then are treated with lysozyme and Triton X-100. After centrifugation of bacterial lysat the supernatant is tested for a presence of site-specific endonuclease activity. The technique enables to analyze about 100 colonies for 3-4 hours.

A broad application of restriction endonucleases in gene-engineering works explains the overgrowing interest of researchers to the properties of these enzymes and methods of their production and discovery [1]. The detection of site-specific endonuclease activities in bacterial strains is a necessary step in the search for restriction enzymes producing strains. However, this problem's solution by biological methods is not always correct due to multiple causes of restriction [2]. Cell destruction and direct analysis of the cell clear lysat ability to hydrolyze DNA may be considered to be the most suitable method for detection of endonuclease activities in a bacterial cell [3]. In some cases, detergents and lysozyme are used in the course of cell destruction and enzyme purification. For instance, lysozyme and Triton X-100 were used to purify Pst I enzyme [4]. Some time later in publications of Grachev and Netesov [5] as well as Zhuravlev and Oreshkina [6] it was shown that the use of Triton X-100 increased the yield of restriction enzymes from 2 to 10 times. However, experimental data do not reveal what this effect is associated with.

The method described by Whitehead and Brown [7] is, probably, most close to the technique described below. These authors cultivated bacteria in 4 ml of liquid nutrient medium, the produced biomass was treated with lysozyme and detergent Bridge-58 and thus obtained cell extract was analyzed for the presence of specific DNase activity. There are some other methods to detect the endonuclease activities in bacteria, but they also include cultivation of bacteria in liquid nutrient medium [4, 8], which considerably increases labor expenditures.

This work describes a simple method for testing bacterial strains for the presence of site-specific DNase activity without preliminary cultivation of bacteria in liquid nutrient medium, which considerably simplifies the screening procedure. The whole analysis takes up 3 - 4 h and allows testing up to 100 individual bacterial colonies in a single experiment.

EXPERIMENTAL PART

Methods

The strains *Bacillus amyloliquefaciens* H (B1664), *B. megaterium* 1884 de Bery (B2506), *Rhodopseudomonas sphaeroides* (B2013), *Flavobacterium okeanokoites* (B2835) were obtained from the collection of the Central Museum of Industrial Microorganisms ARRI of Genetics and Selection of Industrial Microorganisms (Moscow). (*B. amyloliquefaciens* H - producer of Bam HI restrictase; *B. megaterium* 1884 de Bery - producer of Bme 181 restrictase, Ava II isoschizomer; *R. sphaeroides* - producer of Rsa I restrictase; *F. okeanokoites* - producer of Fok I restrictase.)

Lysozyme A (NPO "Biolar", Olaine), phage λ DNA (NPO "Ferment"), Triton X-100 ("Serva", FRG), ethidium bromide ("Sigma", USA), agarose type I ("Sigma", USA) were used in the work. The other reagents were manufactured in the USSR (chemically pure, high purification degree).

The analyzed strains were plated on 1.5% agarose medium containing 35 g/l of sprat hydrolysate and grown up to the formation of colonies of not less than 2 mm in the diameter. *B. amyloliquefaciens* and *B. megaterium* were grown at 37°, and *R. sphaeroides* and *F. okeanokoites* at 30°. Bacterial cells were collected from Petri dishes at the tip of a microbiological loop and transferred into 250 μ l of the incubation mixture containing 100 mM tris-NCl, pH 8.0, 50 mM NaCl, 5 mM EDTA as well as lysing components - 0.1% Triton X-100 and freshly prepared lysozyme at the concentration of 0.1 g/l. The mixture was incubated for 15 min at room temperature at periodic shaking. The bacterial lysat was cleared by centrifugation on "Eppendorf" centrifuge for 1 min (10 000 g). One - four μ l of supernatant was added to samples each containing 0.5 μ l of phage λ DNA in 20 μ l of the incubation mixture and incubated for 1 - 2 h at 30°. The following incubation mixture was used for this purpose: 20 mM tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM 2-mercaptoethanol. The presence of site-specific DNase activity was determined by electrophoresis in an agarose gel. The reaction products were run in 1% agarose gel-electrophoresis. Electrode buffer contained 40 mM tris, 20 mM CH₃COOH, 1 mM EDTA, pH 8.3. Electrophoresis was performed at 10 V/cm for 1 h. After the procedure the gel was stained in ethidium bromide solution - 5 mg/l, 30 min. The DNA bands were seen when the gel was illuminated by ultraviolet light. The presence of clear DNA bands in the band indicates the presence of site-specific DNase activity, and the smeared tail - the presence of nonspecific DNase activities. The gels were photographed with "Zenit" camera through a red filter.

RESULTS AND DISCUSSION

Fig. 1 presents the data of analysis of restriction enzymes activity in the strains *B. megaterium* and *R. sphaeroides* at different cell treatments procedures. Fig. 1 shows that incubation of *R. sphaeroides* with lysozyme did not reveal significant restrictase activity while the presence of Triton X-100 allowed us to clearly observe the picture of DNA fragmentation. In the case of *B. megaterium*, the situation was quite opposite. Incubation with Triton X-100 did not provide any considerable release of restrictase into the

solution while lysozyme produced such an effect.

Simultaneous treatment with lysozyme and Triton X-100 allowed us to detect restrictase activity in both strains.

Fig. 1 The effect of lysozyme and Triton X-100 on the extraction of restrictases into the solution. 1 -3 *B. megaterium* strain; 4 - 6 - *R. sphaeroides* strain. Restrictase test was performed as described in the Methods. Cell treatment: 1, 4 -lysozyme without Triton X-100; 2, 5 - Triton X-100 without lysozyme; 3, 6 - Triton X-100 and lysozyme.

Fig. 2, a shows the dependence of the restriction enzyme activity on the time of incubation of *B. megaterium* cells. The DNA hydrolysis was more complete when cells were incubated for 15 min; the absence of lysozyme considerably decreased the extraction of restrictase. The activity of Bam HI restrictase from *B. amyloliquefaciens* H was noticeable only by the 15th minute of cell incubation (Fig. 2, b). It was impossible to reveal restrictase activity in the absence of Triton X-100.

Fig. 2 The dependence of restriction enzyme Bme18I (a) and Bam HI (b) extraction on the incubation period and the incubation mixture composition. Test on restriction endonuclease activity test was

performed as described in the Methods. Incubation time, min: 1 - 2, 2 - 5, 3 - 15, 4 - 15 (without lysozyme), 5 - 15 (without Triton X-100).

Thus, to widen the range of tested microorganisms, the incubation mixture should contain both Triton X-100 and lysozyme. Cell incubation should be performed for not less than 15 min at room temperature. We used *F. okeanoikoites* - producer of FokI restrictase - to evaluate the method sensitivity. It is known that the yield of this enzyme is not more than 250 U per 1 g of wet cells [9]. Fig. 3 presents the result of the test for FokI restrictase.

Fig. 3 for Fok I restriction endonuclease. The test was performed as described in the Methods.

Let us note the peculiarities of the proposed technique: the strains are tested without special cultivation in liquid nutrient medium; simultaneous incubation of cells with lysozyme and Triton X-100 is performed to widen the range of tested microorganisms.

The use of this technique allows us to reveal restriction endonuclease activity in a large number of microorganism strains. For example, we noticed the presence of restrictase activity in the following genera of microorganisms presenting both natural isolates and museum cultures: *Bacillus*, *Kurtia*, *Paracoccus*, *Vibrio*, *Pseudomonas*, *Rhodopseudomonas*, *Proteus*, *Providencia*, etc.

In some cases, we could not notice the presence of restrictases. For example, we did not reveal restrictase activity using this method in the cells of *Streptomyces* and *Nocardia*. Probably, this is accounted for the structure of their cell membrane, which can't be opened by lysozyme and Triton X-100.

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