DETERMINATION OF RESTRICTION ENDONUCLEASE ACTIVITY IN TOLUENE LYSATES OF BACTERIAL CELLS

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By now more than 350 restriction endonucleases have been described in the literature [7] and they are widely used in molecular biology and in bioengineering research [3]. Selection of strains producing restriction endonucleases with a view to finding new and unique restriction enzymes is continuously in progress. For this reason there is an urgent need for quick and reliable ways of determining restriction endonuclease activity in bacterial cells. The method generally used to estimate restriction endonuclease activity in bacterial cells involves disintegration of the cells with ultrasound followed by high-speed centrifugation in order to obtain a cell-free extract. An essential shortcoming of this method is that the extracts contain activity of nonspecific endo- and exonucleases, in the presence of whose action it is not always possible to reveal specific activity of restriction endonucleases. For instance, according to data in the literature [5], activity of only three of the 16 restriction endonucleases studied can be found in unpurified extracts.

The object of this investigation was to develop a rapid method of determining restriction endonuclease activity in bacterial cells.

EXPERIMENTAL METHOD

A culture of *Bacillus amyloliquefaciens* H, grown as described previously [2], was used. The incubation mixture (60 μl) for determination of activity of restriction endonuclease Bam H1 contained 6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 1 μg DNA of phage λ, and 1-30 μl of bacterial cell extract. Incubation was carried out at 37°C for 30-60 min. The reaction was stopped by addition of glycerin with bromphenol blue and Na_2-EDTA to a final concentration of 10% (v/v), namely 0.02% and 20 mM respectively. The DNA fragments were separated in 1% agarose plates. The electrode buffer contained 40 mM Tris-acetate, pH 8.3, 20 mM sodium acetate, and 2 mM Na_2-EDTA. Electrophoresis was carried out at room temperature for 2-3 h at a voltage of 3 V/cm. The gel was stained for 15 min in a solution of ethidium bromide (10 μg/ml) and DNA fragments were revealed by fluorescence in UV light at 350 nm. The unit of restriction endonuclease Bam H1 activity was taken to be the quantity of enzyme required to produce complete degradation of 1 μg DNA of phage λ at 37°C in 60 min in a standard incubation mixture.

EXPERIMENTAL RESULTS

Considering difficulties connected with testing restriction endonucleases in bacterial cells, several workers have suggested way of extracting these enzymes from the cells, as well as nonspecific methods for determining their activity. This is the case with chromatography of cell-free extracts on columns with affinity ligands [4], the use of an immunosorbent [1], and several other methods. Much of the activity of restriction endonucleases is known to be extractable from cells of Gram-negative microorganisms by means of osmotic shock. This result indicates that the restriction endonucleases are located in the periplasm and (or) on the surface of the cell membrane, which has been demonstrated for restriction endonuclease Pst 1 [8]. Naturally exposure to agents which remove the cell membrane and do not disturb the integrity of the cytoplasmic membrane may lead to extraction of restriction endonucleases

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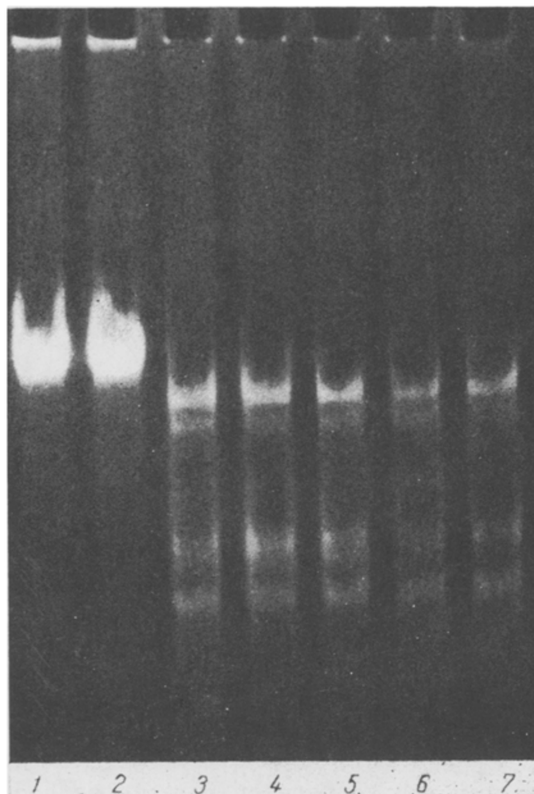

Fig. 1. Electrophoresis of hydrolysis products of DNA of phage λ in agarose gel by extract from cells of *B. amyloliquefaciens* H after treatment with toluene. Quantity of extract added to incubation mixture: 1 μl (1), 5 μl (2), 10 μl (3), 15 μl (4), 20 μl (5), 25 μl (6), and 30 μl (7).

from the cells. As such an agent we chose toluene. The rapid method of determining restriction endonuclease activity which we developed using the Bam HCl enzyme as the example, is as follows. Cells of *B. amyloliquefaciens* H were washed free from culture medium with buffer containing 10 mM NaH_2PO_4 = Na_2HPO_4, pH 7.4, 10 mM 2-mercaptoethanol, 1 mM Na_2-EDTA, and 1 mM NaN_3, after which they were suspended in the same buffer up to an optical density of 15-17 units of 650 nm. To 1 ml of suspension 0.1 ml of toluene was added, the suspension was vigorously shaken in the cold (4°C) for 10 min, after which it was allowed to stand for 15 min to enable separation of the aqueous and toluene phases. To determine activity of restriction endonuclease Bam H1 aliquots from the aqueous phase were incubated with DNA of phage λ and the hydrolysis products were separated and identified, as described in "Experimental Method."

It will be clear from Fig. 1 that after addition of toluene lysate to the incubation mixture in doses of 10, 15, 20, 25, and 30 μl complete hydrolysis of DNA of phage λ, specific for that particular restriction endonuclease, was observed. Meanwhile, in experiments with unpurified extract obtained after ultrasonic destruction of the cells, complete hydrolysis of DNA by nonspecific exo- and endonucleases took place after incubation of 4-5 μl of the extract with DNA for only 10 min, as a result of which specific activity of endonuclease Bam H1 could not be determined. Calculation shows that to estimate activity of restriction endonuclease Bam H1 in bacterial cells 3-6 mg of biomass, equivalent to about 1-2 ml of culture fluid with an optical density of 1.0 unit at 650 nm, is sufficient. For comparison it will be recalled that according to data in the literature [8], to determine activity of restriction endonuclease Pst 1, extracted from cells by the osmotic shock method, 100 ml of culture with an optical density of 1.1 unit at 650 nm was needed.

Other experiments on a larger quantity of bacterial mass (3-4 g) showed that treatment with toluene extracts up to 75-80% of total activity of restriction endonuclease Bam H1.

Meanwhile, during lysis of the cells with toluene only one-quarter of the total protein present in cell-free extracts after disintegration of the cells by ultrasound was liberated into the extract.

The method of determining activity of restriction endonuclease Bam H1 in bacterial cells devised by the writers thus compares favorably with those described in the literature as regards simplicity, speed, small quantity of biomass required for analysis, good reproducibility, and dispensation with the need to obtain a cell-free extract, connected with ultrasonic disintegration of the cells and high-speed centrifugation. The method can probably be used also for preparative extraction of restriction endonucleases from cells, which would greatly simplify the procedure of their subsequent purification.

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POSSIBLE INDUCTION OF RECOMBINATION OF DNA FRAGMENTS FROM DIFFERENT TISSUES BY HYDROCORTISONE

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According to data in the literature, after a single injection of nucleic acid precursor the label is intensively and rapidly incorporated into DNA of proliferating tissues. Later, during a steady reduction in the pool of radioactive precursor, the size of which is almost identical for all tissues, and, correspondingly, with a decrease in specific radioactivity of the DNA of tissues with a high mitotic index, the specific radioactivity of DNA of mitotically less active tissues rises [2]. It can be postulated on the basis of these findings that incorporation of label into DNA of tissues with a low mitotic index after a single injection of radioactive precursor can take place not only at the monomer level, but also by incorporation of entire fragments of DNA of mitotically active tissues. The DNA is evidently transported through the blood stream. Under these circumstances the main source of extracellular plasma DNA is considered to be normocytes, which have lost their Y nuclei and are thus converted into erythrocytes [9]. After injection of exogenous DNA into the blood stream of animals, it can be incorporated into the recipient's DNA [8]; this process, moreover, can take place also in the presence of hydroxyurea, which inhibits semiconservative DNA synthesis. The cells incorporate exogenous DNA, of both high and low molecular weight, but native DNA is incorporated much more efficiently than denatured DNA [12]. Not only homologous, but also heterologous DNA, injected into animals, can be incorporated into the recipient's genome, and this is accompanied by the appearance of the donor's genetic features [14]. We also know that recombination of mobile dispersed genes within the genome can take place in eukaryotes, in the same way as in prokaryotes [3]. Since incorporation of exogenous fragments into DNA

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