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A rapid and simple method for detection of type II restriction endonucleases in cells of bacteria with high activity of nonspecific nucleases

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In this work we describe a novel, rapid and simple microscale procedure for identification of restriction endonuclease activity in bacteria lysates, which contain high levels of non-specific DNA nucleases.

Key words: bacteria screening, site-specific endonuclease purification, type II restriction endonuclease

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INTRODUCTION

Restriction endonucleases are found in many Prokaryota, mainly bacteria (Bickle & Krüger, 1993). They are part of the restriction-modification system which also comprises methyltransferase activity. It is generally considered that their principal biological function is host genome protection against foreign, in particular bacteriophage, DNA (Arber, 1979; Krüger *et al*., 1983).

Type II restriction endonucleases require Mg^{2+} to cleave DNA. They recognize double stranded DNA at palindromic sequences and cleave it at specific sites within or adjacent to their recognition sequences (Pingoud & Jeltsh, 2001).

Site-specific type II endonucleases are utilised in a wide variety of applications (Roberts, 2005). They are important tools in recombinant DNA technology, gene engineering, mapping of genomes and medical diagnostics. Because of their broad applications, the isolation of novel restriction endonucleases is in demand.

Usually, the first step in the production of new commercially useful restriction endonucleases is bacteria screening. It is very important to choose an adequate, easy, quick and highly sensitive method that can provide screening for a large number of strains.

One of the methods that meets these conditions is the one described by Belavin *et al.* (1988). This method allows one to detect the presence of a restriction endonuclease activity in lysates from a single bacterial colony. The bacterial cell walls are disrupted by lysing buffer and the cell-free extract is scanned for the enzyme activity.

This method is usually satisfactory, but fails when high activities of non-specific DNA-degrading nucleases are present in the bacterial lysates. Their presence is the main limiting factor for the successful isolation of restriction endonucleases and their prior removal is necessary (Poch & Somkuti, 1993; 1995; Puchkova *et al*., 2002).

Conventionally, restriction endonucleases can be purified by column chromatography on a variety of matrices (Whitehead *et al*., 1985; Kaida *et al*., 1999). However, these methods require the use of large volumes of both bacterial culture and chromatography matrix (Greene *et al*., 1978; Smith *et al*., 1976), which makes them costly, time-consuming and thus not useful for bacteria screening.

This work describes a quick and simple microscale procedure for preliminary identification of restriction endonuclease activity in lysates of *Enterobacteriaceae*, which are known to contain relatively high levels of nonspecific DNA nucleases. However, our unpublished result indicate that it is universally applicable to a wide variety of bacteria.

MATERIALS AND METHODS

Bacterial strains, culture conditions and identification. Strains of *Enterobacteriaceae* were isolated from sewage samples obtained from the Gdańsk-Wschód Mechanical-Biological Sewage Treatment Plant as well as biological materials from patients of The Mikołaj Kopernik State Hospital in Koszalin, where they were identified according to Isenberg (2007).

Isolation, characterization and primary analysis of *Enterobacteriaceae* from sewage samples were performed on MacConkey agar (Emapol). Both lactose positive and negative bacterial colonies were selected for further tests. Gram staining (Gram PVP kit; Graso) and cytochrome oxidase test (BioMerieux) were performed to exclude other Gram negative but non-fermenting bacilli.

Detailed identification of bacterial strains was performed using Microgen GN-ID test (Graso) (Edberg *et al*., 1979) and Api 20E test (BioMerieux) (Edwards & Ewing, 1972).

Short characteristics of the microbial strains tested is presented in Table 1.

Single bacterial colonies from MacConkey agar were taken to isolate cell lysates according to Belavin *et al.* (1988). To isolate cell lysates by sonication, single bacterial colonies were further cultured for 24 h in 100-ml Erlemayer flask containg 20 ml of Luria–Bertani (LB) medium, at 37°C with shaking.

Preparation of the cell-free extract. To prepare cell free extract according to the Belavin *et al.* (1988) protocol, a single bacterial colony was taken from agar plate and transferred into 40 µl of lysing buffer (100 mM Tris/HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100 and freshly pre-

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Abbreviations: LB, Luria–Bertani medium

Table 1. Characteristics of restriction endonuclease-producing bacteria

Strain	Strain origin	Izoschizomer of	Lactose on MacConkey agar	Urease	Indole	H_2S
Escherichia coli	hospital	EcoRV	+	–	+	–
Escherichia vulneris	hospital	EcoVIII	–	–	–	–
Citrobacter freundii	sewage	BspEI	+	-	–	+
Klebsiella pneumoniae	sewage	BstEII	+	+	–	–

pared lysozyme 0.1 g/l (Sigma)). The mixture was incubated for 15–30 min. at room temperature with periodic shaking.

To prepare cell free extract by sonication, 20 ml of bacterial culture was centrifuged at 4000 × *g* for 10 min. Cells were washed twice with 10 mM K/PO_4 buffer pH 7.0 (Sambrook *et al.*, 1989) (10 mM K/PO_4, 10 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol), resuspended in 3 ml of sonication buffer (10 mM K/PO_4, pH 7.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 15 mM phenylmethylsulfonyl fluoride (PMSF), 5% glycerol) and disintegrated by sonication at 4°C in an ice bath, with 30 × 10-second pulses with one-minute intervals at an amplitude of 12 mm.

Partial purification of restriction endonucleases from bacterial cell free extracts. Bacterial cell free extracts obtained by sonication or the Belavin *et al.* (1988) method were centrifuged at 15000 × g for 30 min at 4°C to remove cells debris. Supernatant was loaded on recycled plasmid mini kit V-spin columns (the ones from A&A Biotechnology were used, but it is possible to use mini columns from another company). The exploited silica-based layer was removed from the glass fiber column and replaced with one of the following chromatography matrices: phosphocellulose P-11 (Whatman), heparin-sepharose (Sigma Aldrich), CM sephadex C-50 (Pharmacia) (cation exchangers), or blue-agarose (Pharmacia) (affinity medium). The columns were filled with 250 µl of a chromatography matrix for purification of the extracts obtained by Belavin method and 1.0 ml for the extracts obtained by sonication. They were washed with 3 × column volume of ice-cold 10 mM K/PO_4 (pH 7.0) buffer. To elute proteins, a stepwise gradient of 0.2–1.0 M KCl (0.2 M increments) was used and 50 µl (from columns with 250 µl of chromatography matrix) or 100 µl fractions (from columns with 1 ml of chromatography matrix) were collected after 30 s. 500 × *g* centrifugation at 4°C. Each fraction was assayed for restriction endonuclease activity as described below.

Endonuclease activity assay. Either the crude bacterial extracts obtained by Belavin *et al.* method (1988) and sonication, or the individual fractions eluted from the chromatography column were added to bacteriophage λ DNA. The digestion was performed for 15–30 min. at 37°C in a total of 20 µl reaction mixture of the following composition: 2 µl of the protein extract, 2 µl of 10 × yellow Tango™ buffer (Fermentas) (330 mM Tris-acetate pH 7.9, 100 mM Mg-acetate, 660 mM K-acetate, 0.1 mg/ml BSA), 1 µg of λ DNA substrate and 15 µl of sterile dH_2O.

The presence of site-specific DNase activity was determined on 1% agarose gel containing 0.5 µg/ml of ethidium bromide, in TBE buffer. The recognition of the restriction endonuclease activity was first established by a comparison of the pattern of digestion with the NEBcutter V2.0 database (New England BioLabs, http://rebase.neb.com/rebase/rebase.html) and followed by comparison of the digest with those of commercially available enzymes.

RESULTS AND DISCUSSION

A major problem that occurs during screening for restriction endonucleases is the presence of nonspecific nucleases in bacterial cells. Their prior removal is advised, as they may mask the presence of restriction endonucleases. We have developed a simple, rapid and low cost method to address this problem.

In the method described here, we used recycled plasmid mini kit V-spin columns, with the silica-based layer removed and replaced with a small volume of a chromatography matrix. Bound proteins were eluted with a stepwise gradient of KCl and the samples were collected by centrifugation.

This method allows one to obtain, within just several minutes, lysate samples purified enough to unmask determine the activity of site-specific endonucleases. Each eluted sample was assayed immediately for the presence of a restriction endonuclease activity by digestion of bacteriophage λ DNA. The endonucleases from individual column fractions remained active for two weeks (data not shown).

In this experiment we used a strain of *E. coli* expressing HindIII endonuclease (Mruk & Kaczorowski, 2003). The cell free extracts obtained with lysing buffer (Fig. 1A, lane 1) as well as sonication (Fig. 1A, lane 2) are rich in non-specific nucleases precluding the visualisation of the site-specific activity. The cell free extracts were purified on mini-columns with phosphocellulose P-11 in accordance with the method described in detail above. Results of the purification are shown in Fig. 1B and C.

The use of phosphocellulose P-11 removed some of the nonspecific endonucleases from the bacterial lysates

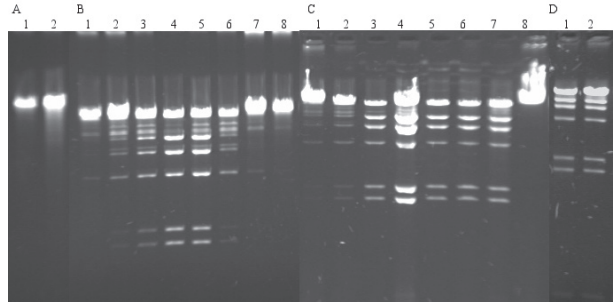

Figure 1. Products of bacteriophage l DNA digestion.
(A) crude *E. coli* extract with HindIII activity obtained by: 1) Belavin method, 2) sonication. **(B)** extract obtained by Belavin method after phosphocellulose purification (nonspecific bands are visible, probably due to inefficient removal of nonspecific nucleases) **(C)** extract obtained by sonication after phosphocellulose purification (nonspecific nucleases were removed, hence specific bands are present and l DNA/HindIII endonuclease digestion pattern is clear; lane 5, 6,7 specific bands; lane 1,2,3,4,8, presence of unspecific bands). **(B)** and **(C)** HindIII eluted with various concentrations of KCl (lanes: 1 0.2 M; 2,3 0.4 M; 4,5 0.6 M; 6,7 0.8 M; 8 1.0 M). **(D)** bacteriophage l DNA digestion pattern obtained by: 1) commercial enzyme 2) sonicate after phosphocellulose purification (0.8 M KCl elution fraction).

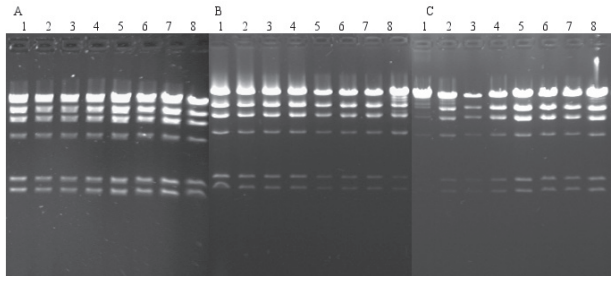

Figure 2. Patterns of bacteriophage l DNA digestion with sonicated extract of *E. coli* with HindIII activity after purification on (A) blue-agarose, **(B)** heparin-sepharose, **(C)** sephadex C-50 and elution with various concentrations of KCl. Lanes: 1 — 0.2 M; 2, 3 — 0.4 M; 4, 5 — 0.6 M; 6, 7 — 0.8 M; 8 — 1.0 M.

obtained according to Belavin *et al.* (1988). After digestion of λ DNA, nonspecific bands were observed in each lane (Fig. 1B), possibly due to an incomplete digestion of the DNA substrate. It may have resulted from small amount of restriction endonucleases present in the samples, as they were obtained from single bacterial colonies. Additionally, restriction endonucleases are lost during the purification process.

In contrast, when we used sonicated bacterial extract purified on phosphocellulose, we got a digestion pattern similar to the one obtained with commercial HindIII (Fig 1 D). This means that the purification on the mini-column removed the nonspecific nucleases (Fig 1C, lane 5, 6 and 7).

Although sonication of bacterial cells is known to release various proteins including non-specific nucleases (Smith *et al.*, 1976) our purification method allowed for their removal and enabled detection of site-specific nucleases.

Usually, to achieve high-purity restriction endonucleases, multistep purification on several matrices is used (Puchkova *et al.*, 2002). However, screening for restriction endonuclease activity requires only partial purification.

We investigated the possibility of using various chromatography matrices. Hence, the crude extract obtained by sonication was also purified on heparin-sepharose, CM sephadex C-50 or blue-agarose. All of them proved to be effective in purifying HindIII restriction endonuclease from the experimental strain of bacteria (Fig. 2A, B, C).

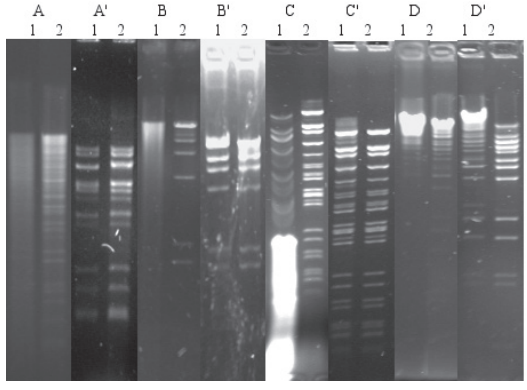

Figure 3. Bacteriophage l DNA digested with endonucleases extracted from various strains of bacteria.
(A, B, C, D) lanes: 1 crude bacterial extract, 2 extract after phosphocellulose purification. **(A', B', C', D')** lanes: 1 extract after phosphocellulose purification, 2 commercial enzyme. Following strains of bacteria were used: **(A, A')** *E. coli* (EcoRV); **(B, B')** *E. vulneris* (EcoVIII); **(C, C')** *Citrobacter freundii* (BspEI); **(D', D')** *Klebsiella pneumoniae* (BstEII).

Our laboratory collection of *Enterobacteriaceae* contains several bacterial strains with high activity of nonspecific nucleases that preclude identification of site-specific endonuclease activity. Sonicated lysates of these bacteria were purified using the method described here. Bacteriophage λ DNA was digested with obtained chromatography fractions. To confirm the digestion specificity, the patterns were compared to those obtained with commercial enzymes (Fig. 3). The restriction patterns of digested λ DNA showed the presence of enzymes with the specificity identical with: EcoRV (Fig. 3A, A') in *Escherichia coli*, EcoVIII (Fig. 3B, B') in *Escherichia vulneris*, BspI (Fig. 3C, C') in *Citrobacter freundii* and BstII (Fig. 3D, D') in *Klebsiella pneumoniae.*

Another method for the removal of nonspecific nucleases before screening for restriction enzymes was described by Puchkova *et al.* (2002). Their method is based on the treatment of crude cell extracts with a temperature above 50°C for 10 minutes. Those authors have shown that in some strains of bacteria this procedure causes inactivation of nonspecific nucleases without reducing the activity of site-specific restriction endonucleases. Although this method is effective for strains producing thermo-resistant restrictases, during bacteria screening one is not aware whether the enzymes to be identified are resistant to a high temperature or not.

Another solution to the problem of nonspecific nucleases was published by Poch & Somkuti (1993; 1995). They presented a simple method, where an affinity gel is placed directly in Eppendorf tubes containing sonicated and centrifuged bacterial extract and then washed with buffers. They showed that this procedure removed DNA, RNA and nonspecific nucleases. That is a quick and simple method, however, when high amounts of nonspecific nucleases are present, there is a possibility that the bacterial extracts will not be purified sufficiently.

The purification method described in the present publication is effective and avoids the pitfalls of the previously published protocols. The procedure is not only very easy, but also does not require any advanced equipment and thus can be used even in laboratories that are not specialised in protein purification. It is particularly useful when screening of numerous bacterial strains is performed. The method may be also applied for bacteria not belonging to the *Enterobacteriaceae* family (data not shown).

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