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COMMUNICATION

Screening of Expression cDNA Libraries for Polysaccharide Hydrolysing Enzymes: Isolation of cDNA Clones Encoding Cellulase, Xylanase, and β-1,3-Glucanase of *Phytophthora parasitica*

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A method to screen cDNA libraries for clones encoding enzymes which hydrolyze macromolecular substrates was established. The method is based on the direct screening of expressed cDNA libraries in phage vectors by plating them at high densities and overlaying the plates with a thin agar layer containing substrates chemically conjugated with Remazol Brilliant dyes. Plaques expressing an enzymatic activity which degrades the substrate are identified by the production of cleared zones in the coloured top agar layer. A cDNA library of the phytopathogenous oomycete *Phytophthora parasitica* was constructed and screened for expression cellulase (EC 3.2.1.4), β-1,3-glucanase (EC 3.2.1.39) and xylanase (EC 3.2.1.8). © 1993 Wiley-Liss, Inc.

METHODOLOGY

Culture Conditions

Phytophthora parasitica DSM 1829 was grown in medium GM7: 3 g L-Asparagine, 1 g KH_2PO_4, 0.5 g $MgSO_4$-$7H_2O$, 50 mg $CaCl_2$, 10 mg $FeCl_3$-$7H_2O$, and 1 mg thiamine were dissolved in distilled water, the pH was set to 5.5 with 2 M NaOH, and the solution was made to 900 ml and autoclaved for 15 min at 120°C. After autoclaving, 100 ml of sterile-filtered glucose solution (200 g/l) was added. Ten milliliters of a stationary *Phytophthora* culture in GM7 minced for 5 s in Waring Blendor was used to inoculate 100 ml GM7 medium in 1 L Erlenmayer flasks. *Phytophthora* cultures were grown at 25°C with gentle shaking for 8 days.

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mRNA Isolation

Total RNA was isolated using a modification of the method of Chomczynski and Sacchi (1987). The mycelium of *P. parasitica* was filtered, washed with distilled water, blotted, weighed, immersed in liquid nitrogen, and grounded to a fine powder under nitrogen with mortar and pestle. Powdered frozen mycelium was slowly poured into GTC buffer (4.5 M guanidium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sodium lauryl sarcosine, and 0.1 M 2-mercaptoethanol) in a beaker under continuous stirring to avoid clumping. Five milliliters of buffer was used for each g mycelium and the suspension was stirred at room temperature for 15 min. Debris was removed by centrifugation for 10 min at 8,000*g*. One tenth vol 2 M ammonium acetate (pH 4.1), 0.5 vol water-saturated phenol, and 0.5 vol of chloroform were added to the supernatant, the mixture was vigorously shaken and placed on ice for 15 min. The emulsion was centrifuged at 15,000*g* for 15 min at 4°C. RNA was precipitated from the upper phase by mixing with 1 vol isopropanol, incubating at –20°C for 1 h and centrifugating for 10 min at 10,000*g* at 4°C. The pellet was dissolved in 1/3 vol of GTC-buffer and, after adding ammonium acetate to give 0.1 M, reprecipitated with isopropanol as above. The pellet was dissolved in 1/6 of the original volume of GTC-buffer and LiCl was added from a 10 M stock solution to give 2 M. The solution was incubated for 4 h on ice and the RNA precipitate was sedimented by centrifugation for 15 min at 15,000*g* at 4°C. The pellet was washed two times with cold 75% ethanol, dried, and dissolved in 10 mM Tris, 1 mM EDTA, 0.5% SDS, pH 7.5.

Polyadenylated RNA (further referred to as mRNA) was isolated from total RNA by chromatography on oligo(dT)-cellulose as described (Sambrook et al., 1989). The quality of the total RNA preparation was assessed by visualizing rRNA-bands on an agarose-formaldehyde gel with ethidiumbromide. The integrity of the mRNA preparation was checked by Northern hybridization using an actin gene from *Phytophthora infestans* (Unkles et al., 1991) kindly provided by J.R. Kinghorn, labeled with digoxigenin as a probe (DNA Labeling and Detection Kit from Boehringer Mannheim, Germany).

cDNA Library Construction

A library of oriented cDNA inserts was constructed in λ Uni-ZAP XR, and all chemicals used in this section were from Stratagene (La Jolla, CA). The vector was a derivative of λZAP (Short et al., 1988) with an EcoRI-XhoI polylinker added; open reading frames inserted into this polylinker can be expressed as fusion proteins with β-galactosidase after induction with isopropylthio-β-D-galactoside (IPTG). mRNA was primed with a 50 bp adaptor containing an XhoI target site and a poly(dT) 3′-terminal region. Methylated cytosine base was used in the first strand synthesis in order to protect XhoI sites in the coding sequence. The second strand was synthesized in the same reaction tube after adding excess of nonmethylated cytidine, so that most XhoI sites in the primer/adapter region were not protected. The next three steps were performed essentially as described by Sambrook et al. (1989): the ends of the cDNA were repaired with T4 DNA polymerase, ligated with EcoRI adaptors, and phosphorylated with T4 polynucleotide kinase.

The reaction product was cleaved with XhoI and cloned into EcoRI/XhoI digested λ UniZAP. The ligation mixture was packaged in vitro and plated on *Escherichia coli* strain SURE (*mcr*A, *mcr*B), which does not restrict cytosine-methylated DNA. The library was amplified using *E. coli* strain XL1-Blue (*rec*A1, *lac, end*A1, *gyr*A96, *thi, hsd*R17, *sup*E44, *rel*A1, {F´ *pro*AB, *lac*I^q, *lac*ZΔM15, Tn10}) (Bullock et al., 1987). Bacteria were grown in L medium with 12 μg/ml tetracycline, and λ phages were plated on LB-plates with 0.2% maltose and 10 mM $MgCl_2$ as described (Sambrook et al., 1989).

Screening of the Library With Coloured Substrates

Refer to the flowchart in Figure 1. The cDNA library was plated using 0.4 ml *E. coli* SURE suspension (optical density 0.5 at 600 nm) in 5 ml of top-agar containing 1 mM IPTG. After 10 h at 37°C, each plate was overlaid with 5 ml of a substrate solution containing 4 to 8 mg of substrate per ml of 0.7% agar at 46°C, and in some cases also containing 0.06% sodium azide. Substrates labeled with Remazol Brilliant Blue R as described (Wolf and Wirth, 1990) were kindly provided by S. Wirth and P. Romanczuk; these substrates are also available from Loewe Biochemica (Otterfing, Germany). The plates had to be poured on an exactly horizontal plane. Overlaid plates were incubated at room

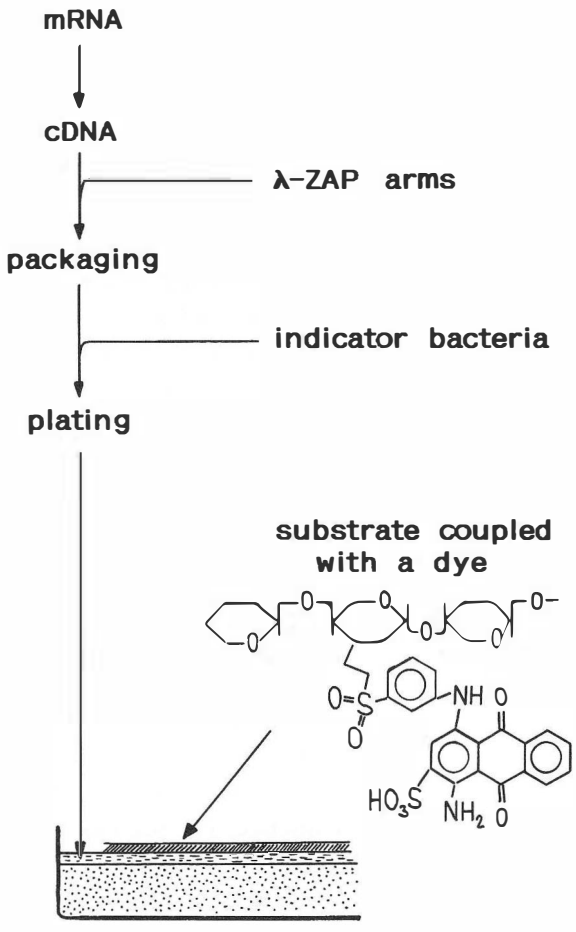
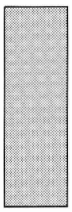


Figure 1. Flowchart. Screening of cDNA library for genes encoding carbohydrolases.



temperature for up to 14 days and inspected regularly in transmitted light. Lytic zones identified in the coloured agar layer were cut out and homogenized in SM buffer (50 mM Tris/HCl pH 7.8, 0.1 M NaCl, 10 mM $MgSO_4$, 0.01% gelatin). Dilutions of these suspensions were plated and overlaid with the substrate. Single positive plaques were isolated and amplified using *E. coli* strain XL1-Blue.

RATIONALE AND DESIGN

Polysaccharide hydrolysing enzymes (carbohydrolases) have experienced the growing attention of the scientific community in the last years. This has been largely motivated by their potential applications in biotechnology, because carbohydrolases are key enzymes in the commercialization of biomass conversion. Moreover, carbohydrolases of plant pathogenic microorganisms are investigated in plant pathology as potential pathogenicity factors.

Two standard methods are used to isolate genes encoding carbohydrolases from fungi: (1) screening cDNA libraries expressing fusion proteins with antibodies raised against the purified enzyme, and (2) screening DNA libraries by hybridization with degenerated oligonucleotide probes designed according to the amino acid sequence of a part of the enzyme. Both methods require purification of the enzyme to homogeneity. Heterologous DNA probes are of no use since the sequences of the enzymes from different organisms are very divergent (Gilkes et al., 1991; Henrissat et al., 1989).

A useful technique for the detection of carbohydrolase activity on plates is the use of substrates coloured by conjugation with reactive dyestuffs (Fernley, 1963). The advantages of the method are the possibility to conjugate different substrates with the same reactive dyestuffs (e.g., Remazol dyes from Hoechst) using standard procedures and the fact that the method is nondestructive: identified clones can be reisolated from plates after the screening. Chromogenic substrates were described for all important carbohydrolases (Biely et al., 1985, 1988a,b; Farkas et al., 1985; McCleary, 1988; Philpot and Chapman, 1977; Wirth and Wolf, 1990, 1992; Wolf and Wirth, 1990; Wood, 1988) and are available commercially (see Methodology). The goal of this work was to exploit the possibility to screen expressed cDNA libraries for carbohydrolases using chromogenic substrates.

A cDNA library of *P. parasitica* that we screened consisted of 10^6 clones, 90% of them with inserts between 0.5 and 2.5 kb. Although only low enzymatic activities may be expected in fusion proteins in cDNA libraries, we thought that this can be compensated by prolonged incubation times since carbohydrolases are exceptionally stable enzymes. Forty plates each with about 20,000 plate forming unit (pfu) were used in every screening experiment. Carboxymethylated and Remazol Brilliant Blue R colored substrates used for the screening were cellulose, curdlan, pullulan, pustulan, and xylan. In our first experiments we tried to incorporate the substrates directly into the solid medium. The method did not work and we have chosen to overlay developed plaques with a thin layer of agar containing the substrate (see Methodology and Fig. 1). This variant has an advantage of enhancing the rate of the reaction in two ways: by shortening the way the enzyme must pass by diffusion to reach the substrate and by increasing the concentration of the substrate which is confined to a small volume in the top agar layer. Since the top layer was very thin, expression of an enzymatic activity resulted in sharp, clearly defined zones.

We have found positive clones on plates with three Remazol Brilliant Blue R-coupled substrates: cellulose, curdlan (β-1,3-glucan), and xylan. Portions of the agar comprising the lytic zones were cut out, homogenized in SM buffer, diluted, plated for single plaques, and screened again. Single plaques containing cDNA sequences for putative cellulase, β-1,3-glucanase, and xylanase were amplified and stored in SM over chloroform at 4°C.

Two problems were encountered during the screening of the library. First, some lots of the coloured substrates used may contain a considerable fraction of low-molecular-weight molecules which diffuse rapidly back into cleared zones. Such substrates should be fractionated by precipitation with acid (Wolf and Wirth, 1990). The second problem was a low enzymatic activity of fusion proteins in plaques. The amount of protein expressed from a recombinant phage is expected to be lower than the amount produced from plasmids since the lysis of the cell interrupts transcription and translation. Moreover, the specific activity of a fusion protein is generally impaired as compared with the native enzyme. Carbohydrolases are exceptionally stable enzymes, that do not need cofactors and generally work well under conditions given in standard agar solid media used for plating λ phages. A high stability in connection with long incubation times makes it possible to

detect very low activities provided that the fusion protein diffuses only slowly from the place where it is produced. Two practical conclusions can be drawn from this reasoning: (1) slowing down protein diffusion by incorporating an inert matrix into the medium may improve the sensitivity of the screening, and (2) the plates should be examined very carefully through a period of up to 10 days since positive clones may reveal themselves by very weak signals.

Hydrolysis of some natural substrates may not be achievable by single cloned enzymes. For example, organisms efficiently digesting insoluble cellulose produce complex enzymatic systems with components processing different catalytic and substrate binding activities. A synergical action of different proteins is necessary for a complete digestion of such substrates. Instead of natural substrates, soluble substances with only one kind of chemical bond as a target for hydrolytic activity should be used for screening of libraries. It may be necessary to combine products of several single genes isolated in this way in order to reconstruct a hydrolytic activity against a natural substrate. In addition to carbohydrolases, the method may be useful in screening for other enzymes hydrolasing soluble macromolecular substrates like proteinases and enzymes degrading nucleic acids.

The screening of gene libraries with the use of macromolecular substrates conjugated with dyes was described for bacterial genomic libraries, which are mostly constructed in plasmid vectors (e.g., concerning xylanases, Wood et al., 1988; Flint et al., 1991; MacKenzie et al., 1989; Whitehead and Hespell, 1989; Mannarelli et al., 1990; Hu et al., 1992). In bacteria, especially gram-negative bacteria, cloned genes may be expressed from their own promoters in *E. coli* and gene products are even properly transported from *E. coli* cells in many cases. On the other hand, screening for products of eukaryotic genes in *E. coli* makes it necessary to screen cDNA libraries instead of genomic libraries. Fusion proteins generated from cDNA copies of eukaryotic genes would not be excreted from *E. coli* cells in most cases. Moreover, representative eukaryotic cDNA libraries have to contain about a thousand times more clones than representative prokaryotic cosmid genomic libraries. Such libraries can be constructed and analysed more easily using λ phage vectors which also guarantee the release of protein products from *E. coli* cells.

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