

CONSTRUCTION OF A RHIZOPUS DELEMAR GENOMIC LIBRARY
AND SCREENING FOR DIRECT LIPASE GENE EXPRESSION

M. J. Haas¹, R. Genuario^{1,2}, and S. H. Fearheller¹

¹U.S. Department of Agriculture
Eastern Regional Research Center, ARS
600 E. Mermaid Lane
Philadelphia, PA 19118, USA

²Department of Food Science
University of Delaware
Newark, DE 19711, USA

ABSTRACT

Recombinant DNA methods were used to begin a molecular biological study of the biotechnically important lipase produced by the fungus *Rhizopus delemar*. Pure, high molecular weight DNA was isolated, mildly digested with *Mbo* I, ligated into pBR322, and introduced into *E. coli* by transformation. Transformants were recovered by ampicillin selection. The frequency of insertional inactivation of tetracycline resistance in the transformants was 95%. The cloned DNA inserts ranged in size from approximately 0 to 14 kilobases (average: 4.7). Colony hybridization using fungal genomic DNA as a probe verified the presence of fungal sequences in the transformed cells. A rapid, sensitive assay for lipase production was developed and applied to a sufficient number of transformants to represent several genomic equivalents of fungal DNA. No lipase-producing clones were detected.

INTRODUCTION

Lipases [E.C. 3.1.1.3] catalyze the hydrolysis of triglycerides to yield fatty acids, glycerol, and partially substituted glycerides (Brockerhoff and

Jensen 1974). The biotechnological utility of these enzymes has been demonstrated in several areas (Kilara 1985, Ratledge 1984, Seitz 1974), including oleochemical hydrolysis for the production of fatty acids and partially substituted glycerides (Kimura et al. 1983), triglyceride modification to yield novel and useful lipids (Yokozeiki et al. 1982, Macrae 1983), organic synthesis for the production of esters (Zaks and Klivanov 1985), the enhancement of food flavors (Kilara 1985), and in the synthesis of flavor compounds (Langrand et al. 1988).

The mycelial fungus *Rhizopus delemar* (Class: Phycomycetes, Order: Mucorales) synthesizes lipases and excretes them into the growth medium. At least three lipases are produced. The crude mixture of these enzymes preferentially hydrolyzes the primary esters of triglycerides (Okumura et al. 1976). These enzymes have been separated, partially purified and characterized (Tsujiisaka et al. 1972, Iwai and Tsujiisaka 1974). Two of them are interconvertible by the addition or removal of noncovalently bound phospholipids (Iwai et al. 1980). The relationships between these lipases and between the genes encoding them have not been further detailed.

The crude lipase mixture from *R. delemar* has been shown to be useful in the modification of triglycerides through the exchange of their primary fatty acyl residues (Macrae 1983). By such fatty acid exchange, it is possible to modify the composition and, therefore, the physical properties and practical applications of a triglyceride. For example, palm oil can be interesterified by the lipase of *R. delemar* to form an analogue of cocoa butter, which has confectionary and cosmetic applications that palm oil does not possess (Macrae, 1983). However, the practical applications of this and other lipases are limited by deficiencies in supply, catalytic specificity, thermal lability, and pH optima. We have adopted a molecular biological approach in order to gain basic knowledge about lipases and the genes encoding them and to foster the modification and biotechnological utilization of these enzymes. We report here the construction of a genomic library of *R. delemar* DNA in *Escherichia coli* and the examination of this library for direct expression of the lipase gene.

MATERIALS and METHODS

Reagents and Microorganisms

Rhizopus delemar, ATCC 34612, was obtained from the American Type Culture Collection. Other microorganisms are from the strain collection of this laboratory.

Cetyltrimethylammonium bromide (CTAB) was obtained from Sigma Chemical Co. 4-Methylumbelliferylaurate (4-MUL) was purchased from United States Biochemical Corporation. Restriction enzyme Mbo I, T4 DNA ligase, and reagents for the synthesis and use of biotinylated DNA were obtained from Bethesda Research Laboratories. BamH I cleaved, dephosphorylated plasmid pBR322 was supplied by New England Biolabs. Nitrocellulose filters (BA-85) were obtained from Schleicher and Schuell.

Isolation of Fungal DNA

Rhizopus delemar, was grown from a spore inoculum in shaken flasks of LB medium (Maniatis et al. 1982) for 24 h at 30 °C. The mycelium was rapidly harvested by filtration over cheesecloth and frozen in liquid nitrogen.

The method of Taylor and Powell (1984), modified to overcome the substantial fungal nucleolytic activity, was used for the isolation of high molecular weight DNA. Frozen mycelia (50 gm, wet weight) were disrupted in liquid nitrogen by 2 min of blending in a Waring blender. The nitrogen was allowed to bubble off and the contents of the cup were transferred into an equal volume of 2X Extraction Buffer (2% CTAB, 200 mM EDTA, 1.4 M NaCl, 100 mM Tris-Cl, pH 8.0) that had been warmed to 55 °C. β -mercaptoethanol was added to 2% weight per volume of the original blended cell mass. The mixture was incubated in a 70 °C bath with gentle swirling until the solution reached 50 °C. An equal volume of chloroform-isoamyl alcohol (24:1) was then added and the solution was mixed by inversion. The phases were separated by centrifugation and the aqueous phase removed with a large bore pipette. One tenth volume of 10% (w/v) CTAB, 0.7 M NaCl was added, and the solvent extraction and centrifugation were repeated. The aqueous phase was removed and mixed with an equal volume of Precipitation

Buffer (1% (w/v) CTAB, 10 mM EDTA, 1% β -mercaptoethanol, 50 mM TrisCl, pH 8.0). After a visible precipitate formed, it was collected by centrifugation. The pellet was dissolved in 5 ml of 1.0 M CsCl, 10 mM EDTA, 200 μ g/ml ethidium bromide, 50 mM TrisCl, pH 8.0 and the DNA isolated by isopycnic banding in an ultracentrifuge. The homogeneity and molecular weight distribution of the resulting DNA were analyzed by electrophoresis in horizontal 0.6% agarose gels followed by staining with ethidium bromide (Shinnick et al. 1975).

Construction of a Genomic Library

One microgram of *R. delemar* genomic DNA was digested with 0.2 units of Mbo I for 15 min in the buffer recommended by the supplier. This solution was split between two tubes, each containing 0.1 μ g of BamH I-cleaved, dephosphorylated pBR322 and 2 units of DNA ligase in the buffer recommended by the enzyme supplier. Following overnight incubation, the ligation mixture was used to transform competent *E. coli* C600 $r^{-}m^{-}$ (Hanahan 1983). Transformation mixtures were plated onto LM medium (Hanahan 1983) containing ampicillin (35 μ g/ml). The insertion of DNA into the BamH I site of pBR322 interrupts the tetracycline gene. Ampicillin resistant transformants, which contained chimeric plasmids, were therefore identified by scoring for the insertional inactivation of their tetracycline-resistance phenotypes.

Analysis of Chimeric Plasmids

Plasmid DNAs were isolated from tetracycline-sensitive transformants (Kado and Liu 1981). The molecular weights of these plasmids were estimated by electrophoresis in 0.6% agarose gels, staining with ethidium bromide, and reference to appropriate standards.

Hybridization Analysis

Genomic *R. delemar* DNA was nick-translated in the presence of biotin-11-dUTP (Anonymous 1985). The product was used as a probe for the detection of fungal sequences in *E. coli* cells containing either pBR322 (control) or the putative *R. delemar* genomic library. Conditions for colony hybridization with this

probe and for determination of the sites of positive hybridization were as described (Haas and Fleming 1988). Hybridization was conducted in 45% formamide for 16 h at 42 °C with a probe concentration of 100 ng/ml.

Screening for Direct Lipase Gene Expression

E. coli transformants containing chimeric DNA were grown on solid rich medium until the colonies were 2 to 3 mm in diameter. The cells were sprayed with a solution containing 10 mg/ml lysozyme, 8% w/v sucrose, 5% w/v Triton X100, 50 mM EDTA, and 50 mM TrisCl, pH 8.0 (Raynal and Guerineau 1984) and incubated 15 min to promote cell lysis and the release of intracellular enzymes. The plates were then sprayed with a solution of 4-MUL (10 mM in ethylene glycol monomethyl ether) and examined after 15 min with a hand-held ultraviolet light for the presence of fluorescent colonies. 4-MUL is a nonfluorescent lipase/esterase substrate, which is enzymatically hydrolyzed to release intensely fluorescent 4-methylumbelliferone (4-MU) (Jacks and Kircher 1967).

RESULTS

Isolation of Fungal DNA

A protocol for the isolation of DNA for genomic cloning should yield significant amounts of high molecular weight product. Our initial attempts yielded preparations that were heterogenous in size and contained large amounts of low-molecular-weight material (Fig. 1, lane 1). Using the method reported here, we were able to obtain DNA preparations in which the majority of the molecules were in excess of 25 kb and the degree of contamination by small fragments was minimal (Fig. 1, lane 2). DNA prepared by this method was uncontaminated by protein and uniformly susceptible to restriction endonuclease digestion. Typical yields were 300 µg per 50 gm of starting material.

Construction and Characterization of a Fungal Genomic Library in *E. coli*

High-molecular-weight fungal DNA was partially digested with Mbo I. Electrophoretic examination indicated that the majority of the DNA was still

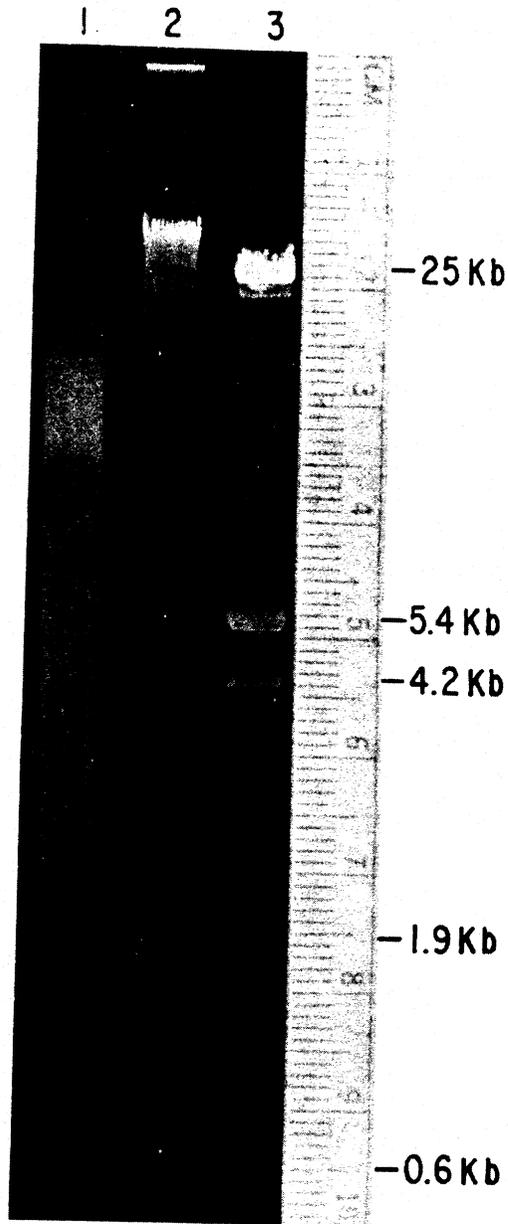


Fig. 1. Electrophoretic analysis of purified *Rhizopus delemar* genomic DNA in a 0.6% agarose gel.

Lane 1: DNA (1 μ g) isolated by means of a protocol (Taylor and Powell 1984) incapable of preventing extensive nucleolytic degradation.

Lane 2: DNA (1 μ g) isolated by means of the modified protocol described here.

Lane 3: Bacteriophage lambda DNA (0.2 μ g) digested with endonucleases Eco R1 and Hind III. The sizes of the lambda fragments are indicated.

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larger than 25 kb. This material was ligated into pBR322 and introduced into *E. coli* by transformation. The efficiency of transformation to ampicillin resistance was 1.7×10^6 per microgram of vector DNA. By comparison, the efficiency of transformation by intact pBR322 was $9 \times 10^7/\mu\text{g}$. The frequency of insertional inactivation of tetracycline resistance among 100 transformants examined was 95%.

The molecular weights of the plasmids in 18 ampicillin-resistant, tetracycline-sensitive transformants were estimated by electrophoresis in an 0.6% agarose gel (Fig. 2). The chimeras ranged in size from 4.3 to 18 kb. Considering the 4.3 kb size of the vector molecule, this indicates that the cloned *Rhizopus* inserts range in size from nearly 0 to approximately 14 kb (average 4.7 kb). Given this average insert size, and estimating the size of the *R. delemar* genome to be approximately 10^4 kb (Storck 1974), it is possible to calculate that 12,800 bacterial transformants are required to obtain a genome equivalent of fungal sequences (Clarke and Carbon 1976).

Colony hybridization using biotinylated fungal DNA as a probe was conducted to establish the presence of *R. delemar* sequences in the transformants. Two thousand bacterial colonies from the putative Mbo I library were grown on a nitrocellulose filter. As a control, *E. coli* C600 $r^- m^-$ containing pBR322 were grown on a separate filter. The cells were lysed in place and their DNAs fixed to the filters. Following hybridization, filter-bound probe was detected by means of an enzymatic colorimetric method. Essentially all ampicillin-resistant transformants developed a significant positive reaction (Fig. 3a). No positive reaction developed on the control filter (Fig. 3b). This verifies the presence of *R. delemar* sequences in the transformed bacterial cells.

Phenotypic Screening for Lipase Gene Expression

The ability of *R. delemar* lipase to convert the non-fluorescent substrate 4-MUL to an intensely fluorescent product was exploited in an attempt to identify members of the genomic library that expressed the lipase gene. Control experiments demonstrated that lipase-producing organisms, including *R. delemar*, gave readily detectable fluorescent reactions when exposed to

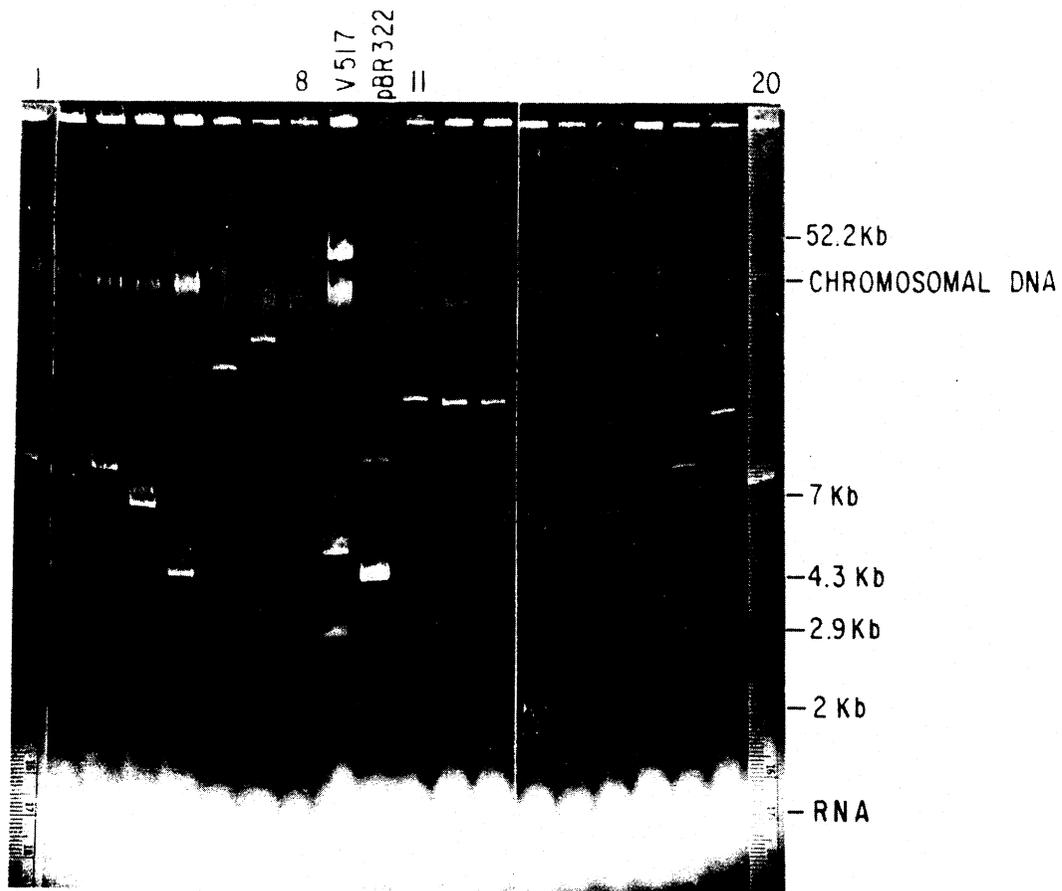


Fig. 2. Agarose gel electrophoresis (0.6% agarose) of alkaline detergent lysates of selected ampicillin-resistant, tetracycline-sensitive *E. coli* derived by transformation with ligation mixtures containing pBR322 and fungal DNA.

Lanes 1-8 and 11-18: Transformants, 100 μ l of lysate.

Lane 9: *E. coli* V517, which harbors several plasmids of known molecular mass, 100 μ l of lysate.

Lane 10: pure pBR322 DNA, 0.05 μ g. Molecular masses of the standards are indicated.

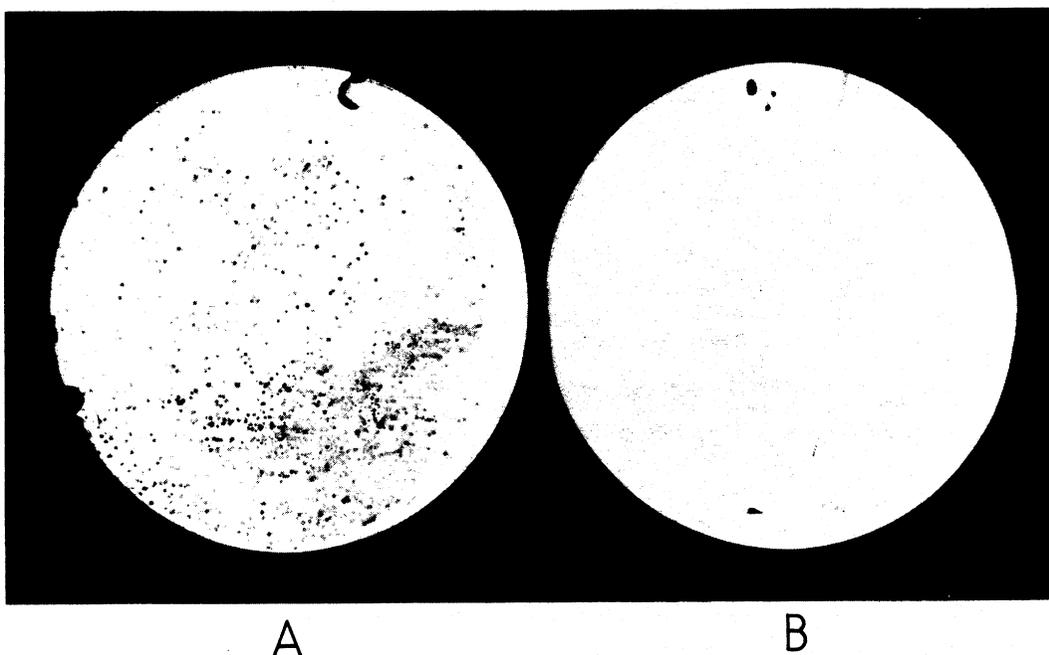


Fig. 3. Detection of fungal nucleic acid in members of the putative genomic library by colony hybridization, with biotinylated fungal DNA as the probe.

(A) *E. coli* bearing chimeric plasmids.

(B) *E. coli* containing pBR322.

4-MUL. The *E. coli* used as the host strain in library construction exhibited minimal 4-MUL hydrolytic capability.

R. delemar excretes its lipase into the growth medium. *E. coli* is a very poor exporter of proteins. In order to screen for expression of the lipase gene in *E. coli*, the cells were treated to release their cytoplasmic contents prior to exposure to 4-MUL. A solution containing a non-ionic detergent, lysozyme, and a cation chelator were used for this purpose. Control experiments demonstrated that this treatment caused the release of 25% of the cellular content of β -galactosidase, a cytoplasmic enzyme. It was also determined that the lysis reagent did not inhibit the lipase.

Transformed *E. coli* sufficient to represent more than five genomes worth of *R. delemar* sequences were examined for lipase production by this method. However, no positively reacting clones were detected.

DISCUSSION

We report here the development of a method for the isolation of high-molecular-weight DNA from *R. delemar*, the production in *E. coli* of a genomic library for this organism, and the attempted detection of lipase production by members of the library.

The direct expression of eukaryotic genes in bacteria is not generally possible, due to the presence of introns that bacteria are not able to process or due to an inability of the bacteria to recognize eukaryotic signals controlling gene expression. However, the direct bacterial expression of some fungal genes has been reported (Kinghorn and Hawkins 1982, Vapneck et al. 1977, Schweizer et al. 1981). Therefore, we attempted the cloning and direct expression of the genomic lipase gene of *R. delemar*.

Initial attempts to isolate high-molecular-weight fungal DNA yielded a heterogenous mixture containing substantial amounts of low-molecular-weight material. The method described here involves the use of high levels of a chelating agent and thereby minimizes the effect of nucleases. This allows the isolation of high-molecular-weight material. The duration of blending of the mycelia greatly affected the yield and size distribution of the product DNA. Blending times longer than that reported here were associated with increased recovery of DNA. However, this material was greatly enriched in small fragments.

The molecular weight of the target lipase is approximately 41,000 (Chiba et al. 1973). This corresponds to a minimum gene size of about 1.3 kb. It was desired to isolate genomic fragments substantially larger than this in order to ensure the isolation of gene-length inserts. Even after partial digestion with *Mbo* I, the average size of our genomic DNA was greater than 25 kb. In spite of this, the average insert size in our genomic library was 4.7 kb. This emphasizes the extent to which the incorporation of small inserts is favored in the ligation event (Dugaiczky et al. 1975).

Colony hybridization using bulk fungal DNA as a probe was used to verify the presence of fungal nucleic acid in the putative genomic library. Virtually all members of the library were found to contain fungal sequences (Fig. 3). There was variation in the extent of reaction of individual colonies with the

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probe DNA. This is seen as a variation in the intensity of the color developed at the positively reacting sites in Fig. 3a. We have not identified the causes of these differences in extent of hybridization with the probe. Perhaps the stronger responses correspond to bacterial colonies containing elevated amounts of fungal DNA, either in the form of larger inserts or of elevated copy numbers for their chimeric plasmids.

Successful use of a phenotypic screen in the detection of gene expression depends upon the sensitivity of the assay. Based on the reported specific activity of the *R. delemar* lipase (Tsujiisaka et al. 1972) and the features of our detection assay, we estimate that the detection limit of this method is approximately 500 lipase molecules per cell. Since pBR322 has a typical copy number of 20 to 50 (Hautala et al. 1979, Boros et al. 1984), it is reasonable to expect that the presence of a functional cloned gene would result in the production of sufficient amounts of lipase to be detected by the assay method used here. In addition, we have recently used a similar screening method to successfully detect the expression of a cloned lipase cDNA (T. Berka and M.J. Haas, in preparation). Our failure to observe direct expression suggests the presence of introns in the lipase gene. Alternatively, *E. coli* may be unable to correctly read the fungal sequences controlling expression of the lipase gene. We are currently investigating these possibilities.

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