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Integrative transformation of the yeast *Yarrowia lipolytica*

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Summary. We have derived a DNA-mediated transformation system for the yeast *Yarrowia lipolytica* based on the lithium acetate method Ito et al. (1983) developed for *Saccharomyces cerevisiae.* The first plasmid used, pLD25, contains the *Y. lipolytica LEU2* gene (coding for the enzyme beta-isopropylmalate dehydrogenase, EC 1.1.1.85) on a 6.6 kb piece of DNA inserted into pBR322. The recipient strain ATCC 20688 contains the rarely reverting mutation *leu2-35.* The *Y. lipolytica LEU2* gene complements *leuB* mutants in *Escherichia coli* and *leu2* mutants in *S. cerevisiae* and it also hybridizes weakly to the *S. cerevisiae LEU2* gene. *Y. lipolytica* transformation frequencies of up to 10^4 Leu^+ cells per microgram of DNA, per 10^8 viable cells have been obtained from plasmids linearized with restriction enzymes. The more than 100-fold increase in transformation frequency obtained by using linearized DNA instead of intact plasmid resembles the situation seen in *S. cerevisiae* for site-directed integrative transformation (Orr-Weaver et al. 1981). The transformants were stable when grown in non-selective medium. We found that pLD25 integrated at the *leu2* region when either linear or intact plasmid was used to transform *Y. lipolytica.*

Key words: *Yarrowia lipolytica* – Integrative transformation – *LEU2* – Genetic recombination

Introduction

The most widely developed systems for expression of genetically engineered proteins have used *E. coli* as the host organism. The *E. coli* systems pose several problems, such as degradation of the protein, possible contamination of the protein with endotoxin and the need to disrupt the cells to recover the protein. Intracellular, "overproduced" proteins are often accumulated in insoluble aggregates called inclusion bodies. These inclusion bodies must be solubilized with denaturants and the protein fractionated and renatured to regain biological activity. In view of these difficulties *B. subtilis* has been employed as an alternative host organism since it secretes proteins and has fewer toxin problems. However, the *B. subtilis* system has some limitations such as instability of transformed strains resulting in loss of plasmids or of heterologous DNA, and degradation of secreted foreign proteins.

In recognition of the above-mentioned difficulties with prokaryotes attention has been focused upon eukaryotes, and specifically yeasts, as host organisms. Yeasts of industrial importance are non-toxic and can be grown to very high densities. Some species are well analyzed genetically and some species can secret proteins.

In the transformation of the yeast *S. cerevisiae* (Hinnen et al. 1978) an *E. coli* plasmid containing a cloned segment of the *S. cerevisiae LEU2* region (Ratzkin and Carbon 1977) transformed a non-reverting *leu2*$^-$ mutant to a Leu^+ phenotype by integrating into the host chromosome. Since the development of this transformation system, the molecular genetics of this organism has progressed very rapidly. Integrative (Orr-Weaver et al. 1981) and autonomously replicating plasmid modes (Beggs 1978; Struhl et al. 1979) of transformation have been developed which offer, respectively, stable transformants and high frequency transformation. The ability to "shuttle" transforming plasmids back and forth between *E. coli* and *S. cerevisiae* has enabled the cloning of *S. cerevisiae* genes by complementation of yeast mutations (Nasmyth and Reed 1980). The cloning and

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characterization of highly expressed yeast genes has resulted in development of efficient systems for expression of heterologous gene products in *S. cerevisiae*, and recently systems have been developed which allow secretion of relatively small heterologous proteins, such as epidermal growth factor and interferon from *S. cerevisiae* (Brake et al. 1984; Hitzeman et al. 1983).

Transformation systems for *Schizosaccharomyces pombe, Kluyveromyces lactis* and *K. fragilis* have been reported by Beach and Nurse (1981) and by Das et al. (1982, 1984), respectively.

The yeast *Yarrowia lipolytica* (formerly *Saccharomycopsis lipolytica* or *Candia lipolytica*) has been used in the industrial production of citric acid (Shah et al. 1982) and single cell protein. It can also be used to produce erythritol, mannitol and isopropylmalic acid (DeZeeuw and Tynan 1973a, 1973b; DeZeeuw and Stasko 1983). This organism has an inherent capability to secrete proteins, including a ribonuclease, acid proteases and most notably an exocellular alkaline protease (Ogrydziak and Scharf 1982; Tobe et al. 1976). It has been studied for its lysine metabolism (Heslot et al. 1979) and for its hydrocarbon metabolism (Bassel and Mortimer 1982). A genetic map of *Y. lipolytica* has been constructed based on tetrad analysis of dissected ascospores (Ogrydziak et al. 1982).

We have developed a transformation system for *Y. lipolytica* to facilitate basic studies of its biology and to improve its industrial potential. For example, to improve upon current *Y. lipolytica* processes, the range of carbon sources *Y. lipolytica* utilizes might be expanded by introduction of the appropriate genes from another species.

Materials and methods

Enzymes

Most restriction enzymes were obtained from New England Biolabs (NEB) as was T4 DNA ligase and *E. coli* polymerase I. ApaI was obtained from Boehringer-Mannheim. SalI, KpnI, XhoI and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratories (BRL). Bacterial alkaline phosphatase was used at approximately 100 units per microgram of linearized plasmid DNA at 65 °C in BamHI assay buffer for 2 h. All other enzymes were used as specified by the respective manufacturers. Restriction digests were analyzed by electrophoresis in submerged 0.8% agarose gels using Tris-Borate-EDTA buffer (Maniatis et al. 1982).

Media and growth conditions

E. coli rich medium was L broth containing, per liter, 10 g Bactotryptone, 5 g Bacto-yeast extract, and 5 g NaCl, adjusted to pH 7.5. *E. coli* minimal medium was 56 salts with 0.33% dextrose (Low 1973). Appropriate amino acids or bases were supplemented to 50 μg/ml.

Yeast rich medium was YPD, containing 1% Bacto-yeast extract, 2% Bacto-Peptone, and 2% dextrose. Yeast minimal medium, SD, contained 0.67% Bacto-yeast nitrogen base without amino acids and 2% dextrose. Synthetic complete medium contained 870 mg/l of powdered stock supplements made by grinding the following together with a mortar and pestle: 2 g each of adenine sulfate, uracil, tryptophan, histidine-HCl, arginine-HCl and methionine, 3 g tyrosine, 6 g leucine, 5 g phenylalanine, 20 g threonine and 3 g lysine. For nutritional testing or selection, the appropriate ingredient was omitted from complete medium.

Y. lipolytica was grown at 28 °C, *S. cerevisiae* at 30 °C and *E. coli* at 37 °C.

Isolation of Y. lipolytica chromosomal DNA

DNA for gene libraries. The wild type strain NRRL Y-1094 was grown to $1-2 \times 10^8$ cells per ml in 4 x 300 ml of YPD in shaking Fernbach flasks. The cells were harvested at 3,000 RPM for 5 min in a Sorvall RC-58 centrifuge with an SA-600 rotor at room temperature, washed in 50 ml 1 M NaCl, and pelleted again. A 15-min "pre-spheroplasting" incubation in 50 ml of 0.2 M tris-(hydroxymethyl)aminomethane-HCl (Tris-HCl) pH 8.5, 0.02 M ethylene-diaminetetracetic acid (EDTA), 1 M NaCl, and 0.1 M 2-mercaptoethanol was followed by pelleting. The cell pellet was resuspended in 40 ml of 1 M NaCl containing 1 mg per ml Zymolyase 5000 (Kirin Breweries, Japan) and incubated for 45 min. At this time greater than 90% of the cells were converted to spheroplasts as detected microscopically by cell lysis upon dilution into water.

The spheroplasts were pelleted and resuspended in 4 tubes in a total of 16 ml 1 M NaCl. A total of 40 ml of lysis buffer [50 mM Tris pH 6.8, 100 mM NaCl, 100 mM EDTA, 0.5% sodium dodecylsulfate (SDS)] containing 0.1 mg/ml proteinase K was added and the lysate incubated at 37 °C for 1.5 h. The lysate was extracted twice with equal volumes of phenol:chloroform (1:1) and two volumes of ethanol were added to the aqueous phase to produce large precipitates of DNA. The DNA was rinsed with 70% and 100% ethanol, then vacuum dried. The dried pellet was redissolved in 8 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) at 65 °C followed by incubation with 300 μl of RNAse A (1 mg/ml, boiled 5 min) for 1 h at 37 °C. The material was extracted twice with phenol-chloroform and ethanol precipitated. The final precipitate was rinsed with ether and dried. The subsequent and final purification step was isopycnic banding in CsCl.

DNA for Southern blots. The SDS spheroplast lysis and potassium acetate treatment from the *S. cerevisiae* mini-prep method described by Sherman et al. (1981) was used to obtain *Y. lipolytica* DNA for Southern blots.

Preparation of plasmid DNA

Bacterial plasmid DNA was prepared by the rapid boiling method of Holmes and Quigley (1981). Subsequent centrifugation in CsCl-ethidium bromide gradients was performed only for large scale preparations. DNA preparations were stored at 4 °C in autoclaved TE buffer.

E. coli transformation

The $CaCl_2$ method of Dagert and Ehrlich (1979) was used. Both 1 to 2 h and overnight $CaCl_2$ treatments of cells were used.