Curr Genet (1985) 10:49–58

Current Genetics

Integrative transformation of the yeast *Yarrowia lipolytica*

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Summary. An *Eco*R1 shotgun of *Yarrowia lipolytica* DNA was inserted into the plasmid YIp333 which carries the *LYS2* gene of *S. cerevisiae*. The resulting plasmid pool was transformed in both *S. cerevisiae* and *Y. lipolytica*. Whereas numerous replicating plasmids could be isolated from the *S. cerevisiae* Lys^+ transformants, all transformants of *Y. lipolytica* so far analyzed were found to result from integrative transformation. This occurred at a frequency of 1 to 10 transformants per μg of input DNA. Cotransformation occurred at high frequency and resulted in tandem integration of 2 to 10 copies of the incoming DNA. Structural and segregational stability of the transforming DNA were both high.

Key words: *Yarrowia lipolytica* – Heterologous gene expression – Integrative transformation – Homologous recombination

Introduction

Yarrowia (Saccharomycopsis) lipolytica is a dimorphic yeast which forms on solid media both yeast-like cells and true mycelium (Wickerham et al. 1969). Its genetics has been developed over the past ten years (Gaillardin et al. 1973; Ogrydziak et al. 1982). Gene enzyme relationships were established for the lysine biosynthetic and catabolic pathways (Heslot et al. 1979), for the n-paraffin oxydative pathway (Bassel et al. 1982) and for extracellular enzymes (Ogrydziak and Sharf 1982). More recently, Beckerich et al. (1984) obtained physiological and genetic evidences for the existence of a transposon in this yeast. In order to develop aspects of the molecular genetics of this yeast, setting up a transformation system was a prerequisite.

This was hampered by several facts. Up to now we found no evidence in this yeast for the existence of a DNA plasmid which could serve as a basis for a transformation vector, such as 2 μm DNA in *S. cerevisiae* (Beggs 1978) or k1 DNA in *Kluyveromyces lactis* (de Louvencourt et al. 1983). Attempts at using vectors developed for *S. cerevisiae* were unsuccessful, either because no corresponding tight mutant was available, and/or because these vectors did not replicate in *Y. lipolytica*, contrary to what happened in *Schizosaccharomyces pombe* (Beach and Nurse 1981) or in *K. lactis* (Das and Hollenberg 1982). Selection schemes based on antibiotic resistance (Jimenez and Davies 1980) could not be used since *Y. lipolytica* was resistant to antibiotics like G418.

We had previously isolated and characterized lysine requiring mutants of *Y. lipolytica* (Heslot et al. 1979). By intraspecific protoplast fusion, we could ascertain that the product of the *LYS2* gene of *S. cerevisiae* cloned on plasmid YIp333 (Eibel and Philippsen 1983) could complement the defect of *lys2*$^-$ mutants of *Y. lipolytica* (J. M. Beckerich et al., unpublished). We therefore looked for Lys^+ transformants in a *lys2*$^-$ background of *Y. lipolytica* using YIp333 derivatives.

Material and methods

Media, strains and plasmids. Standard yeast media were: yeast extract, peptone, glucose (YPD complete medium) and Difco yeast nitrogen base, glucose (YNB minimal medium), as described in Sherman et al. (1979). Media for sporulating and crossing *Y. lipolytica* have been described (Gaillardin et al. 1973).

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Table 1. List of strains and plasmids used

Strain/plasmid	Genotype/description	Source
E. coli		
HB101	*hsd*520, *recA*13, *pro*A, *leu*B, *thi*1	B. Bachman
S. cerevisiae		
P49	*lys2, gal2*	YGSC[a]
1403-7A	*trp1, ura3, suc, MAL4, mel, MGL3, gal3, gal4*	YGSC
DC1-8	*trp1, lys2*	P49 x 1403-7A
Y. lipolytica		
15901-4	*LYS1.5, lyc1.5, ura2*	This laboratory
8601-1	*LYS1.5, lys2.5, ade1*	This laboratory
641	Lys^+ transformant of 8601-1: *Eco*RI shotgun in YIp333	
642	Lys^+ transformant of 8601-1: *Eco*RI shotgun in YIp333	
643	Lys^+ transformant of 8601-1: *Eco*RI shotgun in YIp333	
644	Lys^+ transformant of 8601-1: *Eco*RI shotgun in YIp333	
651	Lys^+ transformant of 8601-1: *Eco*RI shotgun in YIp333	
Plasmids		
YIp333	pBR322 carrying the *LYS2* gene of *S. cerevisiae*	Eibel and Philippsen (1983)
B661	YIp333 + *Eco*RI fragment (*ars*) of *Y. lipolytica*	This work
pINA46	*Eco*RI-*Hind*III fragment (*LYS2*) of YIp333 in pBR322	This work
pINA46S	pINA46 + *Sal*I fragment of B661 (*ars*)	This work
pTG841	*Xyl*E gene of *P. putida* inserted at the *Pst*I site of the *URA3* gene of *S. cerevisiae*, in pBR322	Zukowski et al. (1983)

[a] Yeast Genetic Stock Center, University of California, Berkeley, Calif.

The strains used are listed in Table 1. The *S. cerevisiae* strain DC1-8 (*trp1, lys2*) was selected among the progeny of P49 x 1403-7A as being highly transformable (> 2.10^5 transformants/μg of DNA). Both $lys2^-$ mutations in *S. cerevisiae* DC1-8 and in *Y. lipolytica* 8601-1 destroy alpha-aminoadipate reductase activity.

Plasmid YIp333 carries a 6.9 kb fragment of *S. cerevisiae* DNA inserted between the *Pst*I and *Eco*RI sites of pBR322 (Eibel and Philippsen 1983, see also Fig. 1). The *Eco*RI-*Hind*III fragment of 4.9 kb which carries the *S. cerevisiae LYS2* gene was recloned from YIp333 into pBR322, yielding plasmid pINA46.

Protoplasts of Y. lipolytica (from liquid cultures). Cells were collected by centrifugation and washed twice in TESorb (10 mM TRIS, 1 mM EDTA, 1 M sorbitol). They were resuspended in the same buffer (10 ml/g wet cells) and treated with 2 mM dithiothreitol for 10 min at 30 °C. They were washed twice with protoplasting buffer. This was MESSorb (20 mM MES, 1 M sorbitol, pH 6.0 adjusted with dry TRIS base) in the case of exponentially growing cells, and PAPS (10 mM 2-amino, 2-methyl, 1–3 propanediol, 1 M sorbitol, pH 6.0 adjusted with dry PIPES) in the case of stationary phase cells. The cells were resuspended in the same buffer (5 ml/g wet cells) and treated with cytohelicase (Industries Biologiques Françaises) at a final concentration of 1 mg/ml (exponential phase) or 20 mg/ml (stationary phase). Protoplastisation was followed under a phase contrast microscope and/or by diluting a sample in water. Protoplasts were washed twice with 1 M sorbitol.

DNA extraction from large scale culture of Y. lipolytica. Stationary phase cells were converted into protoplasts using the above procedure, resuspended in a minimum amount of TESorb and gently lysed with three volumes of lysing buffer (3% sarkosyl, 0.5 M TRIS, 0.2 M EDTA, 1 M urea, pH 9). To the lysate 50 μg/ml proteinase K were added and the mixture was incubated for 1 h at 55 °C. Solid CsCl was dissolved in the lysate at a final concentration of 1.7 g/ml and the DNA was banded three times successively. After the last run, DNA was collected at a concentration of about 600 μg/ml, dialysed against TE and kept at 4 °C.

DNA extraction from small scale cultures of Y. lipolytica. The procedure was adapted from Zamir et al. (1981) except that protoplasts were made as described above for stationary phase cells and that precipitation of DNA was made at room temperature with 0.7 volume of isopropanol.

Transformation of Y. lipolytica spheroplasts. We adapted a standard procedure developed for transforming *S. cerevisiae* (Sherman et al. 1979). Protoplasts were made as described above for exponentially growing cells. Protoplastisation was stopped after 5 to 10 min of treatment with cytohelicase, or as soon as osmotic sensitive bodies were detected. Longer treatment resulted in a drastic decline in regeneration frequencies. Under the conditions described, more than 99.99% of the cells were unable to grow on hypotonic medium, whereas 5 to 10% did regenerate on isotonic medium (YNB + 1 M sorbitol).

We occasionally used the LiCl method of Ito et al. (1983) for the transformation of whole cells. Transformation frequencies using either the protoplast or the LiCl method were comparable (1 to 5 transformants/μg of DNA).

Construction of the EcoRI shotgun of Y. lipolytica and DNA manipulations. Of *Y. lipolytica* strain 15901-4 20 μg of DNA were digested with *Eco*RI and ligated with 2 μg of *Eco*RI cut