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Expression of invertase activity in *Yarrowia lipolytica* and its use as a selective marker

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Summary. Few selective markers are available for the transformation of the industrial yeast *Yarrowia lipolytica*, and those that are require the use of specialized hosts (e.g., auxotrophs, antibiotic sensitive). To enable the transformation of any *Y. lipolytica* strain, we used the property that *Y. lipolytica* cannot use sucrose as a sole carbon source. We have constructed a gene fusion where the *Saccharomyces cerevisiae SUC2* gene is placed under the control of the promoter and signal sequence of the *Y. lipolytica XPR2* gene, which encodes an Alkaline Extracellular Protease (AEP). Strains bearing this fusion express invertase activity and grow on sucrose as a carbon source. The activity follows the same regulation as does the alkaline extracellular protease, is secreted into the periplasm and confers a Suc^+ phenotype. It was shown that this chimeric gene could be used as a dominant marker for transformation in a one-step procedure.

Key words: *Yarrowia lipolytica* – Invertase – Secretion – Transformation

Introduction

Transformation of the industrial yeast *Y. lipolytica* has already been accomplished using the *S. cerevisiae LYS2* gene (Gaillardin et al. 1985) and the *Y. lipolytica LEU2* gene (Davidow et al. 1985). Since then, other biosynthetic genes such as *BIO, URA3, HIS1* (Davidow et al. 1987) and *LYS5* (Xuan et al. 1988) have been cloned and isolated, as well as the catabolic gene *LYC1* (Lambert et al. in preparation). These cloned genes have been used as selective markers into suitable recipients, and in all of the cases studied transformation resulted from integration of the vector(s) into the chromosome of the host.

Even as the number of selective markers available increased we believed it worthwhile to attempt the development of an universal marker for transformation. A dominant selective marker would be useful for the transformation of strains where the induction of auxotrophic mutations is either difficult or undesirable. It could also be important in isolating isogenic strains and in avoiding the recovery of conversions and reversions. *Y. lipolytica* was unfortunately found to be resistant to most of the antibiotics commonly used in *S. cerevisiae*, including chloramphenicol or G418 (Cohen et al. 1980; Jimenez and Davies 1980). Consequently, we developed a visual marker using the *lacZ* gene of *E. coli* expressed under the *LEU2* promoter that produces blue colonies in the presence of Xgal as well as the expression of a gene conferring phleomycin resistance to *Y. lipolytica* (Gaillardin and Ribet 1987). Direct selection of $Phleo^R$ transformants was possible on 15 mg/l phleomycin. However, at this concentration at least half of the $Phleo^R$ clones obtained resulted from spontaneous $Phleo^R$ mutations in the recipient strains. The frequency of these false transformants was decreased by using an expression phase before plating; the drawback was a 60-fold reduction in the transformation frequency. Moreover, we were concerned about the possibility of inducing mutations, since phleomycin induces double-strand breaks in DNA (Hecht 1986). Therefore, we decided to develop another type of selection.

Y. lipolytica is a dimorphic yeast which can grow on a limited number of carbon sources, two of which are glucose and glycerol, but not sucrose, paraffins such as *n*-alkanes and alkenes (Klug and Markovetz 1967; 1969; Bassel and Mortimer 1973), lipids and proteins (Ogrydziak et al. 1977). Depending on the growth conditions, *Y. lipolytica* degrades proteins by secreting proteases (Ahearn et al. 1968; Kamada et al. 1972). For

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Table 1. Strains and plasmids used

Designation	Relevant genotype or description	Source or reference
E. coli		
HB101	*hsdR, hsdM⁻, recA13, supE44, lacZ4, leuB6, proA2, thi1, Sm^R*	Boyer and Roulland-Dussoix (1969)
TG1	*supE, thi, (lac-pro* AB), *hsdD5/F⁺, traD*36, *pro* AB, *lacI-ZM*15	
Y. lipolytica		
JM12	*MatB, ura3–18, leu2–35, lys5–12*	JM-M Nicaud, unpublished
JM23	*MatB, ura3-18, leu2–35, lys5–12, xpr2::LYS5*	JM-M Nicaud, unpublished
11104	*MatB, ura2–21, leu2–35, lyc1–100*	This laboratory
W29	*MatA*	Gaillardin et al. (1973)
W29U	*MatA, ura3–18*	This laboratory
TRALI1.1593	*MatB, leu2–35, lys5–12, ade1, xpr2:LYS5:XPR2*	This laboratory
JM58	*JM23* transformed by pINA169 cut by NheI	This work
Plasmids		
pINA62	SalI fragment of *Y. lipolytica* carrying *LEU2* in pBR322	Gaillardin and Ribet (1987)
pRB58	Sau3A fragment of *S. cerevisiae* carrying *SUC2* in YEp24	Carlson and Botstein (1982)
pINA105	BamHI fragment of *Y. lipolytica* carrying part of *XPR2* in pBR322	JM-M Nicaud, unpublished
pINA152	PstI fragment of *Y. lipolytica* carrying *XPR2* in pBR322	JM-M Nicaud, unpublished
pINA150	HindIII-EcoRV fragment from pINA105	This work
pINA151	ClaI deletion from pINA150	This work
pINA165	Insertion of the mutagenized BamHI-SalI fragment of *XPR2* in pINA151	This work
pINA166	EcoRV deletion in pINA165	This work
pINA167	pINA166 + complete *LEU2* gene from pINA62	This work
pINA169	pINA167 + *SUC2* gene from pRB58	This work
Phages		
M13mp18-X8	XhoII-BglII fragment of XPR2 in M13mp18	This work
M13mp18-X4	XhoII-BglII fragment of XPR2 in M13mp18 in the other orientation	This work
M13mp18-X4m	Mutated derivative of M13mp18-X4, carrying PstI and HinDIII sites	This work

example, in alkaline medium it secretes an Alkaline Extracellular Protease (AEP) (Tobe et al. 1976; Ogrydziak et al. 1977; Ogrydziak and Scharf 1982). The corresponding gene, *XPR2,* has been cloned independently by Davidow et al. (1987), Matoba et al. (1988) and in our laboratory.

In this report, we describe the expression and secretion in *Y. lipolytica* of the *S. cerevisiae SUC2* gene under the control of the promoter and signal sequences of the *XPR2* gene. The expression of the invertase activity confers a sucrose- utilizing (Suc^+) phenotype. Invertase activity follows the same regulation as AEP activity and is secreted into the periplasm with only about 18% secreted into the culture medium. The chimeric invertase gene was used as a dominant marker for transformation in a one-step procedure.

Materials and methods

Strains and plasmids. All of the strains, plasmids and phages used are described in Table 1. *Y. lipolytica* strain JM12 was derived from a cross between TRALI. 1593 and W29 *ura*3–18 to be phenotypically $Ura3^-$, $Lys5^-$, $Leu2^-$ and Xpr^+, and highly transformable with pINA62. *Y. lipolytica* strains were constructed using standard genetic techniques (Gaillardin et al. 1973); genetic markers *ade*1 and *leu*2–35 derive from Dr. D. Ogrydziak's collection (Ogrydziak and Scharf 1982) and PFIZER patent culture ATCC20688, respectively. The strain JM23 with no active copy of the *XPR2* gene derives from JM12 and was constructed by gene disruption.

The *Y. lipolytica XPR2* gene was isolated from partial genomic libraries of *Y. lipolytica* DNA in pBR322 using oligonucleotide probes based on the N-terminal amino acid sequence of mature Alkaline Extracellular Protease (unpublished). Plasmid pINA152 carries the complete *XPR2* gene on a 4.2-kb PstI fragment, whereas pINA105 contains only the 5′ half of the coding sequence including promoter and presequences.

Escherichia coli strain HB101 was used for plasmid construction and propagation, and TG1 for phage preparations.

Media. The following media were used: YNB minimal medium is described in Sherman et al. (1979); YNBG and YNBS refer to this medium supplemented with glucose or sucrose (respectively) as carbon sources. $YNBP_oG$, $YNBP_oSa$ and $YNBP_oSf$ are YNB media buffered at pH 6.8 with 50 m*M* phosphate buffer, the carbon source being glucose (G), or autoclaved/filter-sterilized saccharose (Sa/Sf, respectively). Phosphate buffer (P_o) and proteose peptone (1.7 g/l, P_p) were added to induce the AEP promoter. Minimal media were supplemented as required with 50 mg/l amino acids and bases except for leucine (200 mg/l). YPDm corresponds to YPD medium (Gaillardin and Ribet 1987) modified according to A. Franke (personal