Curr Genet (1988) 14:15–21

Current Genetics

Cloning of the *LYS5* gene encoding saccharopine dehydrogenase from the yeast *Yarrowia lipolytica* by target integration

Jian-Wu Xuan, Philippe Fournier, and Claude Gaillardin

Laboratoire de Génétique-INRA, Centre de Biotechnologies Agro-Industrielles, F-78850 Thiverval-Grignon, France

Summary. A *Yarrowia lipolytica* yeast gene bank has been constructed in *E. coli* in an integrative plasmid vector containing the homologous *LEU2* gene, and used to transform a *leu2 lys5* yeast strain. The *LYS5* gene encoding saccharopine dehydrogenase (SDH) has been rescued in *E. coli* from the chromosome of prototrophic transformants, in which the hybrid plasmid had integrated at the *leu2* locus. Evidence that the rescued clone contains the *LYS5* gene comes from complementation tests, genetic crosses, and SDH assay. Further characterization of the gene has been achieved by deletion mapping and subcloning, and by demonstrating the presence of transcripts hybridizing to this sequence.

Key words: *Yarrowia lipolytica* – Targeted integration – Saccharopine dehydrogenase (SDH) – *LYS5* gene – Gene cloning

Introduction

In the industrial yeast *Yarrowia lipolytica,* the biosynthesis of lysine passes through eleven steps known as the aminoadipate pathway (for a review, see Bhattacharjee 1985). The first enzyme of the pathway (homocitrate synthetase) is coded by the *LYS1* gene and is inhibited by lysine (Gaillardin et al. 1976; Gaillardin and Heslot 1979). The last enzyme, saccharopine dehydrogenase (SDH), is coded by the *LYS5* gene and is the single lysine biosynthetic enzyme so far found in *Y. lipolytica* whose synthesis is repressed by lysine (Heslot et al. 1979). This enzyme is also subject to general control, i.e., its activity is repressed upon amino acid starvation (Gaillardin et al. 1979). While both types of control, either specific or general, act independently on SDH expression, it has been suggested by Gaillardin et al. (1979) that there may be a close correlation between SDH specific regulation and the activities of the lysine catabolic enzymes. However, until now little was known about the molecular mechanism of SDH regulation, although several laboratories have purified it to homogeneity, from either *Saccharomyces cerevisiae* (Fujioka 1975; Fujioka and Nakatani 1972; Ogawa and Fujioka 1978), *Candida maltosa* (Schmidt et al. 1985), or *Y. lipolytica* (Gaillardin, unpublished). Therefore, it was of interest to clone the SDH gene in order to elucidate in this yeast the molecular basis of the *LYS5* gene expression.

In the yeast *Y. lipolytica,* a transformation system has been set up (Davidow et al. 1985; Gaillardin et al. 1985) using either the *LEU2* gene of *Y. lipolytica* or the *LYS2* gene of *S. cerevisiae* as selective markers. In both cases transformants resulted from homologous crossing over and integration into a chromosomal target sequence which should be present on the transforming plasmid.

As no *ars* sequence was available to build the gene library (Wing and Ogrydziak 1985; Gaillardin et al. 1985), we used the integrative vector pINA62 described by Gaillardin and Ribet (1987), which contains the *Y. lipolytica LEU2* gene cloned in pBR322. In order to increase the transformation frequency, we linearized (partially or completely) the hybrid plasmids with a restriction enzyme that was afterwards also used to rescue the gene from the chromosomal DNA of the transformants. A similar approach has recently been used by Davidow et al. (1987) to clone the *XPR2* gene coding for an extracellular protease.

Offprint requests to: C. M. Gaillardin

Table 1. List of strains and plasmids used

Strain/plasmid	Genotype/description	Source
E. coli		
HB 101	*hsd*R$^-$, *hsd*M$^-$, *recA*13, *SupE*44, *lacZ*4, *leuB*6, *proA*2, *thi*-1, Sm^R	B. Bachmann
Y. lipolytica		
W29	Wild type	our lab. collection
21501-4	*Mat*B, *lys5*-12, *leu2*-35, *ade1*, *xpr2*	our lab. collection
21604-2	*Mat*A, *leu2*-35, *lys1*-13	our lab. collection
15210-Xc	*Mat*A, *his*-1	our lab. collection
16201-5	*Mat*A, *ura2*-21, *lys5*-12	our lab. collection
447, 443, 445	Different Lys$^+$ transformants of 21501-4	this work
523	Lys$^+$ transformant of 21604-2	this work
Plasmids		
pINA62	A 5.2 kb *Sal*I fragment carrying *LEU2* of *Y. lipolytica* in pBR322	Gaillardin et al. 1987
pINA127	*lys5* complementing DNA cloned in pINA62	this work
pINA127′	Same as pINA127 with an additional *Apa*I genomic fragment	this work
pINA128	*Sph*I deletion of pINA127	this work

Material and methods

Media, strains, and plasmids. Complete medium was YPD, and minimal medium was YNB-glucose as described by Sherman et al. (1986). As our *leu2* mutants have an unknown defect in nitrogen metabolism (unpublished data), they were grown on nitrogen sources other than ammonium sulfate, i.e., either lysine or sodium glutamate (each at 1 g/l). These media are referred to as Ln or Gn, respectively, followed by the letters A or Le according to the needed supplements (adenine 100 mg/l, leucine 200 mg/l). Skim milk plates (SKM) were prepared according to Simms and Ogrydziak (1981). Media for mating and sporulation have already been described (Beckerich et al. 1984). Strains and plasmids are listed on Table 1. The plasmid pINA62 carries a 5.2 kb *Sal*I fragment of *Y. lipolytica* DNA inserted in pBR322.

Construction of the gene library. High molecular weight chromosomal DNA was extracted from *Y. lipolytica* wild-type strain W29 according to Xuan and Kuang (1986). After partial restriction with *Sau*3A, the DNA was fractionated from a sucrose gradient so as to recover 7–15 kb fragments, and was then ligated with *Bam*HI cut and dephosphorylated pINA62 DNA. The *E. coli* HB101 strain was transformed for ampicillin resistance, and five pools representing a total of 6,500 clones were recovered, among which 89% turned out to be hybrids. The mean size of the inserts in the hybrids was about 12 kb. According to the formula of Clarke and Carbon (1976), the DNA of these pools covered more than one *Y. lipolytica* genome with a probability of 0.99. Plasmid DNAs extracted from *E. coli* were transformed into *leu2 lys1* or *leu2 lys5 Y. lipolytica* strains so as to get between 10^4 and 10^5 yeast transformants.

Transformation of Y. lipolytica. We used essentially the protocol published by Davidow et al. (1985), who adapted to *Y. lipolytica* the procedure of transformation described for *S. cerevisiae* by Ito et al. (1983). The few minor changes introduced concern the pH of both the culture (which was set up at 4.0 with 100 m*M* citric acid-sodium citrate buffer) and the lithium acetate solution (adjusted to pH 6.0 with acetic acid), as well as the stage of cell harvest (generally between 8×10^7 and 2×10^8 cells per ml), and will be published in detail elsewhere. With this protocol, we obtained between 10^4 and 10^5 transformants per μg of linearized pINA62 DNA.

Complementation test. After transformation the yeast cells were plated onto the selective LnA medium containing no leucine, and the *Leu*$^+$ transformants obtained were replica plated onto GnA, LnLe or SKM plates to screen for Lys$^+$, Ade$^+$, or Xpr$^+$ transformants respectively.

Deletion construction. Internal deletions within the 8.6 kb insert present in pINA127 were generated by either *Bam*HI, *Bgl*II, *Hind*III or *Sph*I digestion followed by religation at lower concentrations. A *Pst*I partial restriction was also performed on pINA128 (which harbors the *Sph*I deletion of pINA127, see Table 1 and Fig. 2), and we screened for the recovery of two types of deletions. A *Bgl*II-*Xho*I fragment was also recloned in pBR322. All these plasmids were linearized by either *Apa*I or *Kpn*I and transformed into a *leu2 lys5* yeast host for the complementation test.

Hybridization. High molecular weight chromosomal DNA prepared from each transformant was digested, separated on a 0.7% agarose gel, and transferred onto a nylon membrane (Pall France). Preparation of nick-translated ^{32}P-labeled probes and hybridization were performed according to standard protocols (Maniatis et al. 1982). Total RNA was prepared from log phase cultures of the wild-type W29 strain of *Y. lipolytica* grown on YPD. The spheroplasts prepared according to Beckerich et al. (1984) were disrupted by a homogenizer, and the lysate was phenol extracted. The polyA$^+$ fraction was collected from an oligo-dT column. Both total RNA and the polyA$^+$ fraction were transferred onto a nylon membrane, and hybridization was performed according to Williams and Mason (1985). Labeled compounds were from Amersham France.

Saccharopine deshydrogenase assay. We followed the method described by Gaillardin et al. (1979).