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LEU2 directed expression of β-galactosidase activity and phleomycin resistance in *Yarrowia lipolytica*

Claude Gaillardin and Anne-Marie Ribet

Institut National Agronomique, Laboratoire de Génétique, 16, Rue Claude Bernard, F-75231 Paris Cédex 05, France

Summary. The nucleotide sequence of a 968 bp DNA fragment spanning the promoter and the 5′ upstream sequence of the *LEU2* coding sequence of the yeast *Yarrowia lipolytica* has been determined. A *LEU2*::*lacZ* gene fusion has been constructed and expressed in transformed yeast cells, showing that as few as 232 bp of the *LEU2* promotor were sufficient to direct gene expression. In order to develop new markers for transformation of this yeast, the *LEU2* initiation codon was destroyed by in vitro mutagenesis and replaced by a cloning site. A gene confering phleomycin resistance in *E. coli* was attached to the *LEU2* promoter and shown to be efficiently expressed in yeast: direct selection of phleomycin resistant transformants was possible.

Key words: *Yarrowia lipolytica* – *LEU2* promoter – β-galactosidase – Phleomycin resistance

Introduction

Transformation of the industrial yeast *Yarrowia lipolytica* has been achieved using yeast biosynthetic genes cloned in a bacterial plasmid and suitable auxotrophic recipients (Davidow et al. 1985; Gaillardin et al. 1985). In all cases studied, transformation resulted from homologous integration of the vector(s) into the chromosome of the host: isolation of ars sequences have so far failed (Ogrydziak, pers. comm.; Gaillardin et al. 1985). As in *S. cerevisiae*, the frequency of transformation can be dramatically increased (up to 50,000 transformants per μg input vector) if the transforming DNA is linearized within its region of homology with the chromosome (Davidow et al. 1985).

Offprint requests to: C. Gaillardin

Few selective markers are available up to now in this system since heterologous genes from *S. cerevisiae* are often poorly expressed (Gaillardin et al. 1985) or not at all (Davidow et al. 1985) in this host. In order to increase the versatility of the transforming system of this yeast, we tried to develop two additional types of markers. Visual markers, like the *lacZ* gene of *E. coli* which product can be detected easily on colonies, have proven suitable in *S. cerevisiae* for the study of gene expression (Rose et al. 1981; Guarente and Ptashne 1981). Antibiotic resistance genes are useful for the transformation of strains where induction of auxotropic mutations is either difficult or undesirable. *Y. lipolytica* was unfortunately found to be resistant to most antibiotics commonly used in *S. cerevisiae*, including chloramphenicol or G418 (Cohen et al. 1980; Jimenez and Davies 1980). We observed that it was sensitive to the bleomycin/phleomycin group of antibiotics which are described to induce strand scission in DNA (Marugesan et al. 1982). Genes confering phleomycin resistance have been identified in bacteria: one was found in Tn5 in *E. coli*, another one in pUB110 in *B. subtilis*. Both genes have been expressed in *S. cerevisiae* and shown to confer high resistance levels in this yeast (G. Tiraby, pers. comm.).

In this report, we describe the construction of plasmids expressing *lacZ* and *Phleo*^{R} in *Y. lipolytica* under the control of the *LEU2* promoter of this yeast. The *LEU2* gene, first described by Davidow et al. (1985) has been independently recloned in our laboratory by complementation of a *leu2*$^-$ *S. cerevisiae* host. This gene codes for β-isopropylmalate dehydrogenase, and mutants devoid of this activity have been isolated in *E. coli* (*leu*B gene) and *S. cerevisiae* (*leu2* gene). We also report here the structure of the 5′ upstream sequence of this gene and the construction of a portable 290 bp DNA cassette containing the *LEU2* promoter sequences.

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)
JM103	Δlac pro, thi, strA, supE, endA, sbcB15, hsdR4, F′ traD36, proAB, lacI^{q}Z M15	Messing (1981)
S. cerevisiae		
OL1	Matα, leu2-3, leu2-112, his3-11, his3-15, ura3-251, ura3-373	Boy-Marcotte and Jacquet (1982)
Y. lipolytica		
8601-1	MatA, ade1, lys2-5, lyc1-5, LYS1-5	Gaillardin et al. (1985)
DX465-7B	MatB, ade1, leu2-35, xpr2	D. Ogrydziak
21101-9	MatA, ade1, lys2-5, lyc1-5, xpr2, leu2-35	DX465-7Bx8601-1
Plasmids		
pMC1403	lacZ, Y in pBR322	Casadaban et al. (1980)
pUT7	Phleo^R from Tn5 in pBR322	G. Tiraby
pJRD148b	pJRD148 derivative, regulated copy number	Davison et al. (1984)
pINA62	*Sal*I fragment of *Y. lipolytica* carrying *LEU2* in pBR322	This work
pINA62′	Same as pINA62, opposite orientation	This work
pINA92	*lacP, LEU2 promoter*::*Phleo*^{R} pJRD148b	This work
pINA95	pINA92 + complete *LEU2* gene from pINA62	This work
pINA98	*LEU2*::*lacZ* fusion + complete *LEU2* gene in pMC1403	This work
Phages		
M13mp18-M	*Eco*RI-*Stu*I fragment of *LEU2* in M13mp18	This work
M13mp18-M5	Mutated derivative of M13mp18-M, carrying *Asu*II-*Eco*RI sites	This work

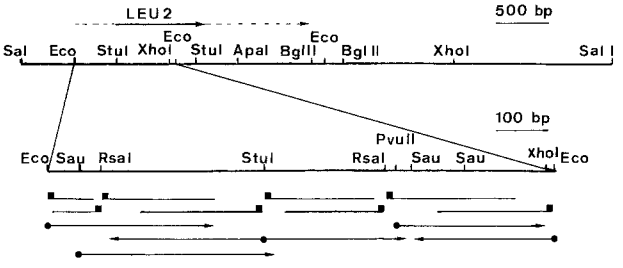

Fig. 1. Structure of the *Y. lipolytica* fragment carrying the *LEU2* gene. *Upper line* restriction map of the 5.6 kb *Sal*I fragment (Eco = *Eco*RI). The minimal (*black arrow*) and maximal (*dotted arrow*) extent of the *LEU2* gene is indicated (see text). The *Sal*I fragment was inserted into pBR322 in the clockwise orientation (pINA62′, the Tet promoter being on the left of the fragment shown) or in the opposite orientation (pINA62). *Bottom line* restriction map of the 5′ upstream fragment and sequencing strategy. Eco, *Eco*RI; Sau, *Sau*3A. Fragments sequenced by the chemical method are indicated by *lines*, the 5′ end labeled extremity is indicated by a *black square*. Fragments sequenced by the chain termination method are indicated by *arrows*

Material and methods

Strains and plasmids (see Table 1). *Y. lipolytica* strain 21109-9 was derived from a cross between DX465-7B and 8601-1 (see Table 1): 10 descendants, phenotypically Ade$^-$, Lys$^-$, Xpr$^-$, Leu$^-$ were checked for transformability with pINA62: 21109-9 was highly transformable (45,000 leu$^+$ transformants per μg of *Apa*I digested pINA62).

The *Y. lipolytica LEU2* gene was isolated from a *Sau*3A library of *Y. lypolytica* DNA in YRp7 (Struhl et al. 1979) transformed into *S. cerevisiae* OL1. Hybrid plasmids were re-isolated from Leu$^+$ transformants and a common 5.6 kb *Sal*I fragment of *Y. lipolytica* DNA was recloned into pBR322 (see Fig. 1). The resulting plasmids contain *LEU2* either in the clockwise orientation (pINA62′) or in the opposite orientation (pINA62): both plasmids confer a Leu$^+$ phenotype to *E. coli* HB101.

Internal deletions of the *Sal*I insert of pINA62 or pINA62′ were generated by *Eco*RI, *Xho*I, *Bgl*II, or *Stu*I digestion followed by religation at dilute concentration.

A *LEU2*::*lacZ* in frame fusion was constructed by inserting at the *Bam*HI site of pMC1403 a 625 bp *Sau*3A fragment of the *LEU2* 5′ upstream region (see Fig. 1). The orientation of the *Sau*3A insert was checked by *Pvu*II digestion (*Pvu*II cuts once within the insert, see Fig. 1): a plasmid containing the desired orientation of the insertion was then digested by *Sal*I and the complete 5.6 kb *Sal*I fragment carrying *LEU2* was inserted into it. The orientation of *LEU2* was checked by *Eco*RI digestion: pINA98 (see Fig. 3) which carries both the *LEU2*::*lacZ* fusion and the *LEU2* gene in the same orientation was saved. Construction of pINA92 and pINA95 is described below.

Construction of pINA92 and pINA95. A 411 bp *Eco*RI-*Stu*I fragment of pINA62 spanning the 5′ upstream region of *LEU2* (see Fig. 1 and 2) was inserted into the replicative form of