Cloning and Disruption of the β-Isopropylmalate Dehydrogenase Gene *(LEU2)* of *Pichia stipitis* with URA3 and Recovery of the Double Auxotroph

Ping Lu[1], Brian P. Davis[2], James Hendrick[2] and Thomas W. Jeffries[1,2]*

USDA Forest Service, Forest Products Laboratory, Madison, WI 53705

[1]Department of Bacteriology, University of Wisconsin–Madison, Madison, WI 53706
[2]U.S. Department of Agriculture, Forest Service, Forest Products Laboratory, One Gifford Pinchot Drive, Madison, WI 53705–2398

Present addresses:

P. Lu: Scriptgen Pharmaceuticals, Inc. 200 Boston Ave, Medford, MA 02155
B.P. Davis: Department of Biochemistry, Biophysics and Genetics, University of Colorado, Health Sciences Center, Denver, CO 80262
J. Hendrick: Ophidian Pharmaceuticals, Inc. 5445 E. Cheryl Parkway, Madison, WI 53711

Key words: *Pichia stipitis,* Gene disruption, Site-specific recombination, Transformation

*Correspondent: Thomas W. Jeffries
USDA, Forest Service
Forest Products Laboratory
One Gifford Pinchot Drive
Madison, WI 53705

Tel: (608) 231-9453
Fax: (608) 231-9262
e-mail: twjeffri@facstaff.wisc.edu

Prepared for publication in *Applied Microbiology and Biotechnology*

September 5, 1997

Summary

Transformation of *Pichia stipitis* is required to advance genetic studies and development of xylose metabolism in this yeast. To this end, we used *P. stipitis URA3 (PsURA3)* to disrupt *P. stipitis LEU2* in a *P. stipitis ura3* mutant. A highly fermentative *P. stipitis* mutant (FPL-DX26) was selected for resistance to 5'-fluoroorotic acid (5' FOA) to obtain *P. stipitis* FPL-UC7 *(ura3-3).* A *URA3:* lacZ "pop-out" cassette was constructed containing *PsURA3* flanked by direct repeats from segments of the lacZ reading frame. The *P. stipitis LEU2* gene *(PsLEU2)* was cloned from a *P. stipitis* CBS 6054 genomic library through homology to *Saccharomyces cerevisiae LEU2,* and a disruption cassette was constructed by replacing the *PsLEU2* reading sequence with the *PsURA3*:lacZ cassette. FPL-UC7 *(ura3-3)* was transformed with the disruption cassette, and a site-specific integrant was identified by selecting for the leu$^-$URA$^+$ phenotype. The *ura3* marker was recovered from this strain by plating cells onto 5'-FOA and screening for spontaneous *URA3* deletion mutants. Excision of the flanked *PsURA3* gene resulted in the leu$^-$ura$^-$ phenotype. The double auxotrophs are stable and can be transformed at a high frequency by *PsLEU2* or *PsURA3* carried on ARS-based plasmids.

Introduction

Pichia stipitis is studied for its ability to produce ethanol from D-xylose (du Preez *et al.* 1986; Ligthelm *et al.* 1988; Grootjen *et al* 1990; Skoog and Hahn-Hägerdal 1990). Basic elements of yeast xylose metabolism are understood, but much remains to be learned about rate-limiting steps and the factors regulating fermentation. To this end, we are engineering xylose metabolism in *P. stipitis* through overexpression and disruption of key genes. Resistance to *kanamycin* works in *P. stipitis* (Ho *et al.* 1991), but the transformation frequencies are very low (Ho *et al.* 1991) which makes the system impractical for the construction of complementation libraries or targeted disruption We previously developed *PsURA3* as a selectable marker in *P. stipitis* (Yang *et al.* 1994). It is a powerful marker for gene manipulation because positive selection systems exist for both auxotrophs and prototrophs of this locus (Boeke *et al.* 1984). However, additional

selectable markers are required to force mating crosses, disrupt genes, and overexpress more than a few genes.

Genetic studies of *P. stipitis* have indicated that wild type strains are haploid and homothallic but that stable diploids can be recovered by cultivation of zygotes on rich medium (Melake et al. 1996). Other studies have indicated that homothallic diploids are prevalent (Gupthar, 1994). *P. stipitis* strains have at least six chromosomes (Passoth et al. 1992).

The yeast *LEU2* gene codes for β-isopropylmalate dehydrogenase (Satyanarayana *et al.* 1968). *Saccharornyces cerevisiae LEU2 (ScLEU2)* was first cloned and sequenced by Andreadis and co-workers (1982, 1984). It has been widely used for transformation and expression in *S. cerevisiae* (Erhart and Hollenberg 1983), and *LEU2* has been used successfully in several other organisms (Berardi and Thomas 1990; Hiep *et al.* 1993; Kimura *et al.* 1995; Piredda and Gaillardin 1994; Saki and Tani 1992). Toh-e (1995) has used *ScLEU2* for disruption in "pop-out" cassette.

The objective of our present research, was to cloned *LEU2* from *P. stipitis* CBS 6054 and disrupt it in a highly fermentative *P. stipitis ura3* strain using a pop-out cassette based on the homologous *PsURA3* after the manner of Alani *et al.* (1987) andToh-e (1995) and to create double auxotrophic *leu2 ura3* mutants. The resulting *P. stipitis ura3-3 leu2D-1* hosts—FPL-LU5, FPL-LU6, and FPL-LU20-can be transformed at high efficiency with either the *LEU2* or *URA3* marker on ARS-based plasmids.

Materials and Methods

Strains and plasmid. *Pichia stipitis* CBS 6054 (=NRRL Y-11545, ATCC 58785) was the source of all DNA and the ultimate origin of all host strains used in this study. *P. stipitis* FPL-061 was derived from CBS 6054 by mutagenesis with nitrosoguanidine and selection for rapid growth on L-xylose in the presence of salicylhydroxamic acid (SHAM) and antimycin A (AA) (Jeffries and Livingston, 1992) and its characteristics have been published (Sreenath and Jeffries, 1997). *P. stipitis* FPL-DX26 (=NRRL Y-21304) was derived from *P. stipitis* FPL-061 by mutagenesis with ethylmethanesulfonate (EMS) and selection for growth on D-xylose in the

1 presence of 1.0 g/12' deoxyglucose (2-DOG) (Pardo *et al.* 1991, Goffrini *et al.* 1995). *Pichia*
2 *stipitis* FPL-UC7, a *ura3-3* mutant derived from FPL-DX26 by selection for resistance to 5'-
3 FOA, was used as the strain for targeted disruption of *LEU2*. *Escherichia coli* DH5α ™ (GibCo
4 BRL, Gaithersburg, MD) and MC1066a *(leuB600 trpC9830 pyrF74::tn5 kan^r ura hsdR hsdM$^+$*
5 *srl::tn10 recA13)* (Sandbaken and Culbertson, 1988) were used for routine recombinant DNA
6 experiments that required bacterial hosts.[1] XL-1 Blue and SOLR™ *E. coli* cells (Stratagene, La
7 Jolla, CA) were used in conjunction with the λ –ZAP™ genomic DNA library. Plasmid
8 Bluescript™ KS(+) from Stratagene was used as a cloning and sequencing vector, pBluescript
9 SK(+) was used as the disrupting and cloning vector, and pUC19 was used to clone the
10 *leu2::URA3* disruption cassette.

11 **Media.** Yeast were routinely cultivated in YPD medium (1% yeast extract, 2% peptone,
12 2% glucose). Standard defined (SD) medium (0.67% Bacto-yeast nitrogen base without amino
13 acids, plus 2% glucose) containing supplemental 20 mg/l uridine or leucine were used as selection
14 medium for disruption and transformation. Fermentation media consisted of 0.17% yeast
15 nitrogen base without amino acids and without ammonium sulfate (Difco), 0.227% urea, 0.656%
16 peptone, and 8% D-xylose or D-glucose.

17 **DNA isolation.** Plasmid DNA was isolated and purified using a QIAprep™ Spin
18 Plasmid Kit (QIAGEN Inc., Chatsworth CA). Yeast genomic DNA was isolated and purified as
19 described previously (Rose *et al.,* 1990).

20 **Transformation.** *Pichia stipitis* was transformed by the lithium acetate protocol as
21 described by Rose *et al.* 1990. *Escherichia coli* was transformed by the calcium chloride method
22 (Sambrook *et al.* 1989).

23 **Genomic DNA library.** Genomic DNA was purified from *P. sripitis* CBS 6054 (wild
24 type), partially digested with *Tsp5091* and fractionated by electrophoresis. The 5 to 10 kb DNA

---

[1] The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

1 fragments were ligated into λ–ZAP (Stratagene) that had been digested with *Eco* RI. The
2 resultant library was calculated to have approximately 1×10^6 individual recombinant phages,
3 with an average insert size of 5 kb. If *P. stipitis* has a genome of equivalent to *S. cerevisiae*
4 (14,000 kb/haploid genome), this library has a complexity of 23 genome equivalents.

5 **DNA sequencing.** Nucleotide sequences *PsLEU2* were determined by the dideoxy
6 method of Sanger *et al.* (1977) using a Sequenase™ kit (United States Biochemical, Cleveland,
7 OH). Sequence analysis was performed according to the method of Devereux *et al.* (1984) using
8 the GCG Sequence Analysis Software Package (GCG, Madison, WI).

9 **Southern blot analysis.** Southern transfer by capillary blotting was performed according
10 to Sambrook *et al.* (1989). DNA hybridizations were done using the Genius™ non radioactive
11 system (Boehringer Mannheim Biochemical, Indianapolis, IN). Nylon membranes were
12 Nytran™ filters (Schleicher & Schuell, Keene, NH). Hybridizations were typically done in 25%
13 formamide at 37°C and washes were performed in 2x SSC at 25°C and 0.5x SSC at 37°C.

14 **PCR analysis.** PCR was used to confirm the genetic structure of the primary *ura3-3,*
15 *leu2::URA3* disruptant, and the *ura3-3, leu2*Δ*-1* reversion mutant. The sequence of the forward
16 primer was 5'-GGAGTTCCTTTGCCAGATG-3', and the sequence of the reverse primer was
17 5'-GCCATTATATTACTGACTAGGCAGC-3'

18 **Results**

19 Cloning of *LEU2* gene in *P. stipitis.* The *P. stipitis* λ–ZAP II genomic library was
20 screened for the *PsLEU2* gene by hybridizing the coding region of *S. cerevisiae LEU2* gene to
21 plaques. Four individual plasmids were recovered from 200,000 plaques screened. All the
22 plasmids bore an identical *PsLEU2* gene sequence as indicated by restriction enzyme mapping
23 and by sequencing all or part of each plasmid insert. Genomic blotting patterns indicated only
24 one *LEU2* gene in *P. stipitis* (Fig. 1).

25 Sequencing of *LEU2* gene. The sequence of the *PsLEU2* coding region and 5'- and 3'-
26 flanking regions were obtained by primer walking from the *Pst* I site within the gene. The
27 sequence contains an open reading frame of 1122 nucleotides encoding a polypeptide of 374

amino acids. A putative upstream RNA initiation site (TATATAAA) is located at -217 to -224. The GenBank sequence accession number for *P. stipitis LEU2 (PsLEU2)* is U83626.

Similarity to other LEU2 genes. A BLAST analysis was performed using the deduced PsLEU2p against the SwissProt database to identify closely related sequences, and the eukaryotic yeast and fungal sequences resulting from this search were aligned using the PileUp progressive sequence analysis method of Feng and Doolittle (1987). As we had previously observed in an analysis of yeast *URA3* sequences, yeast *LEU2* sequences were divided into two major clusters: one with *P. stipitis, Candida maltosa,* and (in this instance) *Pichia ohmeri,* and the other with *S. cerevisiae, Kluyveromyces marxianus,* and *Khyveromyces ;actis.*

ura3 **auxotroph selection.** *P. stipitis* FPL-DX26 was mutagenized with EMS according to the method of Rose *et al.* (1990). Eighty colonies were obtained by selecting for resistance to 5'-FOA in the presence of 100 µg/ml of uridine (Boeke *et al.* 1984). Each strain was suspended in water at concentrations of approximately 10^{9}, 10^{8}, or 10^{7} cells/ml. Small drops 10µl) of each suspension were then plated onto minimal medium without uridine to test for reversion frequency. From this, we selected 15 stable *ura3* auxotrophs for futher transformation testing. Three strains, FPL-UB 1, FPL-UC7, and FPL-UC 16, could be complemented at high frequency by *P. stipitis URA3* carried on pJM6 (Yang *et al.* 1994) and showed low reversion frequencies (<10^{-6} subsepent culture. A trial fermantative experiment was carried out to examine the fermentation characteristics of the mutants. FPL-UC7 *(ura3-3)* exhibited a fermentation ability similar to that of the parental strain, FPL-DX26, and was therefore chosen for *LEU2* disruption.

Construction of URA3 pop-out cassette. A 1.4 kb *Xba* I fragment containing *PsURA3* (Yang *et al.* 1994) was inserted into pUC19 at the *Xba* I site, creating pUC19*/PsURA3.* A *Sac* I/*Pvu* II fragment of about 700 bp from the *lacZ* open reading frame was inserted into the *Sac* I/*Sma* I site in pUC19/*PsURA3,* creating pUC19/*PsURA* 3/a. The ends of the *Sac* I/*Pvu* II fragment from *lacZ* were also blunt-ended with T4 DNA polymerase and inserted into the *Pst* I site of pUC19/*PsURA3/a,* also blunt-ended, creating placURA3 (Fig. 2). The *lacURA3* cassette can be removed by digestion with *Sac* I/*Sph* I.

Construction of disruption vectors. A 2.1 kb *PsLEU2* restriction fragment containing the 1122 bp coding sequence and about 600 bp of the 5' and 3' flanking regions was isolated from a primary clone (number 3), blunt-ended, and then re-ligated into pBluescript KS in which the *Pst* I site had been destroyed by T4 DNA polymerase. To distinguish plasmids bearing the *PsLEU2* gene from those without inserts, the leucine auxotroph, *E. coli* MC 1066a, was transformed with the ligation mixture, and *Leu2* prototrophs with ampicillin (Amp) resistance were identified on B-minimal (leucine-minus) medium supplemented with ampicillin. This plasmid, pLU7, was used for constructing subsequent disruption and complementation vectors. The disruption vector pLU9 (Fig. 3) was based on pLU7. A 900 bp coding sequence within *LEU2* was deleted with *Pst I* and *Bgl II.* The *URA3* disruption cassette was then inserted into the gap by blunt-end ligation. The recombinant vector was introduced into *E. coli* MC 1066a. Ura^+, and Amp^R colonies were screened on B-minimal medium (uracil-minus) supplemented with ampicillin.

Disruption of *LEU2* in *P. stipitis* FPL-UC7 *(ura3-3).* The strategy that was used to disrupt *LEU2* in FPL-UC7 is diagrammed in Fig. 3. The *leu2::URA3 cassette* fragment was removed from pLU9 with *Sma I* and *Apa I* and transformed into FPL-UC7 by the lithium acetate method (Rose *et al.* 1990). Seventy-nine *ura*$^+$ transformants were obtained on SD medium supplemented with leucine. To distinguish random integrants, *ura3* revertants, and *ura3* gene conversion events from site-specific disruptions, the colonies were patched onto YPD, SD plus leucine, and SD medium. Only one strain, FPL-LU75, grew on YPD and SD plus leucine, but not on SD (data not shown), and is a Ura^+Leu^- strain. This low frequency of site-specific integration is characteristic of our experience with *P. stipitis* and is probably due to a low level of homologous recombination (M. Culbertson, personal communication).

To obtain Ura^-Leu^- double auxotrophs, FPL-LU75 was screened on SD supplemented with uridine and leucine and with 0.1% 5'-FOA to select for spontaneous *ura3* eliminations. We obtained 37 colonies that showed stable resistance to 5'-FOA from 7 x 10^8 FPL-LU75 cells. Twelve of the 37 were Leu^-, Ura^- but grew as well as the wild type on complex medium. Fifteen

were Leu⁻, Ura- but grew poorly on complex medium. These were not examined further. We used PCR to confirm the structures of the *leu2* locus in the FPL-DX26, FPL-UC7, FPL-LU75, and four of the double auxotrophs, FPL-LU5, FPL-LU6, FPL-LU11 and FPL-LU20 (Fig. 4). Both FPL-DX26 and FPL-UC7 showed the expected 967 bp fragment resulting from PCR amplification of a portion of the native genomic *LEU2.* The strain FPL-LU75 was apparently heterozygotic, as evidenced by the large band characteristic of the *leu2::URA3* cassette inserted into *LEU2* and the small *leu2*Δ*-1* band characteristic of the *URA3* excised, deleted gene (cf. Fig. 3). Strains FPL-LU5, 6, and 20 are homozygotic *ura3-3, leu2*Δ*-1* mutants that have lost the *URA3* pop-out cassette, leaving behind a single copy of the LacZ fragment. FPL-LU11 also showed the phenotype of Ura⁻, Leu⁻, but it evidently retains the pop-out cassette on one chromosome, so it must have arisen through some other mutation. The *leu2*Δ*-1* mutation is stable. After several passages on minimal medium, no *LEU* revertants of *leu2*Δ*-1* were evident. In contrast, the *ura3-3* mutation, which we assume is a point mutation, did revert occasionally ca. 1×10^{-8}).

Construction of complementary vector of *PsLEU2* gene. In order to determine whether the *leu2*Δ*-1* could be complemented by the *PsLEU2* gene, we constructed a vector containing an autonomous replication sequence (ARS), *PsLEU2.* A 1.5 kb *P. stipitis* ARS fragment was isolated from plasmid pARS4 (Yang *et al.* 1994) and ligated into the *Eco* RI site in pLU7 (Fig. 5). The recombinant vector was cloned in MC 1066a and selected on B-minimal (leucine-minus) medium supplemented with ampicillin. We transformed each of the *ura3-3, leu2*Δ*-1* recipient hosts – FPL-LU5, FPL-LU6, FPL-LU20 – with pLU11, and found transformation frequencies essentially identical to what we obtained using *URA3* as a selectable marker in pJM6 (Yang *et al.,* 1994). In each instance, we obtained approximately 40 to 50 colonies per μg of DNA/10^8 cells using the lithium acetate transformation method (data not shown). This second selectable marker has been introduced into our best fermentative strain, *P. stipitis* FPL-UC7. It provides a good genetic background for further engineering studies.

Discussion

1 Because *P. stipitis* has only one gene for *LEU2,* it is very useful as a selectable marker for
2 genetic studies because it is not necessary to delete two copies in order to obtain an auxotroph.
3 Complementation of auxotrophic mutations with wild type genes such as *URA3* and *LEU2* has
4 an advantage over the use of drug resistance markers because no antibiotic is necessary in order to
5 maintain the plasmids. Furthermore, the recovery of both *leu2* and *ura3 as* selectable markers
6 enables the introduction of multiple genes on different plasmids.

7 In our previous analysis of the *URA3* gene (Yang et al. 1994), *Hansenula polymorpha*
8 *(Pichia angusta)* was distantly removed from but within the same cluster as *P. stipitis.* In the
9 present analysis of *LEU2, H. polymorpha* showed more relatedness to the *S. cerevisiae-*
10 *Kluyveromyces* cluster. As would be expected from other analyses (Kurtzrnan 1994), the *LEU2*
11 sequence of *Schizosaccharomyces pombe* is far removed from the two principal clusters of
12 ascomycetous yeasts.

13 Often genetic manipulation introduces extraneous mutations that decrease complex
14 metabolic processes such as fermentation, so we took care to create the double auxotrophic
15 mutant from a strain that we knew to have high fermentative capacity (FPL-061). Then we
16 introduced a mutation for carbon catabolite resistance (FPL-DX26), and we screened the *ura3*
17 mutants derived from it to identify FPL-UC7 *(ura3-3)* as a highly fermentative recipient host.
18 Our prior *P. stipitis ura3* recipient host, FPL-TJ26 (Yang et al. 1994, Dahn et al. 1966) had been
19 screened only for stability and high transformation frequency, and not for fermentative activity.
20 Further degradation of the fermentative capacities of these strains has been avoided through the
21 use of specific targeted disruption. The URA3 "pop-out" cassette is particularly useful in this
22 respect. The directly repeated *lacZ* flanking regions are highly like to recombine and excise the
23 *PsURA3* gene while leaving behind a single copy of the flanking DNA in the target gene. Thus,
24 *URA3* can be used repeatedly for site specific disruptions (Alani et al. 1987, Toh-e A 1995).

25 The apparent heterozygotic state of FPL-LU75 depicted in Fig. 4 is consistent with both
26 its phenotype (Leu$^-$, Ura$^+$) and its genotype *(ura3-3, ura3-3, leu2::URA3, leu2Δ-1).* Moreover,
27 based on the experience of Melake et al (1996), recovery of stable diploids could be expected. A

synthetic defined (SD) medium supplemented with leucine (but not uridine) was used to recover transformants, so maintenance of the *leu2::URA3* disruption cassette was necessary for cell growth. Site-specific recombination appears to occur at a lower frequency in *P. stipitis* than in *S. cerevisiae.* Among 79 *ura+* transformants, we found only one site-specific integrant (FPL-LU75) which was Leu^- and Ura^+. This suggests that the diploid state prevails under the growth conditions employed. The apparent "pop-out" frequency of the cassette was about 5.6×10^{-8}. Thus this technique provides a convenient means to disrupt additional genes using *URA3* and to recover the *ura3* mutation.

Acknowledgments

Authors Lu and Davis were supported by National Renewable Energy Laboratory subcontract #XAU-4- 11193-02; Hendrick was supported by a National Research Council Fellowship from the Forest Products Laboratory (Madison, WI) and by USDA NRICGP grant No. 96-35500-3172. The authors thank D. Cullen for useful comments and H.K. Sreenath for fermentation screening to identfy FPL-UC7.

References

Alani E, Cao L, Kleckner N (1987) A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics 116: 541–545

Andreadis A, Hsu YP, Kohlhaw GB, Hermodson M, Kohlhaw G, Schimmel P (1984) Yeast *LEU2* repression of mRNA levels by leucine and primary structure of the gene product. J Biol Chem 259: 8059–8062

Andreadis A, Hsu YP, Kohlhaw GB, Schimmel P (1982) Nucleotide sequence of yeast *LEU2* shows 5'-noncoding region has sequences cognate to leucine. Cell 31: 319–326

Berardi E, Thomas DY (1990) An effective transformation method for *Hansenula polymorpha* Curr Genet 18: 169–170

Boeke JD, LaCroute F, Fink GR (1984) A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoroorotic acid resistance. Mol Gen Genet 197: 345–346

Dahn KM, Davis BP, Pittman PE, Kenealy WR, Jeffries TW (1996) Increased xylose reductase activity in the xylose-fermenting yeast *Pichia stipitis* by overexpression of *XYL1*. Appl Biochem Biotechnol 57/58: 267-276

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12: 387–395

du Preez JC, Bosch M, Prior BA (1986). Xylose fermentation by *Candida shehatae* and *Pichia stipitis*: effects of pH, temperature and substrate concentration. Enzyme Microb Technol 8: 360–346

Erhart E, Hollenberg CP (1983) The presence of a defective *LEU2* gene on 2μ DNA recombinant plasmids of *Saccharomyces cerevisiae* is responsible for curing and high copy number. J Bacteriol 156: 625–635

Feng DF, Doolittle RF (1987) Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J Mol Evol 25: 351–360

Goffrini P, Ficarelli A, Ferrero I (1995) Hexokinase activity is affected in mutants of *Kluyveromyces lactis* resistant to glucose repression. Microbiology 141: 441–447

Grootjen DRJ, van der Lans RGJM, Luyben KChAM (1990) Effects of aeration rate on the fermentation of glucose and xylose by *Pichia stipitis* CBS 5773. Enzyme Microb Technol 12: 20–23

Gupthar, AS (1994) Theoretical and practical aspects of ploidy estimation in *Pichia stipitis.* Mycol Res 98: 716-718

Hiep TT, Noskov VN, Pavlov YI (1993) Transformation in the methylotrophic yeast *Pichia methanolica* utilizing homologous *ADE1* and heterologous *ADE2* and *LEU2* genes as genetic markers. Yeast 9: 1189–1197

Ho NWY, Petros D, Deng XX (1991) Genetic transformation of xylose-fermenting yeast *Pichia stipitis.* Appl Biochem Biotechnol 28/29: 369–375

Jeffries TW, Livingston PL (1992) Xylose-fermenting yeast mutants. U.S. Patent. No. 5,126,266. June 30, 1992.

Kimura H, Matamura S, Suzuki M, Sumino Y (1995) Sequencing of the β-isopropylmalate dehydrogenase gene (*LEU2*) from *Acremonium chrysogenum* and its application to heterologous gene expression. J Ferment Bioengineer 80: 534–540

Kurtzman CP (1994) Molecular taxonomy of the yeasts. Yeast 10: 1727–1740

Ligthelm ME, Prior BA, du Preez JC (1988) The oxygen requirements of yeasts for the fermentation of D-xylose and D-glucose to ethanol. Appl Microbiol Biotechnol 28: 63–68

Melake T, Passoth V, Klinner U (1996) Characterization of the genetic system of the xylose-fermenting yeast *Pichia stipitis.* Curr Microbiol 33: 237-242

Pardo EH, Funayama S, Pedrosa FO, Rigo LU (1991) *Pichia stipitis* L-rhamnose dehydrogenase and a catabolite-resistant mutant able to utilize 2-deoxy-D-glucose. Can J Microbiol 38: 417–422

Passoth V, Hansen M, Klinner U, Emeis CC (1992) The electrophoretic banding pattern of the chromospmes of *Pichia stipitis* and *Candida shehatae.* Curr Genet 22: 429-431.

Piredda S, Gaillardin C (1994) Development of a transformation system for the yeast *Yamadazyma* (*Pichia*) *ohmeri*. Yeast 10: 1601–1612

Rose MD, Winston F, Hieter P (1990) Methods in yeast genetics. A laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Saki Y, Tani Y (1992) Directed mutagenesis in an asporogenous methylotrophic yeast: cloning, sequencing, and one-step disruption of the 3-isopropylmalate dehydrogenase gene (*LEU2*) of *Candida boidinii* to derive doubly auxotrophic marker strains. J Bacteriol 174: 5988–5993

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sandbaken, MG, Culbertson MR (1988) Mutations in elongation factor EF-1-alpha affect the frequency of frame shifting and amino acid misincorporation in *Saccharomyces cerevisiae*. Genetics 120: 923–934

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Nat Acad Sci USA 74: 5463–5467

Satyanarayana T, Umbarger HE, Lindegren G (1968) Biosynthesis of branched-chain amino acids in yeast: correlation of biochemical blocks and genetic lesions in leucine auxotrophs. J Bacteriol 96: 2012–2017

Skoog, K, Hahn–Hägerdal B (1990) Effect of oxygenation on xylose fermentation by *Pichia stipitis*. Appl Environ Microbiol 56: 3389–3394

Sreenath HK Jeffries, TW (1997) Diminished respirative growth and enhanced assimilative sugar uptake result in higher specific fermentation rates by the mutant *Pichia stipitis* FPL-061. Appl Biochem Biotechnol 63-65: 109–116

Toh-e A (1995) Construction of a marker gene cassette which is repeatedly usable for gene disruption in yeast. Curr Genet 27: 293–297

Yang VW, Marks JA, Davis BP, Jeffries TW (1994) High-efficiency transformation of *Pichia stipitis* based on its *URA3* gene and a homologous autonomous replication sequence, ARS2. Appl Environ Microb 60: 4245–4254

Legends

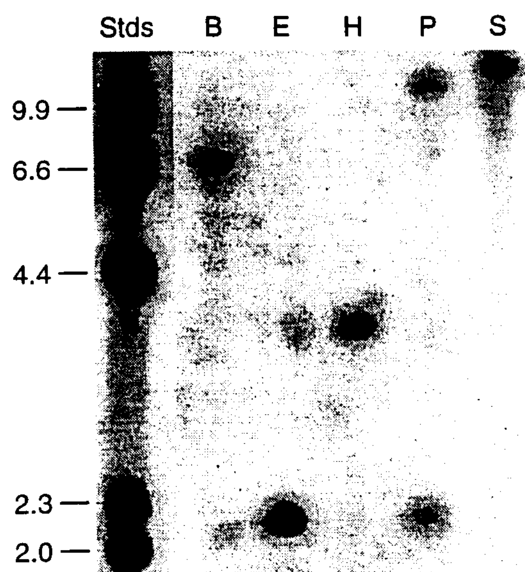
Figure 1. Southern analysis of *Pichia stipitis* CBS 6054 genomic DNA probed with *Xba* I fragment of *PsLEU2.* Restriction enzymes are shown at top of blot. Standard molecular sizes are shown to left of gel. Restriction digestions were *Bam* (B), *Eco* ^{R}*I* (R), *Hind* III (H), *Pst* I (P), *Sal* I (S).

Figure 2. *URA:LacZ* disruption cassette. Arrow indicates reading frame of LacZ. The pUC19 flanking regions are shown by a single line.

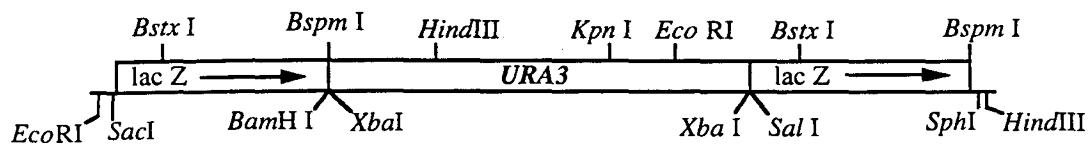
Figure 3. Diagram of strategy for disrupting *LEU2.* The *leu2* gene flanking regions, LacZ repeat regions, and *URA3* gene insert are indicated by gray, striped, and black boxes, respectively. Small arrows represent forward and reverse primers used to amplify region and identify genotypes of auxotrophs. Sizes of expected PCR products from cells at different stages are indicated to left of chromosome diagram.

Figure 4. PCR products from cells at different stages in selection.

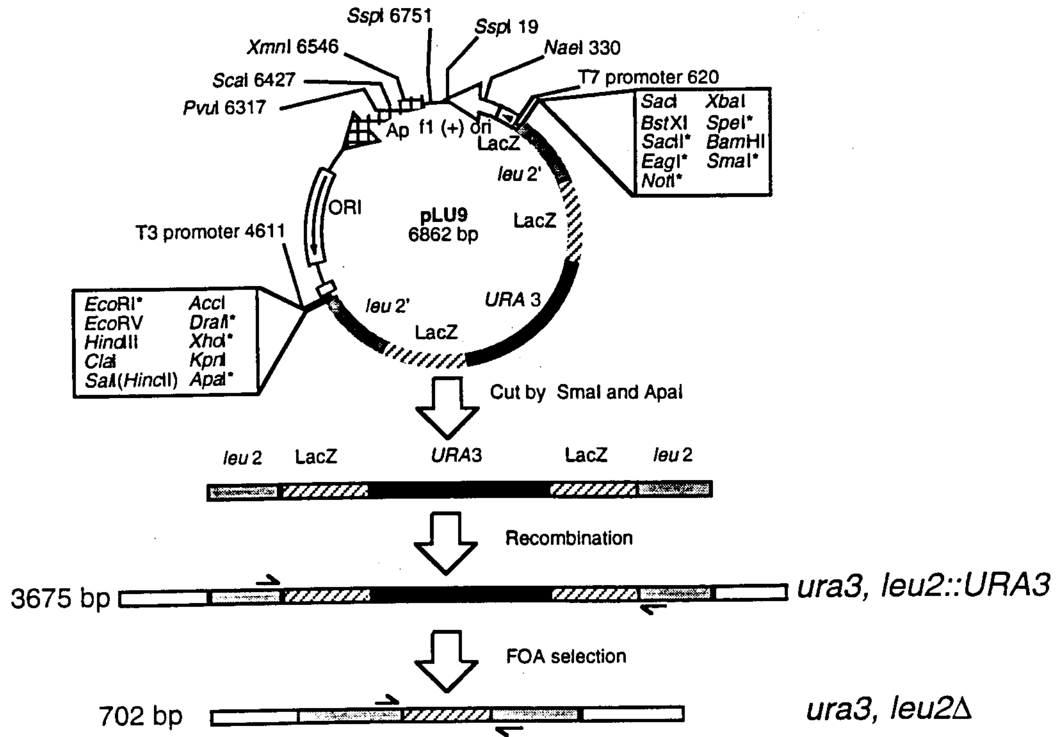
Figure 5. Complementation vector bearing the *PsLEU2* gene. The *PsLEU2* fragment (gray region) and *Pichia stipitis* ARS2 fragment (black region) were inserted into pBluescript KS+ as described in the text.



Lu *et al*. Fig. 1



Lu *et al.* Fig.2



Lu *et al*. Fig. 3

Lu *et al.* Fig. 4

*Ssp*I 6450
*Ssp*I 19
*Nae*I 330
T7 promoter 620
*Xmn*I 6245
*Sca*I 6126
*Pvu*I 6016
Ap
f1 (+) ori
LacZ

SacI*	XbaI
BstXI	SpeI*
SacII*	BamHI*
EagI*	SmaI*
NotI*	

ORI
pLU 11
6561 bp
LEU 2
*Pst*I 1265
T3 promoter 4310

*Eco*RI	*Xho*I
*Eco*RV	*Dra*II*
*Hind*III	*Apa*I*
*Cla*I	*Kpn*I
*Acc*I	

ARS 2
*Bgl*II 2165
*Eco*RI 2765

Lu *et al.* Fig. 5