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Cloning and disruption of the β-isopropylmalate dehydrogenase gene *(LEU2)* of *Pichia stipitis* with *URA3* and recovery of the double auxotroph

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Abstract 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 to obtain *P. sripitis* 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'-fluoroorotate 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 autonomous-replication-sequence-based plasmids.

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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.* 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* its 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 gene products.

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). *Sacccharomyces 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 a "pop-out" cassette.

The objective of our present research was to clone *LEU2* from *P. stipitis* CBS 6054. 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) and Toh-e (1995), and create double auxotrophic *leu2 ura3* mutants. The resulting *P. stipitis ura3-3 leu2*Δ*-1* hosts – FPL-LU5, FPL-LU6, and FPL-LU20 – can be transformed at high efficiency with either the *LEU2* or *URA3* marker on autonomous-replication-sequence (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 and antimycin A (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 ethyl methanesulfonate and selection for growth on D-xylose in the presence of 1.0 g/l 2'-deoxyglucose (Pardo et al. 1991; Goffrini et al. 1995). *P. stipitis* FPL-UC7, a *ura3-3* mutant derived from FPL-DX26 by selection for resistance to 5'-fluoroorotic acid. was used as the strain for targeted disruption *LEU2. Escherichia coli* DH5 *x* (Gibco BRL. Gaithersburg. Md.) and MC1066a *(leub600 trpC9830 pyrF74::tn5 kan*^{r}*ara hsdr hadM*^{r} *srl::tn10 recA13)* (Sandbaken and Culbertson 1988) were used for routine recombinant DNA experiments that required bacterial hosts. [1]XL-l Blue and SOLR *E. coli* cells (Stratagene, La Jolla, Calif.) were used in conjunction with the λ-ZAP genomic DNA library. Plasmid Bluescript KS + from Stratagene was used as a cloning and sequencing vector. pBiuescript SK + was used as the disrupting and cloning vector, and pUC19 was used to clone the *leu2::URA3* disruption cassette.

Media

Yeast were routinely cultivated in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Standard defined (SD) medium (0.67% Bacto yeast nitrogen base without amino acids. plus 2% glucose) containing supplemental 20 mg/l uridine or leucine was used as the selectlon medium for disruption and transformation Fermentation media consisted of 0.17% yeast nitrogen base without amino acids and without ammonium sulfate (Dilco). 0.227% urea. 0.656% peptone, and 8%, D-xylose or mglucose.

DNA isolation

Plasmid DNA was isolated and purified using a QIAprep Spin Plasmid Kit (QIAGEN Inc., Chatsworth, Calif.). Yeast genomic DNA was isolated and purified as described previously (Rose et al. 1990).

[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

Transformation

P. stipitis was transformed by the lithium acetate protocol as described by Rose et al. (19909). *E. coli* was transformed by the calcium chloride method (Sambrook et al. 1989).

Genomic DNA library

Genomic DNA was purified from *P. stipitis* CBS 6054 (wild type), partially disgested with *Tsp* 509I and fractionated by electrophoresis. The 5-to 10-kb DNA fragments were ligated into λ -ZAP (Stratagene), which had been digested with *Eco* RI. Teh resultand library was calculated to the approximately 1×10^{6} individual recombinant phages, with an average insert size of 5 kb. If *P. stipitis* has a genome of equivalent to that of *S. cerevisae* (14 000 kb/haploid genome), this library has a complexity of 23 genome equilavents.

DNA sequencing

Nucleotide sequences of *PsLEL2* were determined by the dideoxy-DNA method of Sanger et al. (1977) using a Sequenase kit (United States Biochemical, Cleveland, Ohio). Sequence analysis was performed according to the method of Devereux et al. (1984) using the GCG sequence analysis software package (GCG, Madison, Wis.).

Southern blot analysis

Southern transfer by capillary blotting was performed according to Sambrook et al. (1989). DNA hybridizations were done using the Genius non-radioactive system (Boehringer Mannheim Biochemical, Indianpolis, Ind.). Nylon membranes were Nytran filters (Schleicher & Schüll, Keene, NH.). Hybridizations were typically done in 25% formamide at 37 °C and washes were performed in 2 × standard saline citrate (SSC) at 25 °C and 0.5 × SSC at 37 °C.

PCR analysis

The polymerase chain reaction (PCR) was used to confirm the genetic structure of the primary *ura3-3, leu2::URA3* disruptant. and the *ura3-3, leu2*Δ*-1* reversion mutant. The sequence of the forward primer was 5'-GGAGTTCCTTTGCCAGATG-3', and the sequence of the reverse primer was 5'-GCCATTATATT-ACTGACTAGGCAGC-3'.

Results

Cloning of the *LEU2* gene into *P. stipitis*

The *P. stipitis* λ-ZAP II genomic library was screened for the *PsLEU2* gene by hybridizing the coding region of the *S. cervisiae LEU2* gene to plaques. Four individual plasmids were recovered from 200000 plaques screened. All the plasmids bore an identical *PsLEU2* gene sequence, as indicated by restriction enzyme mapping and by sequencing of all or part of each plasmid insert. Genomic blotting patterns indicated only one *LEU2* gene in *P. stipitis* (Fig. 1).

Sequencing of the *LEU2* gene

The sequence of the *PsLEU2* coding region and the 5'- and 3'-flanking regions were obtained by "primer

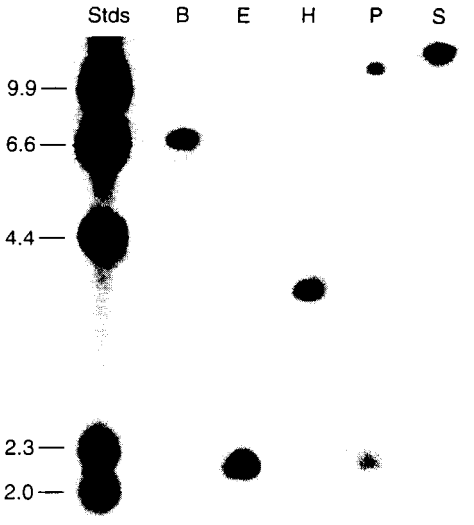


Fig. 1 Southern analysis of *Pichia stipitis* CBS 6054 genomic DNA probed with the *Xba*I fragment of *PsLEU2.* Restriction enzymes are shown at the top of the blot. Standard molecular sizes are shown to the left of the gel. Restriction digestions were *Bam*HI *(B). Eco*RI *(R), Hin*dIII *(H). Pst*I *(P), Sal*I *(S)*

walking" from the *Pst*I site within the gene. The 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 comparing 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 comprising *P. stipitis, Candida malrosa,* and (in this instance) *Pichia ohmeri,* and the other *S. cerevisiae, Kluyveromyces marxianus,* and *Kluyveromyces lactis.*

ura3 auxotroph selection

P. stipitis FPL-DX26 was mutagenized with ethyl methanesulfonate according to the method of Rose et al. (1990). Eighty colonies were obtained by selecting for resistance to 5'-fluoroorotate in the presence of 100 µg/ml 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 (10 µl) 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 further transformation testing. Three strains, FPL-UB1, FPL-UC7. and FPL-UC16, could be complemented at high frequency by *P. stipitis URA3* carried on pJM6 (Yang et al. 1994) and showed low reversion frequencies (< 10^{-6}) in subsequent culture. A trial fermentation 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.

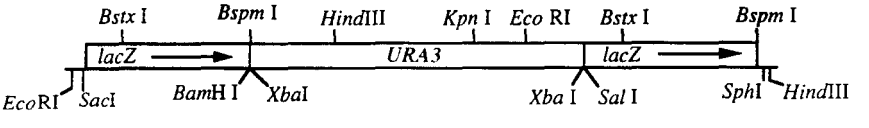
Construction of a *URA3* pop-out cassette

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/*PsURA3/a.* The ends of the *Sac*I/*Pvu*II fragment from *lacZ* were also blunt-ended with T4DNA polymerase and inserted into the *Psf*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 restriciton 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 T4DNA polymerase. To distinguish plasmids bearing the *PsLEU2* gene from those without inserts. the leucine auxotroph, *E. coli* MC1066a, was transformed with the ligation mixture, and *Leu2* prototrophs with ampicillin (Amp) resistance were identified on B minimal (lacking leucine) medium supplemented with ampicillin. This plasmid, pLU7, was used for constructing subsequent disruption and complementation vectors. The disruption vector pLU9 (Fig. 3) was based

Fig. 2 *URA3:lacZ* disruption cassette. Arrow indicates reading frame of *lacZ.* The pUC19 flanking regions are shown by a single line



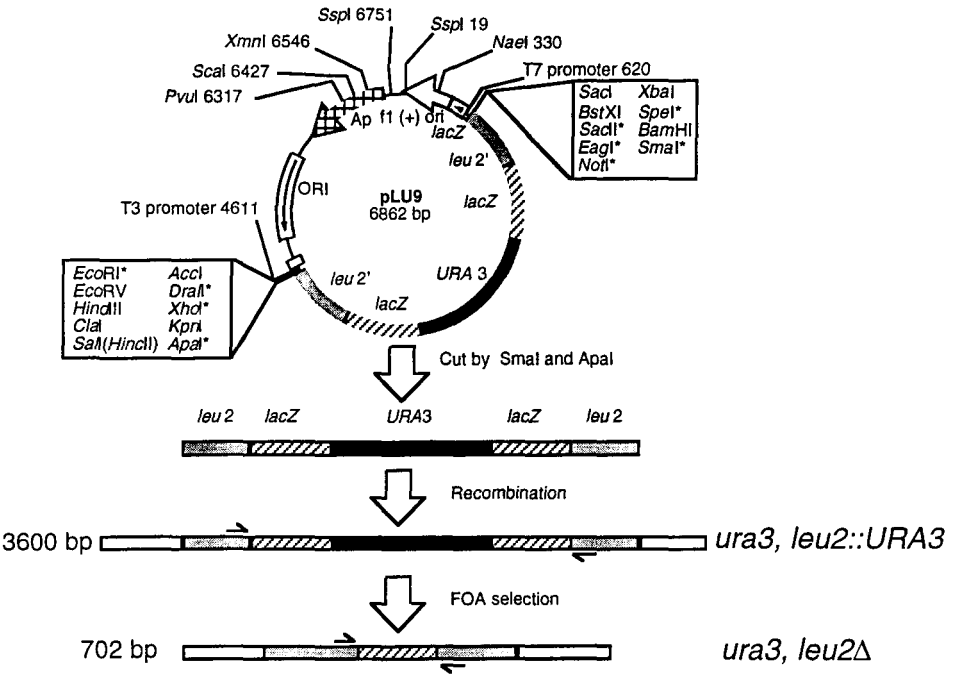


Fig. 3 Diagram of strategy for disrupting *LEU2*. The *leu2* gene flanking regions, *LacZ* repeat regions, and *URA3* gene insert are indicated *(gray, striped,* and *black boxes* respectively). *Small arrows* forward and reverse primers used to amplify the region and identify genotypes of auxotrophs.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

on pLU7. A 900-bp coding sequence within *LEU2* was deleted with *Pst*I and *Bg/*II. The *URA3* disruption cassette was then inserted into the gap by blunt-end ligation. The recombinant vector was introduced into *E. coli* MC1066a. Ura$^+$. and Amp^R colonies were screened on B minimal medium (lacking uracil) supplemented with ampicillin.

Disruption of *LEU2* in *P. stiptis* FPL-UC7 *(ura3-3)*

The strategy that was used to disrupt *LEU2* in FPL-UC7 is illustrated 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). A total of 79 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.

To obtain Ura$^-$Leu$^-$ double auxotrophs. FPL-LU75 was screened on SD supplemented with uridine and leucine and with 0.1% 5'-fluoroorotate to select for spontaneous *ura3* eliminations. We obtained 37 colonies that showed stable resistance to 5'-fluoroorotate from 7×10^8 FPL-LU75 cells. Of the 37, 12 were Leu$^-$, Ura$^-$ but grew as well as the wild type on complex medium; 15 were Leu$^-$, Ura$^-$ but grew poorly on complex medium. These were not examined further. We used the polymerase chain reaction (PCR) to confirm the structures of the *leu2* locus in FPL-DX26, FPL-UC7, FPL-LU75, and in 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 shown 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 (approx. 1×10^{-8}).

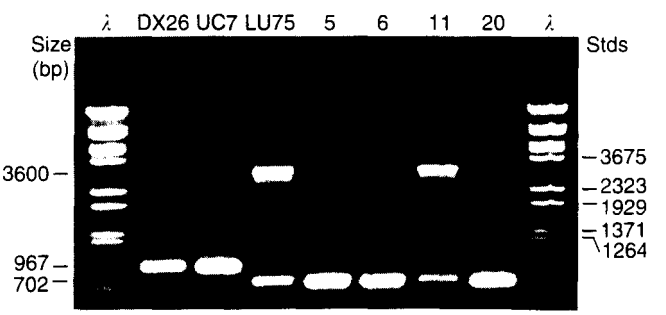


Fig. 4 PCR products from cells at different stages in selection

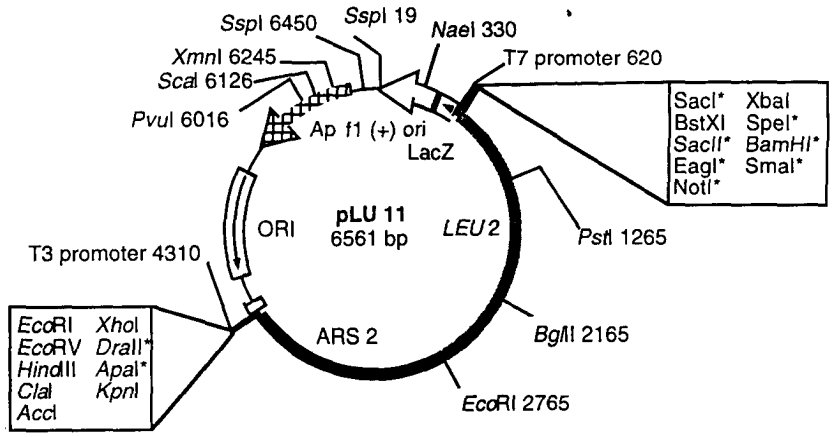


Fig. 5 Complementation vector bearing the *PsLEU2* gene. The *PsLEU2* fragment *(gray region)* and the *Pichia stipitis* ARS2 fragment *(black region)* were inserted into pBluescript KS + as described in the text

Construction of a complementary vector of the *PsLEU2* gene

In order to determine whether *leu2*Δ*-1* could be complemented by the *PsLEU2* gene, we constructed a vector containing an 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 MC1066a and selected on B minimal (lacking leucine) 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 40-50 colonies/μg DNA for 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

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

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

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

The apparent heterozygotic state of FPL-LU75, depicted in Fig. 4, is consistent with both its phenotype (Leu^-, Ura^+) and its genotype *(ura3-3, ura3-3, lea2::URA3, leu2*Δ*-1).* Moreover, on the basis of 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.

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