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High-Efficiency Transformation of *Pichia stipitis* Based on Its *URA3* Gene and a Homologous Autonomous Replication Sequence, *ARS2*

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This paper describes the first high-efficiency transformation system for the xylose-fermenting yeast *Pichia stipitis*. The system includes integrating and autonomously replicating plasmids based on the gene for orotidine-5'-phosphate decarboxylase *(URA3)* and an autonomous replicating sequence (ARS) element *(ARS2)* isolated from *P. stipitis* CBS 6054. Ura⁻ auxotrophs were obtained by selecting for resistance to 5-fluoroorotic acid and were identified as *ura3* mutants by transformation with *P. stipitis URA3*. *P. stipitis URA3* was cloned by its homology to *Saccharomyces cerevisiae URA3,* with which it is 69% identical in the coding region. *P. stipitis* ARS elements were cloned functionally through plasmid rescue. These sequences confer autonomous replication when cloned into vectors bearing the *P. stipitis URA3* gene. *P. stipitis ARS2* has features similar to those of the consensus ARS of S. *cerevisiae* and other ARS elements. Circular plasmids bearing the *P. stipitis URA3* gene with various amounts of flanking sequences produced 600 to 8,600 Ura⁺ transformants per μg of DNA by electroporation. Most transformants obtained with circular vectors arose without integration of vector sequences. One vector yielded 5,200 to 12,500 Ura⁺ transformants per μg of DNA after it was linearized at various restriction enzyme sites within the *P. stipitis URA3* insert. Transformants arising from linearized vectors produced stable integrants, and integration events were site specific for the genomic *ura3* in 20% of the transformants examined. Plasmids bearing the *P. stipitis URA3* gene and *ARS2* element produced more than 30,000 transformants per μg of plasmid DNA. Autonomously replicating plasmids were stable for at least 50 generations in selection medium and were present at an average of 10 copies per nucleus.

Fermentation of hemicellulosic sugars is required for the economic bioconversion of lignocellulose to ethanol. *Pichia stipitis is* one of the best xylose-fermenting yeasts (15, 58, 68). In addition to metabolizing all common monosaccharides, *P. stipitis* uses xylan (35), both enzymatic and acid hydrolysates of hemicellulose (69, 73), and spent sulfite waste liquors (8). P. *stipitis* produces ethanol but little or no xylitol from xylose. However, wild-type strains of *P. stipitis* will not ferment xylose at rates or with yields that enable commercial ethanol production from hemicellulosic sugars (47). To be economically feasible, fermentation rates need to be several times faster with greater yields than now achievable (27, 31).

Some research groups have attempted to improve yeast xylose fermentations through heterologous gene expression. *P. stipitis* genes for xylose reductase *(XYL1),* and xylitol dehydrogenase *(XYL2)* have been cloned and expressed in *Saccharomyces cerevisiae.* (2, 22, 66). When only *XYL1* is expressed, *S. cerevisiae* forms large amounts of xylitol. When both genes are expressed, *S. cerevisiae* transformants grow and carry out a restricted fermentation of xylose (32, 33, 67).

Manipulation of *P. stipitis* genes to improve fermentation in *P. stipitis* has been limited by the systems employed. Ho et al. have described the transformation of *P. stipitis* based on kanamycin resistance (28), and Morosoli et al. have expressed genes in *P. stipitis* by using *S. cerevisiae HIS4* as a selectable marker on an *S. cerevisiae* - based shuttle vector (42), but transformation rates were low in both instances. Transformation systems based on *URA3* genes (9) and autonomously replicating sequences (ARSs) (65) are very powerful in *S. cerevisiae,* other yeasts (12, 52, 57), and fungi (19, 59). These *URA3* - based systems use both positive and negative selection and can be manipulated to introduce multiple disruptions in the genome (l).

The present work describes an efficient transformation system for *P. stipitis* based on integrating vectors carrying the *P. stipitis URA3* gene and autonomously replicating vectors bearing both the *URA3* gene and a *P. stipitis* ARS *(ARS2).* Transformation by electroporation is efficient, with frequencies for integrating vectors at about 10,000 transformants per μg of DNA and more than 30,000 transformants per μg of DNA for *ARS2* - bearing vectors. This system allows random and site-specific integration and stable autonomous replication. The *URA3* gene and *ARS2* from *P. stipitis* are closely related to a number of other yeast *URA3* genes and ARS elements.

MATERIALS AND METHODS

Strains. *P. stipitis* CBS 6054 (NRRL Y-11545, ATCC 58785) was the parental strain for mutagenesis and the source of all cloned DNA. We obtained the *P. stipitis ura3* auxotroph TJ26 *(ura3-1)* by selecting for resistance to 5-fluoroorotic acid (FOA) in the presence of 100 mg of uridine per ml by the method of Boeke et al. (9). *S. cerevisiae* SHY-3 (10) (a *ste-VC9 ura3-52 trp-289 leu2-3 leu2-112 his3-D1 ade1-101 can1-100)* was obtained from the Yeast Genetic Stock Center at the University of California, Berkeley. .*Escherichia coli* CSH28 [Δ(*lac pro) supF trp pyrF strA thi*], which has a defect in

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orotidine monophosphate decarboxylase (39), was a gift from J. M. Cregg (Oregon Graduate Institute of Science and Technology, Beaverton). *E. coli* DH5 α (Gibco BRL, Gaithersburg, Md.) [F^- *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*] was used for all recombinant DNA experiments that required a bacterial host.

Media. Yeasts were routinely cultivated in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or YMA (1% yeast extract, 2% malt extract). Yeast nitrogen base (YNB) without amino acids and with 5 mg of ammonium sulfate per ml (Difco, Detroit, Mich.) was used as the basal defined medium for all auxotrophic selections and transformations. Auxotrophs were grown in YNB (0.17%) with 2% glucose (YNBG), and required nutrients were supplied according to Rose et al. (48), except that occasionally uridine was supplied at 100 mg/ml in place of uracil. *E. coli* was cultivated in Luria-Bertani medium with 50 mg of ampicillin per ml in liquid media or 100 mg of ampicillin per ml in solid media.

Plasmids. Plasmid UT701, which consists of the *S. cerevisiae URA3* gene (9) on a l-kb Bg/II fragment from a bacterium-fungus shuttle vector, was obtained from D. Cullen (Forest Products Laboratory, Madison, Wis.). YEp351 and YEp352 (26), which bear the *S. cerevisiae LEU2* and *URA3* genes, respectively, were both obtained from the American Type Culture Collection (Rockville, Md.). YCp50 (49) was provided by D. Tolervay and E. Hurt of the European Molecular Biology Laboratory (Heidelberg, Germany). Plasmid Bluescript KSII$^+$ was obtained from Stratagene (La Jolla, Calif.).

DNA isolation. Plasmid DNA was isolated and purified by the alkaline extraction method of Birnboim and Doly (7) or with the Magic Prep kit of Promega Corp. (Madison, Wis.), Yeast genomic DNA was isolated and purified as described by Specht et al. (61) or Rose et al. (50). Yeast plasmid DNA was prepared by the method of Hoffman and Winston (29).

Enzymes and chemicals. Restriction enzymes and other DNA modification enzymes were obtained from New England Biolabs (Beverly, Mass.), Stratagene, or Promega and were used as recommended by the suppliers. FOA was obtained from PCR, Inc. (Gainesville, Fla.). SeaKem GTG and SeaPlaque GTG agarose were obtained from FMC BioProducts (Rockland, Maine).

Mutant isolation. Spontaneous *P. stipitis ura3* auxotrophs were isolated by their ability to grow on media containing FOA (9). Cells from a 3-week-old culture of *P. stipitis* CBS 6054 were suspended in distilled water to a density of ca. 10^8 cells per ml, and 0.1 ml was spread onto YNBG agar plates supplemented with 100 mg of uridine per ml plus 0.5, 1.0, or 1.5 mg of FOA per ml. From these plates, 36 strains were obtained and inoculated onto YNBG agar plates with FOA (0.75 mg/ml) and uridine, YNBG plus uridine, and YNBG alone. Ura$^-$ strains were examined further. *ura3* mutant strains were identified by complementation with *S. cerevisiae URA3* on YEp352 and with *P. stipitis URA3* on pVY1.

Transformations. *P. stipitis* TJ26 and *S. cerevisiae* SHY-3 were transformed by the electroporation method described by Becker and Guarente (3). Freshly grown, young cells (50 μl; about 2×10^8 CFU) were aliquoted into a 0.2-cm cuvette for electroporation in a Gene Pulser together with a Gene Controller apparatus and cuvettes from Bio-Rad (Hercules, Calif.). Electrotransformation rates were optimum with 200 ng of DNA at a voltage of 1.5 kV (7.5 kV/cm) with a capacitance and resistance of 25 μF and 200 W, respectively. Following electroporation, cells were plated on YNBG without uracil plus 1 M sorbitol for osmotic support. Colonies were visible after 2 to 4 days of growth at 30°C. Transformation frequencies varied among different preparations of competent yeast cells. Electrocompetent *P. stipitis* TJ26 could be frozen in 1 M sorbitol and stored at –80°C, but transformation frequencies decreased as much as 100-fold. All transformation frequencies reported here were obtained with freshly prepared cells. The reversion frequency was estimated from the number of colonies that arose from a control electrophoresis without DNA.

Cloning the *P. stipitis URA3* gene. Genomic DNA from *P. stipitis* CBS 6054 was cut with *Xho* I and *Bam* HI and electrophoresed through 0.7% agarose. DNA corresponding to ca. 4.4 kb was electroeluted from the gel slice. The purified DNA fragment was ligated into the corresponding sites of pBluescript KSII$^+$. Three transformants of *E. coli* DH5 α contained plasmids with an identical 4.4-kb insert that hybridized to the *S. cerevisiae URA3* probe. One of these was designated pVY1. To locate the *URA3* gene on this fragment, we restricted the insert and again hybridized with the *S. cerevisiae URA3* probe. The insert was subcloned by restriction digestion with *Not* I and *Sac* I (pVY2), *Sal* I and *Xba* I (pVY3), *Hin* dIII and Xba I (pVY4), and *Hin* dIII and *Eco* RI (pVY5) and inserted into the corresponding sites of pBluescript KSII$^+$ (see Fig. 1). YIpPs1 was constructed by excising 62 bp from the cloning cassette in pVY2 to eliminate inconvenient restriction enzyme sites (*Bam* HI to *Apa* I). We constructed pVY351 by inserting the *Sac* I - *Bam* HI fragment from pVY1 that contains the *P. stipitis URA3* gene into YEp351. The *URA3* gene was sequenced from the *Bam* HI site at position –327 to position + 1167, using the dideoxy chain termination method of Sanger et al. (54) (Lofstrand Labs, Ltd., Gaithersburg, Md.).

Cloning the *P. stipitis ARS2* sequence. We cloned *P. stipitis ARS2* by taking advantage of its ability to maintain *URA3* as an episomal, circular plasmid in *P. stipitis* TJ26. We partially digested *P. stipitis* genomic DNA with *Sau* 3AI, ligated the fragments into the *Bam* HI site of pVY2, and transformed *P. stipitis* TJ26 with the resulting ligation mixture. We pooled the cells from several plates (ea. 5×10^3 independent transformants) and cultivated them in YNB xylose broth for 18 h at 30°C with agitation at 125 rpm. We then prepared plasmid DNA from the fresh broth culture to transform *E. coli* DH5 α cells by the method of Hoffman and Winston (29). The nucleotide sequences of *ARS2* in pJM6 were determined by the dideoxy method (54), using a Sequenase kit supplied by United States Biochemicals (Cleveland, Ohio). Exonuclease III was used to make nested sets of deletions for nucleic acid sequencing by the method of Henikoff (23) as described by Sambrook et al. (53).

Southern blot analysis. Southern transfer (60) by capillary blotting was performed as described by Sambrook et al. (53). DNA hybridizations were done with a Genius 1 kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as described by the manufacturer. Nylon membranes were either positively charged microporous nylon 66 membranes (Boehringer Mannheim) or Nytran filters (Schleicher & Schuell, Keene, N.H.). Hybridizations were typically done in 25% formamide at 37°C, and washes were in 2× SSC (l× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 22°C and 0.5× SSC at 37°C for 5 to 10 min.

Computer analysis. The Genetics Computer Group programs (13) developed at the University of Wisconsin—Madison were used to evaluate DNA and derived amino acid sequences. Densitometry was done with a Hewlett-Packard Desk Sanner with the Quantitative Images program (National Institutes of Health) on a Macintosh II computer.

Nucleotide sequence accession numbers. The GenBank sequence accession number for *P. stipitis URA3* (PSURA3.fe) is U08629, and that for *P. stipitis ARS2* (PSARS2.fe) is U08628.

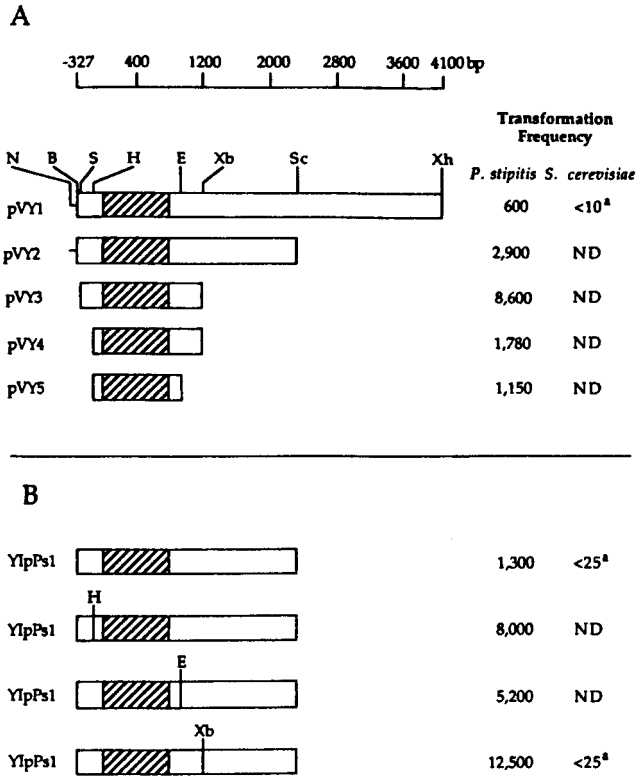

FIG. 1. Transformation frequencies of *P. stipitis* TJ26 with *URA3* gene-bearing plasmids. (A) Transformation frequencies of circular plasmids bearing the *URA3* gene from *P. stipitis*. Plasmids pVY1 to pVY5 bear deletions in the genomic DNA fragment containing the *URA3* gene. Restriction enzyme sites are *Not*I (N), *Bam*HI (B), *Sal*I (S), *Hin*dIII (H), *Eco*RI (E), *Xba*I (Xb), *Sac*I (Sc), and *Xho*I (Xh). (B) Transformation frequencies of *P. stipitis* with linearized *URA3*-bearing plasmids. YIpPs1 plasmid bearing the *P. stipitis URA3* gene either undigested or digested with *Hin*dIII (H), *Eco*RI (E), or *Xba*I (Xb), all of which cut on either side of the structural coding region (▨) of the *URA3* gene, was transformed into *P. stipitis* TJ26 (*ura3-1*). Hatched boxes indicate the amino acid-coding sequence of the *URA3* gene. a, below the level of detection in this experiment; ND, not determined. Units for transformation frequencies are number of Ura$^+$ colonies per milligram of DNA per 2×10^8 cells transformed. Control experiments showed that approximately 30% of the cells survived electroporation.

RESULTS

Isolation of *P. stipitis* TJ26 *(ura3).* Of the 36 *P. stipitis* Ura$^-$ mutants originally obtained, eight strains grew well on YNBG with FOA and uridine and on YNBG with uridine but not on YNBG alone. TJ26 exhibited an absolute requirement for uracil for growth and reverted to uracil prototrophy at a low frequency ($<1.5 \times 10^{-8}$). *P. stipitis* TJ26 could be transformed by *P. stipitis URA3*- bearing vectors at high frequencies (Fig. 1). We did not identify the mutation in the *ura3* gene in strain TJ26 and therefore designated it *ura3-1*.

Transformation with *S. cerevisiae* and *P. stipitis URA3.* Two different *S. cerevisiae* vectors, YEp352 and YCp50, transformed TJ26 but with only low efficiency (0 to 100 Ura$^+$ transformants per μg of DNA). These grew poorly or not at all when subcultured to plates or liquid, and genomic Southern blots of transformants that did grow showed no extrachromosomal or integrated plasmid sequences (data not shown). Transformants obtained with *S. cerevisiae URA3* apparently arose either from gene conversion events or from transient expression of *S. cerevisiae URA3* on the episomal vectors, because no integration of exogenous DNA was observed, and some transformants did not grow following subculture. To obtain higher transformation frequencies and site-specific integration, we cloned the *P. stipitis URA3* gene for use as a homologous selectable marker.

A 1.0-kb *Bgl* II fragment of the structural *URA3* gene of *S. cerevisiae* from the vector UT701 hybridized specifically to a 4.4-kb *Xho* I - *Bam* HI fragment from *P. stipitis.* To determine the optimum fragment for integrative transformation, we made subclones at convenient restriction sites (Fig. 1A). Plasmid pVY3, which bears approximately 1.43 kb of *P. stipitis* genomic DNA, transformed TJ26 at a rate higher than that of any of the other plasmids (Fig. 1A). When transformed into *E. coli* CSH28, pVY2 complemented the *E. coli pyrF* mutation (data not shown). This indicated that pVY2 contained the *URA3* gene and not some nonallelic suppressor. When the *P. stipitis URA3* gene was introduced into the *S. cerevisiae* high-copy-number vector YEp351, the resulting vector, pVY351, transformed *S. cerevisiae* SHY3 essentially as well as YEp352, which bears *S. cerevisiae URA3.*

In *S. cerevisiae* (46) and other yeasts (52), a double-strand break in homologous DNA increases site-specific integration of the plasmid. To determine the effect of double-strand breaks on our integrative transformation vectors, we electrotransformed *P. stipitis* TJ26 with supercooled YIpPsl or YIpPsl that had been digested and linearized with *Hin* dIII, *Eco* RI or *Xba* I, all of which cut within the flanking regions of the *URA3* gene (Fig. 1B). Linearization increased the transformation frequency about 4-to 10-fold, depending on the restriction site.

Figure 2 shows a representative result for 6 of the 30 transformants tested. The banding pattern shown is typical of three different types of transformation. Lanes 3,5, and 8 show the endogenous *URA3* band of TJ26 (10.3 kb, shown in lane 1) and one other band. These extra bands were not the same size as linearized YIpPsl (Fig. 2, lane 2), nor were they characteristic of uncut, episomal plasmid. This result indicated that the plasmid had integrated into the genomic DNA. Because the extra bands had different sizes, this pattern corresponded to integration into different, nonhomologous sites within the genome. Lanes 4 and 7 of Fig. 2 show only one band of 16 kb, and it is different in size from the endogenous *ura3* gene in TJ26 by the 5.3-kb size of the YIpPsl plasmid. This pattern indicated that the entire YIpPsl plasmid had integrated into the *ura3* gene of TJ26 by homologous recombination. Lane 6 shows the endogenous *ura3* gene as a single band at 10.3 kb present in TJ26 and a series of higher-molecular-weight bands. These bands are typical of plasmids that have not integrated into the genome. In one transformant of 30 analyzed by Southern hybridization, we observed what appeared to be YIpPsl—which does not possess a known ARS-replicating autonomously. This may indicate that plasmids without obvious replication origins can propagate for some time in *P. stipitis,* like plasmids described for *Schizosaccharomyces pombe* (24, 41).

We also isolated genomic DNA from 16 individual colonies transformed with uncut plasmids pVY2 and YIpPsl. Southern analysis showed that in all cases but one, only one band hybridized to a *URA3* probe, and this band corresponded in intensity and size to the endogenous *ura3* gene in the auxotroph TJ26 (data not shown). A second hybridization to the same membrane with pBluescript as a probe revealed no hybridization signal. In one case (of 16), the entire pVY2 plasmid integrated into the *ura3* gene of TJ26.

Sequence of the *URA3* gene of *P. stipitis* and comparison with other yeast *URA3* genes. The nucleotide sequence and

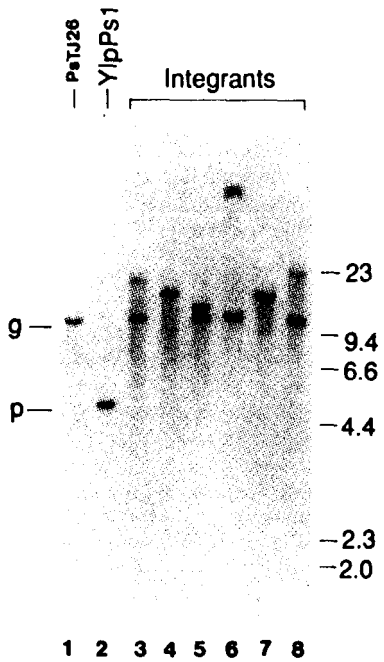

FIG. 2. Genomic Southern analysis of *P. stipitis* transformed with linearized plasmid bearing the *URA3* gene. The transformants described in the legend to Fig. 1B were grown in minimal liquid medium overnight at 30°C, and genomic DNA was isolated. Each lane represents 15 μg of DNA cleaved with *Pst*I, which does not cut within the *URA3* gene or in the YIpPs1 plasmid, except lane 2, which represents 100 pg of plasmid YIpPs1 digested with *Bam*HI. The *URA3* gene from plasmid pVY3 was used as a probe. Lane 1 is the untransformed parent strain, TJ26. Lanes 3 to 8 are *P. stipitis* TJ26 transformed with YIpPs1 linearized with *Hin*dIII at position −124 (lanes 3 and 4), *Eco*RI at position +909 (lanes 5 and 6), or *Xba*I at position +1161 (lanes 7 and 8). Position numbers are relative to the start of translation (see Fig. 3). Molecular size standards are shown to the right (in kilobase pairs). g, genomic; p, plasmid.

deduced amino acid sequence of the *P. stipitis URA3* gene are shown in Fig. 3. The original *Bam* HI - *Xho* I fragment cloned from *P. stipitis* contained 327 bp upstream from the start of translation, 801 bp of protein-coding sequence, and approximately 3.25 kb of 3' flanking sequence.

The *P. stipitis URA3* gene encodes a 267-amino-acid protein that has a high level of sequence identity to other yeast *URA3* genes and proteins, as shown in Fig. 4. Among the *URA3* genes available for sequence comparisons from the GenBank and EMBL data banks, the genes most similar to *P. stipitis* are from *Candida maltosa* (protein, 85% identical; nucleotide, 74% identical), *Candida albicans* (protein, 81%; nucleotide, 73%), and *Candida boidinii* (protein, 81%; nucleotide, 74%). The *P. stipitis URA3* gene is more distantly related to *URA3* genes from *S. cerevisiae* (protein, 71%; nucleotide, 67%) and *Kluyveromyces lactis* (protein, 73%; nucleotide, 69%). In this analysis the yeasts were found in two clusters, except for *Schizosaccharomyces pombe,* which did not appear to be closely related to the other yeast *URA3* genes.

In comparison to the 5' flanking sequences of the *S. cerevisiae URA3* gene (51), the sequences in the *P. stipitis* gene centered at positions – 91, –75, and/or –59 are likely to bind TATA-binding factor. Other sequences that may be necessary for transcriptional control include the long tracts of dA-dT, as indicated in Fig, 3, The core sequence GTAATCT, which binds the PPR1 protein in *S. cerevisiae,* is also conserved in the *P. stipitis URA3* gene and is centered at position –201. However, without transcript or footprint analysis, the significance of these similarities is unknown.

Isolation and sequencing of ARS elements. *E. coli* transformants obtained with autonomously replicating vectors rescued from *P. stipitis* carried plasmids having two nonidentical inserts. One was 6.2 kb in length *(ARS1),* and another was 4.1 kb in length *(ARS2). ARS1* was unstable when propagated in *E. coli* and was not studied further. Sequential transfers between *P. stipitis* TJ26 and *E. coli* showed that the vector containing *ARS2* could replicate in both hosts. The 4.1 -kb *ARS2* sequence was subjected to deletion analysis, and the sequence(s) responsible for autonomous replication was located by its functionality in TJ26. A 1.15-kb fragment in pJM14 is necessary and sufficient for high-level transformation, presumably as a result of episomally maintained plasmids (Fig. 5).

Sequences similar to the *S. cerevisiae* consensus ARS (A/T TTTAT A/G TTT A/T) (36, 72) are found in *P. stipitis ARS2* (underlined regions in Fig. 6). Within the first 600 bp of the 1,157-bp fragment, there are seven sequence motifs similar to the 11 -bp consensus *S. cerevisiae* ARS (one or two mismatches). These motifs are surrounded by extensive regions of similarity with *S. cerevisiae ARS2* (54 and 63% identity in 154- and 60-bp overlaps, respectively) (70) and with other ARS elements (data not shown).

Stability and copy number of *ARS2* - bearing plasmids in *P. stipitis.* We assumed that the increased transformation frequencies of plasmids bearing the putative ARS elements were due to the plasmids being maintained episomally. To test this, we performed Southern analysis on 14 independent transformants. *P. stipitis* TJ26 was transformed with plasmids bearing 4.1 kb (pJM2), 1.8 kb (pJM6), and 1.15 kb (pJM14) of the putative ARS and was maintained in minimal liquid media for 7 days with subculturing to approximately 10^6 cells per ml each day. Under these conditions, the cells grew through at least 50 generations. Genomic DNA was prepared, and the DNAs were cleaved by restriction enzymes. Southern analysis of six transformants with the labeled *P. stipitis URA3* gene as a probe is shown in Fig. 7.

The enzyme *Bam* HI does not cleave plasmid pJM2; therefore, lanes 4 to 6 of Fig. 7 show multiple high-molecular-weight bands characteristic of uncut and concatenated plasmid bands as well as the genomic *P. stipitis ura3* on a *Bam* HI fragment. The enzyme ***Pst*** I cuts plasmids pJM2 and pJM6 once, and the prominent bands in lanes 8 to 13 have the characteristic sizes expected from a single cleavage. Southern analysis of pJM14 showed identical results (data not shown). Of the 14 *P. stipitis* transformants tested, all maintained the *ARS2* - bearing plasmids as extrachromosomal elements for at least 50 generations in liquid minimal medium under selective conditions. Under nonselective conditions, the plasmids were lost within 10 to 20 generations (data not shown). This mitotic instability is characteristic of episomally maintained, ARS-bearing plasmids.

Figure 7 further shows that the plasmids were maintained at a moderately high copy number. By comparing the nuclear URA3 band with the plasmid band by densitometry analysis, we estimated that the copy numbers were 8 to 10 copies of plasmid per nucleus.

DISCUSSION

This report describes a new transformation system for the xylose-fermenting yeast *P. stipitis.* The system is based on a *P. stipitis ura3* auxotrophic strain (TJ26 [*uru3- 1*]) and on the orotidine-5'-phosphate decarboxylase *(URA3)* gene and an ARS *(ARS2),* both isolated from *P. stipitis* CBS 6054. Plasmids

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                                 BamH I
-327                             GGATCCG GAGCCGTTGT CTGAGAAGAA TAAAGAGAAT ATAGTCGACA

-280 CGGCTGCTGA GGGTGGAGAA GAAAAGAAAA TCGAAACAGA CGGAGATAAC TAATCTGCAA TAAATATTAC

-210 ACTTATGTAA TCTAATGGTT TCTTAAGAAA GTAGTCGTTA TGGAGAAGTA GTATTTTGTA TTTTAGTTAC
                                                          *** *            ****
-140 TTTCATAAAT ATAGCAAGCT TATGTTGCAT CTCAAACAGT GCGAAAAATA TTTTAGTCTA TTTTATAGTA
                 ****
 -70 TCTACAAACA AATTTTCAAT TCATCACAGA CTTTCTTTGT GTACTAGCAA CACCCTAAAA ACATTCGACA

   1  M   V   N   V   Q   T   Y   T   Q   R   A   E   S   H   Q   S   P   V   A   Q
   1 ATG GTC AAC GTC CAA ACC TAT ACA CAA AGG GCC GAG TCT CAC CAG TCA CCG GTA GCA CAG

  21  R   L   F   K   L   M   D   S   K   K   T   N   L   C   A   S   V   D   V   K
  61 CGT CTC TTC AAA TTA ATG GAC AGC AAG AAA ACT AAC TTG TGT GCT TCA GTG GAT GTG AAA

  41  T   T   S   E   F   L   S   L   I   D   K   L   G   P   Y   I   C   L   V   K
 121 ACA ACC TCT GAG TTC TTA TCT TTG ATT GAT AAG TTG GGT CCT TAC ATT TGT TTG GTT AAG

  61  T   H   I   D   I   I   D   D   F   S   Y   E   G   T   I   V   P   L   L   E
 181 ACC CAT ATC GAC ATT ATC GAC GAT TTT TCG TAT GAA GGT ACC ATT GTT CCT TTG TTG GAA

  81  L   S   K   K   H   N   F   M   I   F   E   D   R   K   F   A   D   I   G   N
 241 TTG TCC AAG AAG CAC AAC TTC ATG ATC TTT GAA GAT CGT AAA TTC GCA GAC ATT GGA AAC

 101  T   V   K   S   Q   Y   S   G   G   V   Y   K   I   A   Q   W   S   D   I   T
 301 ACT GTC AAG TCT CAG TAT TCA GGT GGA GTC TAC AAG ATT GCC CAA TGG TCA GAT ATC ACC

 121  N   A   H   G   V   T   G   S   G   I   V   Q   G   L   K   E   A   A   R   E
 361 AAT GCT CAC GGT GTT ACT GGT TCT GGT ATT GTT CAG GGA TTA AAG GAA GCT GCA AGA GAA

 141  T   T   D   E   P   R   G   L   L   M   L   A   E   L   S   S   K   G   S   I
 421 ACT ACC GAC GAA CCA AGA GGT TTA TTG ATG CTT GCC GAA CTA TCT TCT AAG GGC TCG ATT

 161  A   H   G   K   Y   T   E   E   T   V   E   I   A   K   T   D   K   E   F   V
 481 GCT CAT GGT AAG TAT ACT GAA GAA ACT GTG GAA ATC GCC AAA ACT GAC AAA GAG TTT GTT

 181  I   G   F   I   A   Q   R   D   M   G   G   Q   D   E   G   F   D   W   I   V
 541 ATT GGG TTC ATT GCC CAA AGA GAC ATG GGT GGA CAA GAT GAA GGT TTC GAT TGG ATT GTT

 201  M   T   P   G   V   G   L   D   D   K   G   D   S   L   G   Q   Q   Y   R   T
 601 ATG ACT CCT GGT GTC GGT TTG GAT GAC AAG GGT GAC AGT TTA GGT CAA CAA TAT AGA ACA

 221  V   D   E   V   V   S   T   G   T   D   I   I   I   V   G   R   G   L   F   G
 661 GTA GAT GAA GTT GTT TCC ACA GGA ACC GAT ATT ATC ATT GTT GGT AGA GGC TTA TTC GGC

 241  K   G   R   D   P   D   V   E   G   Q   R   Y   R   E   A   G   W   N   A   Y
 721 AAG GGT AGA GAT CCT GAT GTA GAG GGC CAA AGA TAC AGA GAA GCT GGA TGG AAC GCT TAC

 261  L   K   K   T   S   Q   V   *
 781 TTG AAG AAG ACA AGC CAA GTG TAA  AATATGTAAA ATTTTGTAAA AATCGTAAAA CTGTAATGTA

 845 CATCGTTAAG TATTTACAAA CAACTATATG ATTATTTACA AGCAGAGGCC TATTCCAAAA TTTCGAATTC
 915 CAGTAAGTCG TCAGTCAATC TGGTTCTCAT TATTTCAAAA ACCAAGAGGG CCACGGATGT AGCCGGTATT
 985 GTTGTCAATG CATTGCGGAC AAAACCTTTG TATGCCATTT GATACCAAGT CAACTTTGAT TTTGTCTTCA
1055 TCCTAAGGAT TTGGTTATAT GTATCAATGT ATGAGTTGTA GTACAAGGTT ATGGAGGGTT TGTATGAACG
1125 CGTAGCTGAT GCATTGTAGA AATCAAGAGC TTCAAGTCTA GA
                                            Xba I
```

FIG. 3. Nucleotide and derived amino acid sequences of the *URA3* gene from *P. stipitis*. The putative TATA boxes (at positions −91, −75, and −58) are indicated by asterisks. The dA-dT regions are underlined, and the area of sequence similarity to the PPR1-binding sequence of *S. cerevisiae* is double underlined. Unique restriction sites for *Bam*HI and *Xba*I are indicated at positions −324 and +1161.

are transformed into *P. stipitis* by electroporation. When introduced into *P. stipitis*, *URA3*-bearing plasmids give rise to Ura$^+$ colonies by gene conversion or by integration into homologous or apparently random sites within the genome. Plasmids bearing both the *URA3* gene and *ARS2* are maintained exogenously for at least 50 generations at an average copy number of 8 to 10 per nucleus. Both integrating and autonomously replicating plasmids transform *P. stipitis* at high frequencies.

The results presented here show that circular plasmids not carrying *ARS2* are most likely to transform *P. stipitis* by gene conversion (15 of 16), but not by integration of the entire plasmid, and that these plasmids when linearized are most likely to transform by integration into the genome (29 of 30).

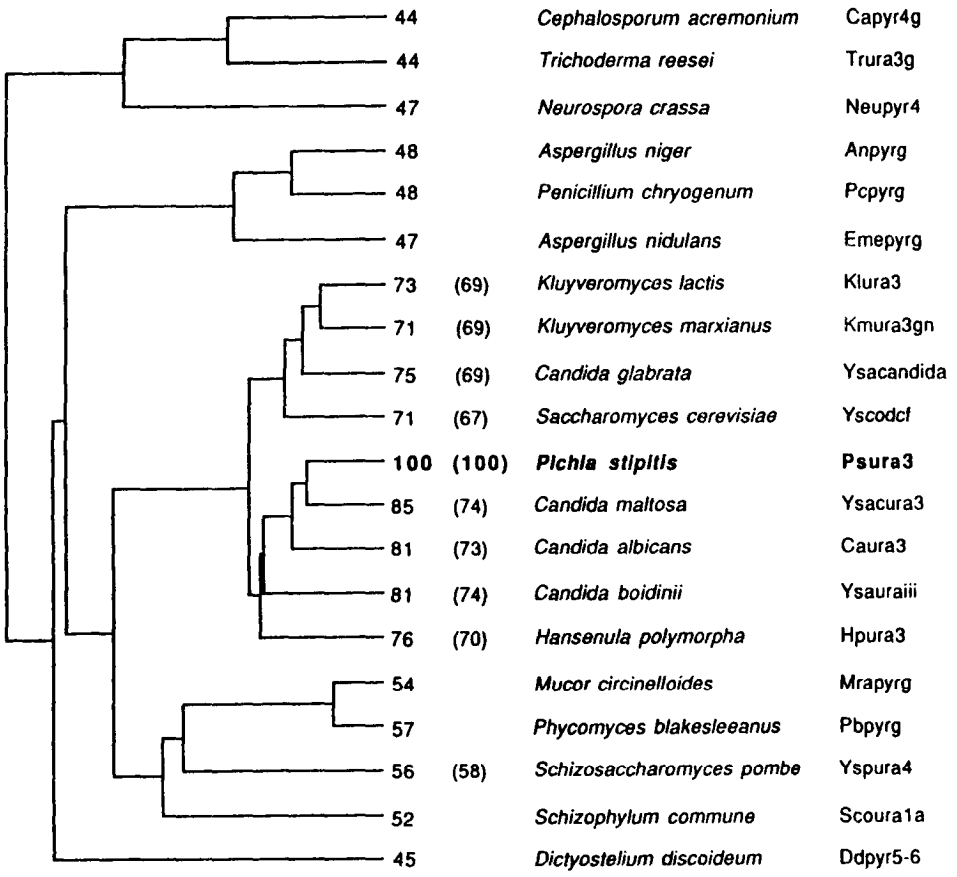

FIG. 4. Similarity among different fungal *URA3* genes. The sequences of 20 different *URA3* genes available from the GenBank and EMBL data banks were compared by using the Genetics Computer Group Pileup program. The organism name, percent similarity to *P. stipitis* URA3 protein, and percent similarity to *URA3* DNA coding sequences (in parentheses) are shown in the dendrogram. Pileup is a simplification of the progressive alignment method of Feng and Doolittle (17) and is similar to the method described by Higgins and Sharp (25). References: Capyr4g, 71; Trura3g, 5; Neupyr4, 43; Anpyrg, 74; Pcpyrg, 11; Emepyrg, 44; Klura3, 40; Kmura3gn, 6; Ysacandida, 75; Yscodcf, 50; Ysacura3, 45; Caura3, 16; Ysauraiii, 52; Hpura3, 38; Mrapyrg, 4; Pbpyrg, 14; Yspura4, 20; Scoura1a, 18; and Ddpyr5-6, 30.

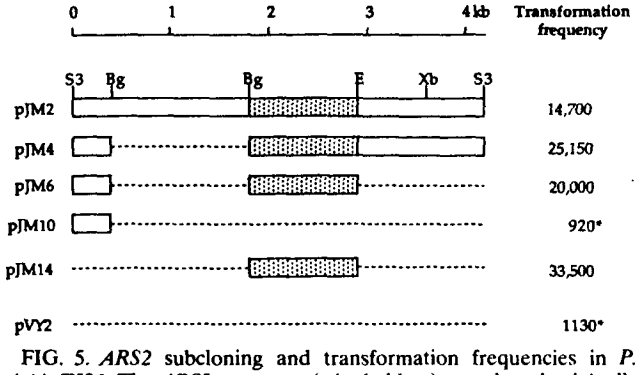

FIG. 5. *ARS2* subcloning and transformation frequencies in *P. stipitis* TJ26. The *ARS2* sequence (stippled box) was cloned originally into pJM2 as described in Materials and Methods. Deletions (dotted lines) were made within the original pJM2 vector at convenient restriction enzyme sites or by subcloning. pVY2 was included as a non-*ARS2*-bearing plasmid control. The asterisk indicates that these values were obtained in a separate experiment with a different preparation of cells. Abbreviations for restriction sites: S3, *Sau*3AI; Bg, *Bgl*II; E, *Eco*RI; Xb, *Xba*I.

Surprisingly, only 20% of integrations (6 of 29) were site specific to *ura3-1*. Because all secondary bands were different sizes, we tentatively concluded that about 80% (23 of 29) of integrated transformation events were by nonhomologous and apparently random integration into the genome. Finally, because we never saw a banding pattern corresponding to a homologous recombination band (at 16 kb) and an endogenous *ura3* band (at 10.3 kb), we also tentatively conclude that *P. stipitis* TJ26 is primarily haploid.

P. stipitis has been described as a homothaliic diploid yeast (34). Isolation of auxotrophic mutants of diploid strains is sometimes difficult, and other investigators have used reagents, such as nystatin, to improve the recovery of mutants following mutagenesis and enrichment (21, 55). We obtained *P. stipitis* TJ26 *(ura3-1)* from a YMA culture that had been held for several weeks at 3 to 5°C, and we now know that *P. stipitis* sporulates at low frequencies under these conditions (unpublished data). We may have fortuitously isolated a recessive *ura3* mutation as the parent strain CBS 6054 was undergoing sporulation. The metabolic analog, 5'-FOA, provides a strong positive selection, so a *ura3/URA3* heterodiploid could become a *ura3/ura3* homozygous diploid by mitotic gene conversion, and these strains could have been readily detected under the conditions employed. Alternatively, *P. stipitis* may be haploid or exist in culture as a mixture of diploid and haploid cells. The

```
                10         20         30         40         50         60         70         80
   EcoR I       |          |          |          |          |          |          |          |
   1 GAATTCAGTA TAGGATATGG TGTTTAGCAA AATATGCCTC TGTTTTTTTG GTAAGTTGTA GTAATTCGGA ATTTTTCGCA

  81 ACTTTTCCAG AAATGCCTTT AGTCCAAACA TATTATCGCA AATTATTTAT AATCAGTTAA TATGTTTAAG TCTCTGGAAT

 161 TTCATGTCAG CAGTGAAACG AAGTTGCACA GTCTATAGCA CCCTTAACGC TGAAGTATTG ACTAAATGTG TCTATTTTTC

 241 TCTTCGTACA TTTTAGTCAG ATTGTCGAAA TCTAAATCTC TACAGGCTGC AACCAAAGAC ATCTTGCTGA TTCGTATCTC

 321 ATTTCTAACT CAGCAAAATT TTATTATCCA GCAGACAACA ATTATCTCCT TTCTTACATG ATACTTAGAC TTCTAATCTC

 401 CTAGTGGATA AGAAAAATAA ATCCAAAGAA TGGAAAATGT AAAGCTGCGC TGGTGATGTC CGCTAAGGTC ATAAACTCAA

 481 GCTACATTGC GTCGAATATT TTGCTACGAT TGCTCAGAAA TAAAATCCAT TGTATATCAT GAGGGTCAAT AATAATAATA

 561 ATCAACGAGT CCGATTGCTG AACCACCCAA TAAATATCAA TCATGGTCTT ACAAGCAAAC ACAACATACA ATAATTATCG

 641 GGGCGGTCCA ACAATGTCGG CTCGATTGTA CTGTATGCAC CTAGCGTTTT TCATCTGGCA TTTATCCGAG TACCCCTAAA

 721 CGCTAAAGCG TAGAGAATAA TGTAACCTTA TCTTGCCGGA GCAACTAACA ATCGCCTTGA ACACAAACTG GGTTGCTCCC

 801 CTGTTCTCGA AGTCGAAGAG TAGCTCCTTC TAGCTCCAGT GAAAATGTAC AGATACGCAG AGATGGTGAC TGTACCTAAA

 881 CAGACTCCGT TTGCACCTAC TCCGTCAGAA CAATAGCTCT ACAATAAAGC CTCCCACCAC GCTCCACTTA CATATGCATA

 961 TGCACATACA TTGTAAGAGA AGTATGTTGG CCATGTAGAC ACAAACAAGA TGTCGGCGAC TACAGATTCT TTTATCCAGC

1041 CAATGGAGAG AGAAGGTACT TGTCAGAGAA GAATGCATGC ACCGGGAAAT AATGCGGTGT CTGGGGCTTT TGGATGAGTT

1121 AAAATACTTC TGACCTTGTA GACACCGCAG AAGATCT
                                    Bgl II
```

FIG. 6. Sequence of the *ARS2* element of *P. stipitis*. The 1,157-bp (*Eco*RI site to *Bgl*II site) sequence bearing ARS activity present within the 1.8-kb element in pJM6 was sequenced (described in Materials and Methods). The sequence similarities (one or two mismatches) to the consensus *S. cerevisiae* ARS (A/T TTTAT A/G TTT A/T) are underlined. Sequences similar to other ARS elements are double underlined (see text).

observation that Southern analysis of homologous integration transformants never showed one disrupted and one endogenous allele (Fig. 2) reinforces the idea that strain TJ26 is haploid.

In *S. cerevisiae* (46) and other yeast (52) transformation systems, site-specific homologous integration occurs at high frequencies; in most cases the frequency approaches 100%. In contrast, we found only 20% homologous integration into the *P. stipitis ura3* locus, and the other 80% of integration events occurred at apparently random sites within the genome. These results demonstrate that site-directed gene disruption might be slightly more difficult to achieve in *P. stipitis* than in other yeasts, but in practice it requires screening of only a few more transformants. From a practical perspective, random integration may prove advantageous. Expression is often site dependent, and some integrative transformants bearing heterologous DNA may prove more useful than others as a result of higher or lower levels of expression.

The 5' flanking sequence of the *P. stipitis URA3* gene reveals some similarity with the 5' flanking region of the *S. cerevisiae URA3* gene. The *P. stipitis URA3* gene contained extensive tracts of dA-dT from position –307 to –128, and sequences similar to these have been shown to be important for basal-level transcription of some *S. cerevisiae* genes (62,64). Although the TATA sequence at position –75 seems to be the best candidate for binding TATA-binding factor protein, there are two other TATA-like sequences, at –91 and –58. Similar to the ease for the *URA3* and *HIS3* (63) genes of *S. cerevisiae*, there may be more than one TATA element used for basal-level and induced transcription.

The *ARS2* element isolated from *P. stipitis* bears many of the hallmarks of ARSs isolated from other organisms. The sequence is 61% A or T, and the consensus ARS A/T TTTAT A/G TTT A/T is repeated seven times as 9 of 11 and 10 of 11 matches (Fig. 6). However, initial exonuclease III deletion analysis at the 5' end of the sequence shown in Fig. 6 indicates that as little as 350 bp at the 3' end of the sequence insufficient for high-frequency transformation of *P. stipitis* (unpublished data). Comparisons with other yeast ARSs showed only two regions of similarity in the 350-bp 3' terminus. *P. stipitis ARS2* shared a 15-bp region (100% identity from position 820 to 834) with YL4RSlDNA from *Yarrowia lipolytica* (37) and a 13-bp sequence (100% identity from position 1104 to 1116) with YSCARSS5 from *S. cerevisiae* (56). Neither block of identity is surrounded by regions of general similarity as observed with *S. cerevisiae ARS2* and other yeast ARS elements in the first half of *P. stipitis ARS2*. Identification of the sequences that are necessary and sufficient for autonomous replication will rely on more precise deletion experiments, which are in progress in our laboratory.

We recently found that the vector pJM6 was able to transform *S. cerevisiae* at high frequencies. These results suggest that the *URA3* gene and *ARS2* sequences isolated from *P. stipitis* are able to function in *S. cerevisiae*. This is surprising because we found that *ARS1* or 2μm replication origins of *S. cerevisiae* together with the *URA3* gene of *S. cerevisiae* were not

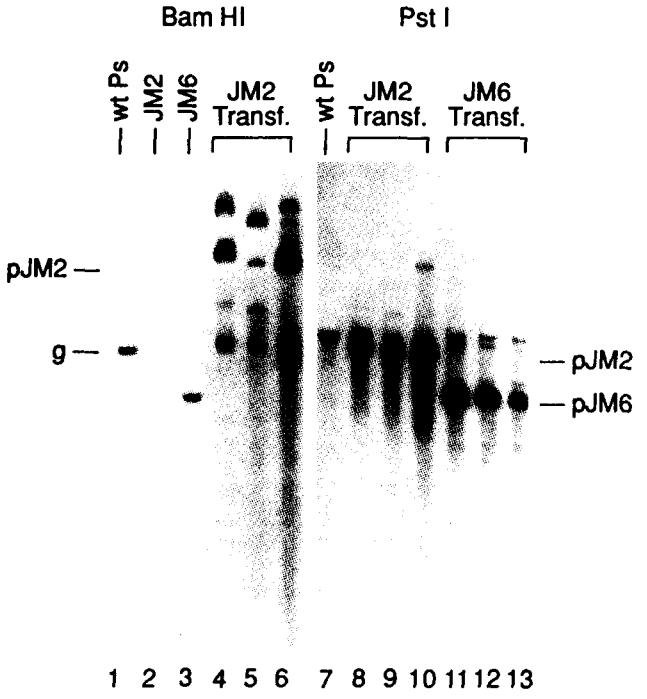

FIG. 7. Stability of *ARS2*-bearing plasmids in *P. stipitis*. Southern blot analysis of *P. stipitis* TJ26 transformed with pJM2 or pJM6 is shown. Transformants were grown for 50 generations in minimal medium (described in Materials and Methods), and total genomic DNA was isolated and digested with *Bam*HI (lanes 1 to 6) or *Pst*I (lanes 7 to 13). All lanes contain approximately 15 mg of DNA, except the control plasmid lanes (JM2 and JM6), which contain approximately 100 pg of plasmid DNA. The blot was hybridized to a labeled *URA3* gene fragment from pVY3. Lanes 1 and 7, wild-type *P. stipitis* 6054 DNA; lane 2, pJM2 plasmid DNA; lane 3, pJM6 plasmid DNA; lanes 4 to 6 and 8 to 10, DNAs from three individual colonies transformed with pJM2; lanes 11 to 13, DNAs from three individual colonies transformed with pJM6. g, genomic *ura3* gene.

able to transform *P. stipitis* at high frequencies (unpublished data).

The *ARS2* - bearing plasmids exist episomally in *P. stipitis*, rather than integrating into the genome, and are stable when propagated in minimal selective media for at least 50 generations. The extrachromosomal nature and stability of the plasmids provide a powerful molecular genetic system in *P. stipitis*, facilitating complementation analysis, mutational analysis of genes, and expression of heterologous proteins.

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