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Sh ble and Cre adapted for functional genomics and metabolic engineering of *Pichia stipitis*

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Abstract

Pichia stipitis is widely studied for its capacity to ferment D-xylose to ethanol. Strain improvement has been facilitated by recent completion of the *P. stipitis* genome. *P. stipitis* uses CUG to code for serine rather than leucine, as is the case for the universal genetic code thereby limiting the availability of heterologous drug resistance markers for transformation. Development of a modified selectable marker for resistance to bleomycin (*Sh ble*) and efficient excision of the marker after integration (*lox*P/Cre) should facilitate functional genomics and metabolic engineering in this yeast. The *Sh ble* marker did not code for an active protein in *P. stipitis* until four CUG codons were mutagenized to TTG, which is properly translated as leucine in yeasts that use the alternative yeast nuclear genetic code. The 18 CTG codons in Cre were mutagenized in a similar manner and the system was used to delete *XYL2*. The resulting *xyl2Δ* mutant did not use xylose as a carbon source. Published by Elsevier Inc.

Keywords: Transformation; Genetic engineering; Yeast; Expression; Mutagenesis; *Sh ble*; Cre; Alternative yeast nuclear genetic code; CUG

1. Introduction

The ascomycetous yeast, *Pichia stipitis*, is one of the best xylose fermenting organisms yet described [1,2]. For the last 15 years *P. stipitis* has been used as a source of genes for expression in *Saccharomyces cerevisiae* and it has been genetically manipulated for improved fermentation of xylose and glucose [3]. Fermentation of both glucose and xylose is essential for economical conversion of biomass into ethanol [4]. While *P. stipitis* ferments xylose very well, further improvements are necessary for development of commercial strains. Such improvements can best be accomplished through metabolic engineering, which requires genetic tools for transformation and marker recovery.

At least three transformation systems have been previously described for *P. stipitis*. Ho et al. reported protoplast transformation of *P. stipitis* CBS 7126 using a plasmid vector based on the *S. cerevisiae* 2μ vector using Km^R as the selectable marker and the antibiotic genticin to select for putative transformants, but because most cells are partially resistant to this antibiotic, its use is cumbersome [5]. Piontek et al. transformed a *P. stipitis trp5* mutant with *S. cerevisiae* derived vectors containing *S. cerevisiae TRP5* and a *Schwanniomyces*-derived autonomous replication sequence (ARS) [6]. Neither of these researchers reported targeted gene deletion in *P. stipitis*. In 1994 Yang et al. reported the high frequency transformation of *P. stipitis* TJ26 (*ura3*) using electroporation along with the native *PsURA3* gene as a selectable marker and a *P. stipitis* DNA sequence that served as an autonomous replication sequence (*ARS2*) [7]. Lu et al. extended this system by using *URA3* to knock out the gene for β-isopropylmalate dehydrogenase [8]. This latter system has been used successfully to disrupt *ADH1*, *ADH2* [9,10] and *XYL3* [11] in order to understand the function of these genes. It has also been used to disrupt *CYC1* and *STO1* in *P. stipitis* to reduce respiration and result in better strains producing ethanol [12,13] (Table 1).

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Table 1
Plasmids used in this study

Plasmid name	Features
pJML457	*ARS2, URA3*
pJML533	*ARS2, pTEF1-Sh ble* (adapted) – t*XYL2*
pJML545	pJML533 + p*XYL1*-Cre rec (adapted) – t*XYL1*
pJML585	pJML457 + *XYL2*

Although *P. stipitis* ferments biomass sugars, development of this yeast strain has been hindered by the limited utility of available genetic tools. A few auxotrophic markers have been cloned and described. Only two strains have been reported with two auxotrophic mutations, *P. stipitis* PLU20 and pJH43, which are *ura3 leu2* and *trp5-10*, *his 3-1*, respectively [8,6]. Only *TRP5* and *URA3* are useful in selecting for the presence of plasmids [14,15]. The most commonly used plasmid, pJM6, is based on pUC19 but due to the presence of both the *ARS* and *URA3* in the middle of the multiple cloning site (MCS) [7] its use is laborious. The problem is compounded by the presence of four common restriction enzyme sites in wild type *URA3*. In addition, most available promoters are either from the carbon metabolism genes *PsXYL1*, *PsADH1*, and *PsTKL1* or they come from a heterologous system, which can cause problems in comparing effects of heterologous gene expression when cells are grown on glucose or xylose. Moreover, *S. cerevisiae* promoters from the *PGK1*, *PDC1* and *ADH1* genes have shown only partial success in driving the expression of genes in *P. stipitis* [6,15]. Aside from the earlier report of Km^R, no selectable marker had been developed that would enable the introduction of genes into prototrophic strains. An improved transformation and expression system is essential to make use of the recently completed *P. stipitis* genome [http://genome.jgi-psf.org/euk_cur1.html] (Table 2).

The *lox*P/Cre recombination system, which enables the rescue of auxotrophic markers, has been adapted for use in *S. cerevisiae*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe* [16–18]. We thought a similar system would be very useful for the genetic manipulation of *P. stipitis*. Such a system would require a native promoter, an antibiotic resistance marker for use in various strains, and the construction of improved vectors. In the current paper we report the engineering of a similar system for use in *P. stipitis*, which uses the alternative yeast nuclear genetic code. We further demonstrate the use of this system for the disruption and subsequent complementation of *XYL2*.

P. stipitis had not been previously reported to use the alternative yeast nuclear code, but since its close relative, *C. shehatae*, had been found to use it, we hypothesized that *P. stipitis* probably did so as well, and we decided to test this possibility by transforming cells with a heterologous drug resistance marker that was engineered for alternative codon usage. Our findings support the alternative use of the CUG codon by *P. stipitis*.

Table 2
Yeast strains used in this study

Yeast strain	Genotype	Reference
Pichia stipitis CBS 6054	Wild type strain	[7]
P. stipitis UC7	*ura3*	[8]
P. stipitis yJML111	*Δxyl2::lox*P-*URA3-lox*P	This study
P. stipitis yJML123–127	*Δxyl2::lox*P	This study

2. Materials and methods

2.1. Yeast media

Synthetic defined medium without uracil (ScD-ura), yeast peptone dextrose (YPD), and ScD with 5′ fluoroorotic acid (ScD + FOA) were made as described in [19] except that all sugars were autoclaved separately from the basal media. Yeast peptone xylose (YPX) was similar to YPD but replaced dextrose with xylose. For YPD + Zeocin, 10 g of yeast extract and 20 g of peptone were dissolved in water. The pH was adjusted to 7.5 using NaOH, and 20 g of agar was added along with water to a final volume of 900 ml. After autoclaving, 100 ml of a 20% dextrose solution was added and the medium was cooled to 65 °C. Zeocin was added to a final concentration of 100 μg/ml, and the medium was quickly poured into plates.

2.2. EST library construction and sequencing

P. stipitis CBS 6054 [7] was grown at 30 °C in 200 ml of either YPD or YPX in either a 2.8 l flask shaken at 300 rpm or a 500 ml flask shaken at 50 rpm. Cells were collected by centrifugation at 4 °C and 9279 × *g*. Cells were suspended in water and centrifuged at 835 × *g* for 5 min. Cells were then frozen in liquid N_2. Total RNA was extracted using RNeasy Maxi Kit (Invitrogen) and polyA mRNA was extracted using Oligotex mRNA Maxi Kit (Invitrogen). Equal amounts of mRNA from each condition were pooled. An EST library was constructed using the Smart cDNA Library Construction Kit (Clontech) from the pooled mRNA. Individual plaques were used to inoculate 2 ml of LB + $MgSO_4$ media that had been inoculated 3 h earlier with 10 μl of a XL-1 Blue overnight culture. This reaction mixture was incubated 15 min at 37 °C with shaking then without shaking for 30 min and finally with shaking overnight. The *E. coli* debris was removed by centrifugation at 539 × *g* for 10 min and 1 μl of each supernatant containing phage DNA of was used as template.

2.3. PCR and sequencing

The inserts in individual transformants were amplified using PCR and 5′ pTRiplEX2 and 3′ pTRiplEX2 (Clontech) as primers. The PCR reaction mixture was then treated with Exo-Sap1 (USB) to digest and dephosphorylating unused primers. Sequencing was performed using the PCR product as template using the dideoxy method and 5′ pTriplEX2 as primer. A total of 965 individual phages were sequenced, and 678 readable sequences were obtained.

2.4. Cloning of the promoter of TEF1

A genome walker library was constructed following the method in the Ultimate Genome Walker Kit (BD Biosciences Clontech) and additional libraries were constructed using *Hap*I, *Mac*I, *Pml*I, *Sma*I, *Ssp*I and *Stu*I (New England Biolabs) as restriction enzymes. A sequence 700 bp downstream of the *TEF1* promoter was amplified as described in the Ultimate Genome Walker Kit using primers 5′ GTGGACTTACCAGAATCGACGTGACCG 3′ and 5′ GAACCCTTACCCAATTCAGCGGCTTCC 3′ and AmpliTaq Gold (Applied Biosystems). Sequencing was performed using the dideoxy method at the University of Wisconsin, Madison Biotech Center.

2.5. Vector construction

pBluescript KS II− (Stratagene) was modified by adding *Nco*I, *Bgl*II, and *Nsi*I sites flanking the *Kpn*I of the multiple cloning site to create pJML295. Oligos [5′ TCGAGGGGGGGCCCGGTACCATGGAGATCTATGCATCGTAC 3′ and 5′ CGATGCATAGATCTCCATGGTACCGGGCCCCCCC 3′] were combined, phosphorylated with T4 polynucleotide kinase (New England Biolabs), and ligated into the *Xho*I–*Kpn*I sites of pBluescript KS II−. The vector pJML457 (Fig. 1) was constructed by ligating *PsARS2* flanked by *Pst*I–*Xho*I and a modified *PsURA3* flanked by *Nsi*I–*Sal*I into the *Kpn*I *Nsi*I restriction sites of pJML295. The vector pJML533 (Fig. 1) was constructed in the same manner as pJML457 but instead of *PsURA3* an *Nsi*I–*Sal*I fragment with the adapted *Sh ble* gene in fusion with 700 bp of the *TEF1* promoter, and 458 bp of 3′ *PsXYL2* untranslated region was used.

2.6. Disruption of the XYL2 gene in P. stipitis

A disruption cassette was constructed containing −350 to 161 and 935–1490 of the 5′ and 3′ regions, respectively, of the *XYL2* gene in *P. stipitis* with the *PsURA3* flanked by the *lox*P sites. The cassette was transformed into *P. stipitis* UC7 (*ura3*) [8] using a modified Li-acetate transformation protocol [20] in which the heat shock was at 42 °C for 5 min, and ura$^+$ colonies were selected on ScD-ura plates. A secondary screen was performed on xylose plates. Putative site-specific disruptants were identified by their slow growth on xylose. A *xyl2::lox*P-*URA3-lox*P disruptant was identified by amplification of the *XYL2* loci using primers (5′ GAACTCGAGAATGGTGGGCTCATC 3′ and 5′ GAGGCCAAGGACATTTTGTTTGGC 3′) that anneal to the outside of the disruption cassette. The mutated Cre-recombinase was placed under the control of 393 bp *XYL1* promoter in and inserted into pJML533 to create pJML535. The plasmid was transformed using a modified bicine method [21] and transformants selected in YPD (pH 7.5) with 100 μg/ml of Zeocin. Transformants were then grown in YPX overnight and ura$^-$ colonies selected by plating in ScD + FOA plates. Removal of the *URA3* gene from the *XYL2* loci was verified by PCR amplification.

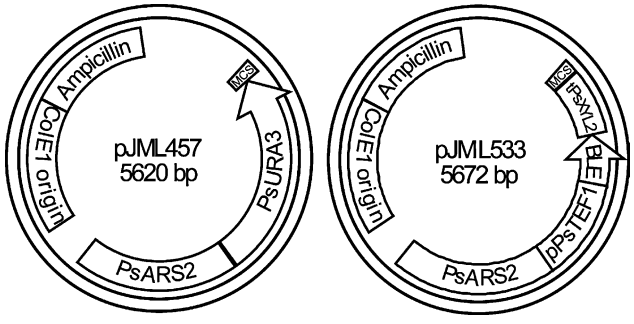

Fig. 1. Diagram of vectors pJML457 and pJML535 showing location and orientation of *PsARS2*, and the modified selectable markers *PsURA3* and *Sh ble* (BLE).

2.7. Mutagenesis of PsURA3, Sh ble, and Cre recombinase

Using site-specific mutagenic PCR the *Bam*HI, *Eco*RI, *Kpn*I and *Sal*I restriction enzyme sites were removed in the *PsURA3* gene. The 3 and 18 CTG codons present in *Sh ble* and Cre recombinase, respectively, were mutagenized to TTG codons using PCR mutagenesis. Furthermore, codons 51, 59, 69, 209, 113, and 116 of *Sh ble* were modified to codons UCC, CCA, AGA, AGA, and GUG, respectively, which are more frequently used by *P. stipitis*. For Cre-recombinase, codons 16, 24, 32, 34, 36, 45, 95, 100, 101, 106, 119, 164, 216, 223, 234, 263, 270, 333, 337, and 342 were changed to the more frequently used codons CCA, AGA, AGA, AGA, GCT, TTA, TTG, AGA, CGT, AGA, AGA, GGT, GGT, AGA, CCA, GGT, AGA, GGT, AGA, and GGT, respectively.

2.8. Cloning of XYL2

The *XYL2* gene was amplified using *Pfu* polymerase (Stratagene) and primers 5′ GGATCCAGCTTGCTGAATGCTTCTTTAGAGGCC 3′ and 5′ CTCGAGCACCAATTAATTGGTGGACAAGTGTGTG 3′ and cloned into pCR4.0 using Zero blunt Topo PCR Cloning Kit for Sequencing (Invitrogen) as described. The sequence was verified by the dideoxy method at the University of Wisconsin Biotech Center, and the *XYL2* insert was cloned into pJML457 using *Bam*HI and *Not*I restriction enzyme sites.

2.9. Deposition

The vectors pJML457 [=NRRL B-30775], pJML533 [=NRRL B-30776] and pJML545 [=NRRL B-30777] (see below) were deposited in the Midwest Area, National Center for Agricultural Utilization Research culture collection in Peoria, Illinois.

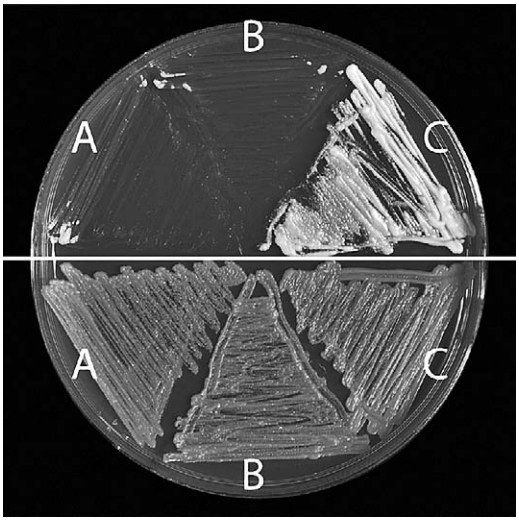

Fig. 2. The adapted *Sh ble* confers resistance to Zeocin. *Pichia stipitis* transformed with either an empty vector (A), a vector expressing the native (B) or adapted (C) *Sh ble* were plated in either ScD-ura (lower panel) or YPX supplemented with 100 μg/ml Zeocin (upper panel).

3. Results

In the search for new potential genetic markers, *P. stipitis* was found to be sensitive to 100 μg/ml Zeocin on YPD plates at pH 7.5 (data not shown). A plasmid containing the native *Sh ble* gene under the control of the *XYL1* promoter and *URA3* was transformed into *P. stipitis* UC7 (*ura3*) and transformants were selected on ScD-ura plates. Isolated colonies from the resulting transformant were tested for resistance to Zeocin on YPX pH 7.5 Zeocin plates. The gene did not confer resistance to Zeocin under the conditions tested (Fig. 2). One explanation for the lack of resistance was the presence of five CTG codons in the open reading frame of *Sh ble*. If *P. stipitis* were to use the alternative yeast nuclear genetic code, it would decode this codon as serine rather than leucine and it could result in the expression of a non-active protein.

P. stipitis is closely related to *C. shehatae* [22] a yeast that uses the alternative yeast nuclear genetic code [23]. A blast search of GenBank using the Ser $tRNA_{CAG}$ gene of *C. albicans* identified the Ser $tRNA_{CAG}$ gene from *P. stipitis* that decodes CTG [24]. This *P. stipitis* tRNA gene is identical to that of *C. guillermondii* and is closely related to the same genes in *D. hansenii*, *C. albicans*, *C. zeylanoides*, and *C. parapsilosis* (Fig. 3). All these yeasts use the alternative yeast nuclear genetic code [23,25]. Presence of the Ser $tRNA_{CAG}$ in *P. stipitis* provides genomic evidence that *P. stipitis* uses the alternative code which could explain the inability of the native *Sh ble* to confer resistance to Zeocin.

To test this hypothesis, all five CTGs in the native *Sh ble* were mutated to TTGs. In addition other codons not used very frequently in *P. stipitis* were also mutated to codons with higher frequency of usage in order to increase the level of expression. The adapted *Sh ble* was placed under the control of the *XYL1* promoter, inserted into a plasmid containing *URA3* and transformed into UC7. The resulting strain was resistant to Zeocin whereas the host strain transformed with an empty vector or the unmodified *Sh ble* was not resistant to Zeocin (Fig. 2).

Having a novel genetic marker gave us the opportunity to create new a plasmid for use in *P. stipitis*. To facilitate later studies in carbon metabolism, we placed the *Sh ble* gene under the control of a constitutive promoter from a gene that is not involved directly in carbon metabolism. To identify a strong promoter, we created an expressed sequence tag (EST) library from mRNA isolated from *P. stipitis* growing under aerobic or oxygen limited condition and using either xylose or glucose as a carbon source. Out of 678 sequences, 16 clones were from a gene with high identity to *S. cerevisiae* transcription elongation factor 1 alpha (*TEF1*). This sequence has been identified as a strong constitutive promoter in *S. cerevisiae* [26]. We named the *P. stipitis* gene *PsTEF1*. A 700 bp fragment of the 5′ untranslated region of *PsTEF1* was cloned by PCR. This promoter was fused to the open reading frame of the mutagenized *Sh ble* gene, which was followed by the *XYL2* terminator. The cloned fragment was inserted along with the *PsARS2* into a modified pBluescript II KS vector. The final plasmid, pJML535, was successfully transformed into *P. stipitis* FPL-UC7 (*ura3*) and it conferred resistance to Zeocin (Fig. 2).

We determined whether we could adapt the *lox*P/Cre recombinase system for use in *P. stipitis*. In a test of the system, we disrupted the gene that encodes xylitol dehydrogenase (*XYL2*). The resulting strain should be unable to use xylose as a carbon source. This system would facilitate our screening process, and to our knowledge the physiological consequences of *xyl2* disruption had not previously been

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P. stipitis:          1 GATACGATGGCCGAGTGGTTAAGGCGAAGGATGCAGGTTCCT 42
C. guilliermondii:    1 GATACGATGGCCGAGTGGTTAAGGCGAAGGATGCAGGTTCCT 42
D. hansenii:          1 GATACGATGGCCGAGTGGTTAAGGCGGAGGATGCAGGTTCCT 42
C. albicans:          1 GATACGATGGCCGAGTGGTTAAGGCGAAGGATGCAGGTTCCT 42
C. zeylanoides:       1 GATACGATGGCCGAGTGGTTAAGGCGATGGATGCAGGTTCCA 42
C. parapsilosis:      1 GATGCGATGACCGAGTGGTTAAGGTGAAGGATGCAGGTTCCT 42

P. stipitis:         43 TTGGGCTCTGCCCGCGCAGGTTCGAACCCTGCTCGTGTCG 82
C. guilliermondii:   43 TTGGGCTCTGCCCGCGCAGGTTCGAACCCTGCTCGTGTCG 82
D. hansenii:         43 TTGGGCTCTGCCCGCGCAGGTTCGAACCCTGCTCGTGTCG 82
C. albicans:         43 TTGGGCATTGCCCGCGCAGGTTCGAACCCTGCTCGTGTCG 82
C. zeylanoides:      43 TTGGGCTTCGCCCGCGCAGGTTCGAACCCTGCTCGTGTCG 82
C. parapsilosis:     43 TTGGGCATTGCCCGCGCAGGTTCGAACCCTGCTCGCGTCG 82
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Fig. 3. Comparison of *P. stipitis* Ser-tRNACAG gene (AC152121) with the orthologous genes from *Candida guillermondii* (D17533), *D. hansenii* (CR382134), *C. albicans* (X64564), *Candida zeylanoides* (D12941), and *Candida parapsilosis* (D14890). The anticodon CAG is underlined.

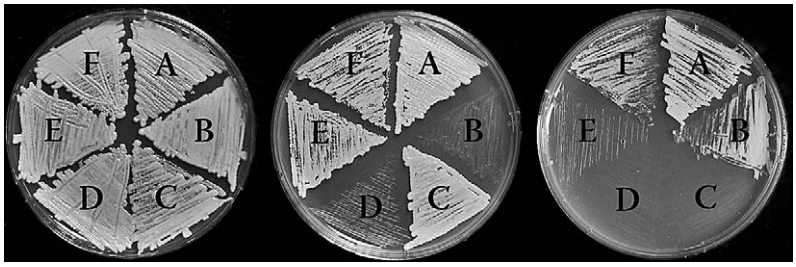

Fig. 4. Removal of *URA3* allows complementation of the phenotype. CBS6054 *XYL2 URA3* (A), UC7 *XYL2 ura3* (B), yJML111 *xyl2*:*lox*P-*URA3*-*lox*P (C), yJML123 *xyl2::lox*P (D), yJML123 + pJML457 (*URA3*) (E), yJML123 + pJML585 (*URA3 XYL2*) (F) plated either in YPD (left). ScD-ura (center) or ScXylose (right).

examined. We disrupted *XYL2* in *P. stipitis* UC7 (*ura3*) using *PsURA3* flanked by the 34 bp *lox*P sites to create *P. stipitis* yJML111. This strain could not grow on xylose as a carbon source (Fig. 4C). Disruption of *XYL2* was confirmed by the amplification of the *URA3* ORF using primers outside the disruption cassette (Fig. 5, lane B).

Each of the 18 CUG codons in Cre was converted into appropriately translated codons, as detailed in the Materials and Methods. The modified gene was placed under the control of the *XYL1* promoter and *XYL1* terminator and cloned into pJML535. The resulting plasmid (pJML545) was then transformed into yJML1111 (*Δxyl2::lox*P-*URA3*-*lox*P), and transformants were selected for resistance to Zeocin. A Zeocin positive strain was then grown overnight in xylose and strains that had lost the *URA3* were selected by growth on ScD + FOA plates. Four different strains (yJML123–127) were collected. Loss of the *URA3* marker from the *XYL2* locus was verified by the inability to grow in uracil deficient media (Fig. 4D) and by PCR (Fig. 5, lanes C–F).

To confirm the phenotype, *XYL2* was cloned into a plasmid containing *URA3* (pJML457) to create pJML585 and transformed into yJML123 (*xyl2Δ ura3*). This transformant was tested for growth using xylose as a carbon source. The resulting strain yJML123 + pJML585 (*URA3 XYL2*) could grow in xylose-containing medium (Fig. 4F), which was in marked contrast to the strain transformed with the parental plasmid pJML545 without *XYL2* (Fig. 4E). This finding confirmed that we had created a *xyl2* deletion, recovered the *URA3* selectable marker using a modified Cre gene and complemented the xylose utilization deficiency phenotype with *XYL2*.

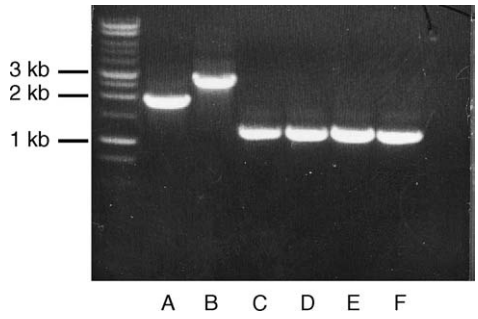

Fig. 5. Amplification of the XYL2 loci from genomic DNA. PCR fragments were amplified using primers outside of the target gene. Lane A: UC7 *XYL2 ura3*, parental; 1.9 kbp. Lane B: yJML111 *xyl2::lox*P-*URA3*-*lox*P *ura3*, insert strain; 1.8 kbp. Lanes C–F: yJML123 127 *xyl2::lox*P *ura3*, deleted; 1.2 kbp.

4. Discussion

In the last 20 years, some ascomycetous yeasts have been shown to use the alternative yeast nuclear genetic code in which the CUG codon has been reassigned from leucine to serine [27]. *Debaryomyces hansenii* [28], *C. albicans* [29,30], *Candida guilliermondii*, *Candida tropicalis* [31] *Candida membranifaciens*, *Candida shehatae* and at least 75 other yeast species [23] appear to assign serine to CUG using Ser $tRNA_{CAG}$ [25,24], so this is a significant problem—especially given the increased interest in using unusual yeasts for the synthesis of novel products.

Our discovery that *P. stipitis* uses the alternative yeast nuclear genetic code could explain the lack of functional antibiotic resistance markers for this yeast. Even though CUG is rarely used in *P. stipitis* and other yeasts [32] many bacterial genes that confer drug resistance contain significant numbers of CUG codons, and the erroneous translation of CUG as serine could render these proteins useless. The blasticidin resistance gene, *bsd*, contains four CTGs [33]. The native kanamycin (Kan^R) resistance gene [34,35], which was reportedly used by Ho et al. [5], contains four CTGs. Modification of these codons to other leucine encoding codons could increase the ability of these genes to confer resistance to kanamycin and geneticin and allow the use of higher antibiotic concentrations in selecting for transformants. This change would help avoid the appearance of spontaneous drug resistant mutants that often arise at low antibiotic levels.

Codon usage could affect heterologous expression of other proteins in *P. stipitis*. Xylanases from *Cryptococcus albidus* and *Trichoderma reesei* (*XYL2*) have been expressed in *P. stipitis* [36,14,15]. Each of these genes contains four CTGs. Although is hard to predict a priori, it is possible that the activities of these proteins could increase significantly with modification of the CTG codons. Previous studies have shown only minor structural changes when only two such translational changes were introduced through heterologous expression

[37]. However, minor changes are not always the rule. The *lacZ* gene of *E. coli* contains 54 CTGs. This number represents over half the codons for leucine in β-galactosidase. Not surprisingly, Weierstall et al. could not use *lacZ* as a reporter gene to study the promoters of *SUT1*, *SUT2*, and *SUT3* in *P. stipitis* [38]. Cho and Jeffries used a *PsADH1::lacZ* fusion to study regulation of the *ADH1* promoter. β-Galactosidase increased 10-fold under oxygen limiting conditions, but the low activity observed could have been due to an endogenous protein because the activity of a control without the *lacZ* insert was not reported [10]. In addition, a BLAST analysis [39] on the *P. stipitis* genome suggests the presence of a gene with high similarity to the β-galactosidase of *K. lactis* [40].

The use of the alternative yeast nuclear genetic code by *P. stipitis* would introduce an additional barrier to successful protoplast fusion and karioduction with yeasts such as *S. cerevisiae* that use the standard code. Use of the alternative code could explain in part the tendency of *S. cerevisiae*/*P. stipitis* fusants to segregate rapidly into the parental strains [41,42], and the general lack of improved strains obtained by this approach. Finally, although CTG is rarely used in *P. stipitis* (for example, there is no CTG codon in *SUT1*, *XYL1*, *XYL2*, and *XYL3*—all of which have been successfully expressed in *S. cerevisiae*)—the use of genomic libraries to complement *S. cerevisiae* mutants could be impaired. Hamacher et al. [43] screened 18,000 genomic transformants of *P. stipitis* to complement a sugar transporter deficient mutant. No transformant was found that was able to utilize and to grow on xylose. On the other hand, several *P. stipitis* genes have been cloned by complementation in *S. cerevisiae*. For example, Passoth et al. isolated two genes for alcohol dehydrogenase by complementation in *S. cerevisiae* [44], and Weierstall et al. cloned three *P. stipitis* genes for sugar transporters by complementation in *S. cerevisiae* [38]. None of these open reading frames contained the CUG codon.

In contrast to *S. cerevisiae* where various plasmids are available for transformation, *P. stipitis* lacks efficient transformation vectors. The vector pJM457 is a vast improvement over pJM6. In contrast to pJM6, it keeps the same multiple cloning sites as in pBluescript II KS (Strategene), which facilitates cloning from this vector into the *P. stipitis* shuttle vector. The same approach was used in constructing the pRS4XX series for *S. cerevisiae* [45,46]. pJML533 provides the same overall multiple cloning sites as pJML545, but employs a novel marker for use in strains in which an auxotrophic marker is not available. Either pJML545 or pJML457 has more unique restriction sites available than pJM6. Both vectors were used in this study to either express *XYL2* or the Cre-recombinase. The use of the modified *lox*P/Cre recombinase will facilitate the production of double mutants and the ability to complement mutants, which is essential to verify the phenotype.

The *lox*P/Cre recombinase system can also be used for repeated genomic integrations. Johansson and Hahn-Hägerdal successfully overproduced pentose phosphate pathway enzymes in *S. cerevisiae* [47]. The adapted system will be even more useful for such an approach in *P. stipitis*, because homologous recombination occurs at a much lower frequency in this organism. In implementing the *lox*P/Cre system in *P. stipitis*, we have observed that with repeated use in cells with several *lox*P sites, inhibition of cell growth was encountered following transformation of the recipient strain with Cre. Wieczorke et al. have observed similar effects in *S. cerevisiae* [48].

In summary, these tools will facilitate the construction of *P. stipitis* mutants in a wide variety of pathways. which will in turn improve our understanding of xylose metabolism in this yeast. In addition these tools could also be used for other closely related yeasts that use the alternative yeast nuclear genetic code such as *C. albicans*, *C. tropicalis* [49], and *D. hansenii*.

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