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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.