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Research Article

Development and characterization of a vector set with regulated promoters for systematic metabolic engineering in *Saccharomyces cerevisiae*

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Abstract

A set of vectors was constructed that enable combined and systematic testing of metabolic pathway genes in *Saccharomyces cerevisiae*. The vectors are available as CEN/ARS and 2 μ-based plasmids with a choice of three inducible promoters, P_{GAL1}, P_{CUP1} and P_{ADH2}. These features offer control over the initiation and level of gene expression. In addition, the vectors can be used as templates to generate PCR fragments for targeted chromosomal integration of gene expression cassettes. Selection markers are flanked by *lox*P elements to allow efficient *Cre*A-mediated marker removal and recycling after genomic integration. For each promoter, expression of a bacterial *lacZ* reporter gene was characterized from plasmid-based and integrated chromosomal cassettes, and compared to that of the glycolytic P_{PGK1} promoter. Plasmid stabilities were also determined. The promoters showed distinct activity profiles useful for modulating expression of metabolic pathway genes. This series of plasmids with inducible promoters extends our previous vector set carrying the constitutive promoters P_{PGK1}, P_{TEF1} and $P_{HXT7\text{-}391}$.

Keywords: ***Saccharomyces cerevisiae*; vectors; integration; inducible yeast promoters; *Cre* recombinase; metabolic engineering**

Introduction

Saccharomyces cerevisiae is an important research and industrial microorganism widely utilized for metabolic pathway engineering and protein expression. Many vector-based tools and genomic integration techniques have been designed to facilitate the genetic programming of this yeast. For introduction and expression of multiple pathway genes, we have previously constructed a set of pXP vectors that enable combined and systematic testing of multiple pathway genes in *S. cerevisiae* (Fang *et al*., 2011). The vectors are available with six different yeast selection markers (*CAN1*, *TRP1*, *MET15*, *HIS3*, *URA3* and *LEU2-d8*), three different promoters (P_{PGK1}, P_{TEF1} and $P_{HXT7\text{-}391}$), and in both high-copy 2 μ and low-copy CEN/ARS versions. They are compact in size and can serve as templates for amplification of integration fragments by PCR, enabling seamless transition from plasmid-based testing to genomic integration of pathway genes. Furthermore, the selection markers are flanked by *lox*P elements to allow efficient *Cre*A-mediated marker removal and recycling. With its unique combination of features, this set of plasmids has proven to be very useful for yeast metabolic pathway engineering.

The strong promoters available on the pXP series are known to allow high and relatively constant levels of expression in media with glucose as the carbon source. However, controlled initiation of gene expression is often desired in metabolic engineering studies, and inducible promoters are superior

in such applications. P_{GAL1}, P_{CUP1} and P_{ADH2} are three inducible promoters routinely used in yeast research; the promoters respond to specific inducers and possess distinct induction profiles. The tightly regulated and widely used *GAL1* promoter is induced 1000-fold in the presence of galactose and is repressed by glucose (Johnston, 1987). The *CUP1* promoter is induced 20-fold by copper ions in glucose media (Butt *et al*., 1984; Etcheverry, 1990). The late-phase *ADH2* promoter is strongly repressed by up to 200-fold in the presence of glucose, but produces high-level expression as glucose levels drop and ethanol consumption begins (Price *et al*., 1990; Lee and Da Silva, 2005). These promoters are very useful for regulating gene expression for metabolic engineering and other applications.

We have expanded the pXP vector set to include these three important promoters (P_{GAL1}, P_{CUP1} and P_{ADH2}), while retaining the other useful features of the original vectors. Expression was characterized using the *Escherichia coli lacZ* reporter gene, and strength and induction profiles of the promoters were compared with those of the *PGK1* promoter in batch culture. For the CEN/ARS and 2 μ-based vectors, plasmid segregational stabilities were determined under expression conditions. PCR fragments containing the promoter–*lacZ* expression cassette were amplified from the new vector templates and integrated into identical loci in the chromosomes. Expression and induction profiles in the integrant strains were also determined and compared. The characterization of the vectors, and the comparison of P_{GAL1}, P_{CUP1}, P_{ADH2} and P_{PGK1} from both plasmid-based and integrated expression cassettes, will facilitate their application for protein expression and metabolic pathway engineering in yeast.

Materials and methods

Strains and cultivation

Escherichia coli strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q}ZΔ*M15* Tn*10*(Tet^{r})]) was used for plasmid maintenance and amplification. *Saccharomyces cerevisiae* strain BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Open Biosystems, Huntsville, AL) was used for expression studies.

E. coli XL1-Blue was grown in Luria-Bertani (LB) medium (Sambrook and Russell, 2001). LB was supplemented with 0.1 mg/ml ampicillin for the selection of plasmid-containing strains. Selective SDC(A) and SGC(A) media (1% dextrose or galactose, 0.67% yeast nitrogen base, 0.5% Bacto casamino acids and 100 mg/l adenine) and complex YPD or YPG media (1% dextrose or galactose, 1% Bacto yeast extract and 2% Bacto peptone) were used for cultivating yeast strains. YPD and SDC(A) agar plates contained 2% Bacto agar. The yeast and *E. coli* strains were cultivated in 3 or 5 ml medium at 30°C and 37°C, respectively, at 250 rpm in an air shaker (New Brunswick Scientific).

Vector construction

PCR reactions were performed using the Novagen KOD Polymerase Kit (EMD Chemicals, San Diego, CA, USA). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Oligonucleotide primers were purchased from IDT DNA (San Diego, CA, USA); all primer sequences are given in the Supporting Information (Table S1).

The *GAL1* promoter was amplified from the pYES2 plasmid (Invitrogen) using primers FF2455 and FF2456. The PCR product was digested with *Nde*I and *Spe*I and used to replace the *HXT7-391* promoter in pXP522 and pXP622 (Fang *et al*., 2011). *Renilla* luciferase (Rluc) was excised from plasmid pXP116-Rluc (Fang *et al*., 2011) by *Spe*I and *Xho*I and inserted between P_{GAL1} and T_{CYC1} in the above plasmids to give pBF3174 (CEN/ARS) and pBF3175 (2 μ-based). The Rluc-T_{CYC1} segment on vectors pBF3174 and pBF3175 was then replaced by a *CYC1* terminator sequence amplified by PCR from pXP118 (Fang *et al*., 2011), using primers CYC1TFwd (containing an internal *Xho*I site) and CYC1TRev, and digested with *Spe*I and *Hin*dIII. The *ADH2* promoter was amplified from plasmid pKOS12-122C(*Nde*-) (KOSAN Biosciences, Hayward, CA, USA), using primers ADH2PFwd and ADH2PRev. The *PGK1* promoter sequence was PCR-amplified from pXP118 using primers PGK1PFwd and PGK1PRev. The *CUP1* promoter sequence was PCR-amplified from p*δ*-neo-*LacZ*-neo (Lee, 1996), using primers CUP1PFwd and CUP1-PRev. The PCR products were digested with *Nde*I and *Xho*I and inserted into pBF3174 and pBF3175, replacing the P_{GAL1}-Rluc sequences. To construct additional vectors with *URA3* markers, the *URA3* cassette was excised from pXP118 and inserted into the *Sma*I sites to replace the *LEU2-d8* markers. The final

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vectors, pXP711 to pXP842, are described in Table 1 and Figure 1.

Vectors containing P_{PGK1}, P_{ADH2}, and P_{GAL1} were configured with *Spe*I and *Xho*I restriction sites between the promoters and T_{CYC1} elements. Because the *CUP1* promoter sequence contains an internal *Spe*I site, vectors with P_{CUP1} were configured with *Avr*II and *Xho*I restriction sites between the promoter and terminator. To insert the *lac*Z reporter gene, the native *lac*Z gene sequence was amplified from pdUBlacZ (Lee and Da Silva, 1997), using primers lacZFwdSpeI and lacZRevXhoI. The PCR product was digested with *Spe*I and *Xho*I and inserted into each of the *URA3*-marked CEN/ARS and 2 μ-based vectors containing P_{PGK1}, P_{ADH2} or P_{GAL1}. For P_{CUP1}, the *Spe*I- and *Xho*I-digested PCR product was inserted into the vectors cut with *Avr*II and *Xho*I. The coordinates of the promoters used are given in the Supporting information.

Table 1. List of vectors constructed

Name	Promoter	Marker	Type
pXP711	*PGK1*	*LEU2-d8*	CEN/ARS
pXP712	*PGK1*	*URA3*	CEN/ARS
pXP721	*GAL1*	*LEU2-d8*	CEN/ARS
pXP722	*GAL1*	*URA3*	CEN/ARS
pXP731	*CUP1*	*LEU2-d8*	CEN/ARS
pXP732	*CUP1*	*URA3*	CEN/ARS
pXP741	*ADH2*	*LEU2-d8*	CEN/ARS
pXP742	*ADH2*	*URA3*	CEN/ARS
pXP811	*PGK1*	*LEU2-d8*	2 μ
pXP812	*PGK1*	*URA3*	2 μ
pXP821	*GAL1*	*LEU2-d8*	2 μ
pXP822	*GAL1*	*URA3*	2 μ
pXP831	*CUP1*	*LEU2-d8*	2 μ
pXP832	*CUP1*	*URA3*	2 μ
pXP841	*ADH2*	*LEU2-d8*	2 μ
pXP842	*ADH2*	*URA3*	2 μ
pXP712–*lacZ*	*PGK1*	*URA3*	CEN/ARS
pXP812–*lacZ*	*PGK1*	*URA3*	2 μ
pXP722–*lacZ*	*GAL1*	*URA3*	CEN/ARS
pXP822–*lacZ*	*GAL1*	*URA3*	2 μ
pXP732–*lacZ*	*CUP1*	*URA3*	CEN/ARS
pXP832–*lacZ*	*CUP1*	*URA3*	2 μ
pXP742–*lacZ*	*ADH2*	*URA3*	CEN/ARS
pXP842–*lacZ*	*ADH2*	*URA3*	2 μ

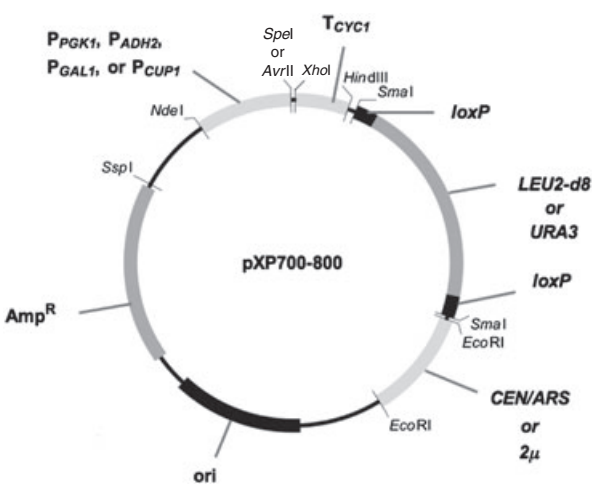

Figure 1. Schematic of vectors constructed. Plasmids pXP731, pXP732, pXP831 and pXP832 (with the *CUP1* promoter) have an *Avr*II site rather than a *Spe*I site following the promoter sequence. All vectors are described in Table 2

Yeast strain construction

Strain BY4741 was transformed with vectors pXP712-*lacZ* to pXP842-*lacZ* (Table 1), using a lithium acetate protocol (Ito *et al*., 1983). For construction of the integrant strains, the cassettes were first amplified by PCR from low-copy vector templates, using forward (FF2322, ADH2intfwd, CUP2intfwd and GAL1intfwd) and reverse (FF2321) primers, each with a 50 bp complementarity to genomic sequences flanking the *S. cerevisiae* Ty1 element yBLWTy1-1. The PCR fragments were then transformed into the yeast. Integrant colonies were selected on SDC(A) plates and confirmed by PCR using primers FF2323 and FF2324, which are complementary to sequences flanking the integration site.

β-Galactosidase assay

Seed cultures (3 ml) were inoculated from –80°C glycerol stock and grown for 18 h in selective SDC (A) medium. SGC(A) medium was used for the *GAL1* promoter strains. Seed cultures were used to simultaneously inoculate four identical test tubes, each containing 5 ml SDC(A) or YPD medium, to an initial optical density (OD_{600}) of 0.02. Cell OD was measured using a Pharmacia Novaspec II spectrophotometer, with an OD_{600} of 1.0 equivalent to a dry cell weight of approximately 0.6 g/l. $CuSO_4$ (1 mM) was included as an inducer for the *CUP1* promoter. For the *GAL1* promoter strains, SGC(A) and YPG medium with 1% galactose as the carbon source was used. A tube was removed at 12, 24, 36 and 48 h for β-galactosidase assays. The cells were permeabilized using the protocol from

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Srienc *et al*. (1983), and β-galactosidase activities of the permeabilized cells were determined using an ONPG assay (Da Silva and Bailey, 1989). One unit of activity is defined as the change in the absorbance reading at 420 nm/min/g cells (dry weight) (Shimadzu UV-2450 spectrophotometer). Three independent time-course experiments were performed for each strain.

Plasmid stability determination

The segregational stability of the yeast vectors was evaluated using replica plating. The 24 and 48 h yeast cultures were diluted with sterile water and plated onto YPD plates. Two hundred colonies were transferred to SDC plates (lacking uracil) and YPD plates (to verify viability). The percentage of plasmid-containing cells was determined as the number of colonies on the SDC plates divided by the number of viable colonies transferred.

Results and discussion

Vector constructs

A series of 16 new plasmid vectors were constructed (Figure 1, Table 1). Each vector was configured with 1 of 4 promoters (P_{PGK1}, P_{GAL1}, P_{CUP1} and P_{ADH2}), with either *URA3* or *LEU2-d8* selection markers, and in multicopy 2 μ or low-copy CEN/ARS versions. The vectors were constructed using the pXP522 and pXP622 plasmid backbones published in Fang *et al*. (2011), and were thus truncated by approximately 300 nucleotides in the non-coding region compared to the pXP100 to pXP400 vector series. These new vectors were designed to enhance a seamless transition from plasmid-based expression to chromosomal integration and expression. The vectors can serve as templates to directly generate integration cassettes by PCR with reusable selection markers for easy marker recycling.

To characterize the new vectors, the *lac*Z reporter gene was PCR-amplified and inserted between the promoters and T_{CYC1} sequences on the *URA3*-marked 2 μ-based and CEN/ARS plasmids (Table 1). The resulting eight vectors were transformed into strain BY4741 and activity levels compared during batch culture. In addition, single-copy integrants were constructed, allowing controlled promoter comparisons. β-Galactosidase activity was used as a general indicator of protein expression from the various vectors and promoters, recognizing that variation will likely be seen with other protein products, due to multiple factors including plasmid copy number variation and protein turnover rates.

Plasmid-based expression

Expression using the 2 μ-based and CEN/ARS vectors was evaluated under conditions for high levels of induction. Yeast strains carrying plasmids with the P_{PGK1}-*lac*Z, P_{ADH2}-*lac*Z or P_{CUP1}-*lac*Z cassette were cultivated in selective SDC(A) medium. For strains with P_{CUP1}-*lac*Z, the medium was supplemented with 1 mM $CuSO_4$ for P_{CUP1} induction (Lee and Da Silva, 1997, 2005). The strains carrying plasmids with P_{ADH2}-*lac*Z were also cultivated in YPD to compare expression with that in SDC(A). The *ADH2* promoter is known to provide higher expression levels in complex media relative to selective media (Price *et al*., 1990; Lee and Da Silva, 2005). For strains carrying P_{GAL1}-*lac*Z, SGC(A) medium containing galactose as the carbon source and inducer was used. For characterization of promoter activity at different times during batch culture, samples were taken at 12 h (late exponential phase), 24 h (early stationary phase), 36 h (stationary phase) and 48 h (stationary phase). The three inducible promoters in the new vector set were compared to the *PGK1* promoter. Growth curves for all strains can be found in Figures S1–S6 (see Supporting information).

Expression under P_{PGK1}

The BY4741 strains carrying the P_{PGK1}-*lac*Z expression cassette on pXP712 (CEN/ARS) and pXP812 (2 μ) plasmids were cultivated, and specific β-galactosidase activities were measured at four time points between 12 and 48 h (Figure 2). With both plasmids, specific activity was highest at 12 h, with subsequent activity levels comparable or slightly lower. At 12 and 24 h, the specific β-galactosidase activities with the 2 μ-based plasmid were approximately four times those with the CEN/ARS plasmid. This activity ratio is similar to that observed for exponential phase luciferase activities using the similar pXP218/118 plasmids in our previous study (Fang *et al*., 2011). For pXP712-*lacZ*, the percentage of the population carrying the plasmid was 89% at 24 h and 82% at 48 h (Table 2). For the multicopy pXP812-*lacZ*,

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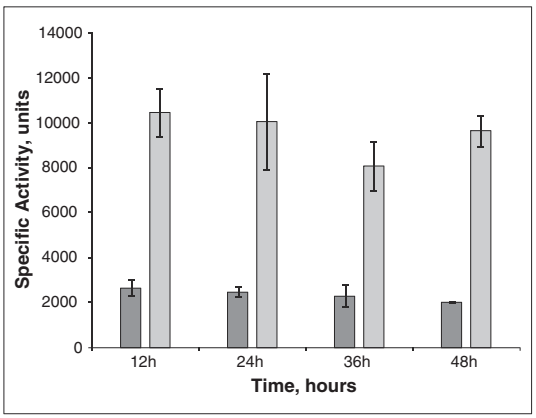

Figure 2. Expression from *PGK1* promoter. Specific β-galactosidase activity for strains carrying pXP712-*lacZ* (CEN/ARS; dark grey bar) and pXP812-*lacZ* (2 μ; light grey bar). Cells were cultured in 1% SDC(A). The activities are averages (with SDs) of triplicate experiments. One unit of activity is defined as the change in the absorbance at 420 nm/min/g cells (dry weight)

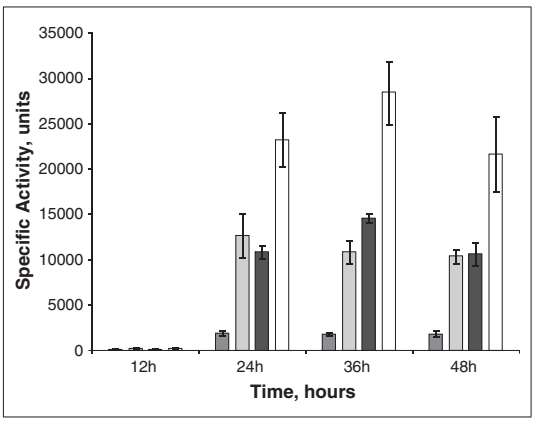

Figure 3. Expression from *ADH2* promoter. Specific β-galactosidase activitiy for strains carrying pXP742-*lacZ* (CEN/ARS) cultured in 1% SDC(A) (dark grey bar) and in 1% YPD (black bar), and carrying pXP842-*lacZ* (2 μ) cultured in 1% SDC(A) (light grey bar) and 1% YPD (white bar). The activities are averages (with SDs) of triplicate experiments

Table 2. Plasmid stability

Expression cassette	Medium	Percentage plasmid-containing cells			
		24 h		48 h	
		CEN/ARS	2 μ	CEN/ARS	2 μ
P_{PGK1}–*lacZ*	SDC(A)	89	76	82	75
P_{ADH2}–*lacZ*	SDC(A)	84	82	82	76
P_{ADH2}–*lacZ*	YPD	76	70	66	64
P_{GAL1}–*lacZ*	SGC(A)	81	69	80	63
P_{CUP1}–*lacZ*	SDC(A), Cu^{2+}	84	70	82	67

the values were lower: 76% at 24 h and 75% at 48 h. Therefore, plasmid stabilities did not decrease significantly.

Expression under P_{ADH2}

Expression from the *ADH2* promoter was evaluated using both YPD and SDC(A) media. Strong repression of P_{ADH2} was observed at 12 h in both YPD and SDC(A), with specific β-galactosidase activities of only approximately 100 U (Figure 3). Sharp increases in specific activity were observed at 24 h in all strains, as P_{ADH2} was derepressed with depletion of the glucose. At 24 h in SDC (A), specific β-galactosidase activity in the strains carrying pXP742-*lacZ* (CEN/ARS) had increased nearly 19-fold from the level at 12 h. Specific β-galactosidase activity in the strain carrying pXP842-*lacZ* (2 μ) increased approximately 80-fold between 12 and 24 h. The activities for both strains remained approximately constant in subsequent samples at 36 and 48 h. In YPD media, specific β-galactosidase activity in the strain carrying pXP742-*lacZ* was 100 times higher at 24 h than at 12 h. The specific activity in the strain with pXP842-*lacZ* was greater than 200 times higher than at 12 h. At 36 h, additional increases of 20–25% were observed for both plasmids. The much higher activities seen in YPD relative to SDC(A) is due to the regulation of this promoter, and better derepression in complex media (Price *et al*., 1990; Lee and Da Silva, 2005).

In selective SDC(A) medium, the plasmid stability of pXP742-*lacZ* (CEN/ARS) was 84% at 24 h and 82% at 48 h; the segregational stability of pXP842-*lacZ* (2 μ) was 82% at 24 h and 76% at 48 h (Table 2). When the strains were grown in non-selective YPD medium, the percentage of plasmid-containing cells with pXP742-*lacZ* was 76% at 24 h and 66% at 48 h; for pXP842-*lacZ*, the values were 70% at 24 h and 64% at 48 h. The plasmid stabilities in YPD were lower than those in SDC(A), but still remained relatively high in the absence of selection, due to the late onset of expression using this promoter. These results confirm higher P_{ADH2} activity in complex medium despite lower plasmid stability. Specific β-galactosidase activity in strains carrying pXP742-*lacZ* was nearly 8.5 times higher

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in YPD than in SDC(A) at 36 h. In strains carrying pXP842-*lacZ*, specific activity was approximately 2.6 times higher in YPD than in SDC(A).

Expression under P_{CUP1}

To evaluate expression from the *CUP1* promoter, we added 1 mM $CuSO_4$ to SDC(A) medium at time zero for induction. Continuous increases in specific β-galactosidase activity were observed between 12 and 36 h, with levels approximately 2.7-fold higher at 36 h (Figure 4). The induction profile was similar for the strains carrying pXP732-*lacZ* (CEN/ARS) and pXP832-*lacZ* (2 μ), although activities were three- to four-fold higher with the multicopy plasmid. The percentage of cells carrying pXP732-*lacZ* was 84% at 24 h and 82% at 48 h (Table 2). The segregational stability of pXP832-*lacZ* was lower: 70% at 24 h and 67% at 48 h.

Expression under P_{GAL1}

Because the *GAL1* promoter responds strongly to galactose and is repressed by glucose, strains were grown in SGC(A) galactose medium to obtain full induction. With both pXP722-*lacZ* (CEN/ARS) and pXP832-*lacZ* (2 μ), specific β-galactosidase activity was highest at 12 h and decreased at the subsequent time points (Figure 5); this is due to the consumption of the galactose inducer. With both plasmids, specific activity at 48 h was approximately 40% of that at 12 h. Interestingly, activities were only approximately two-fold higher for the strain with the 2 μ-based plasmid relative to the CEN/ARS plasmid. The plasmid stabilities stayed relatively constant over the duration of the experiments. For the yeast strain carrying pXP722-*lacZ*, the percentage of plasmid-containing cells was 81% at 24 h and 80% at 48 h (Table 2). For pXP822-*lacZ*, the values were lower at 69% at 24 h and 63% at 48 h.

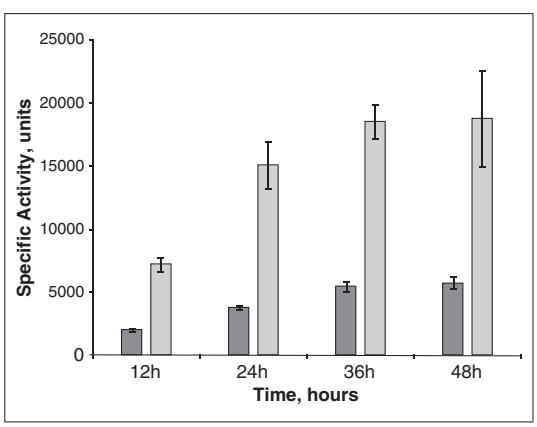

Figure 4. Expression from *CUP1* promoter. Specific β-galactosidase activity for strains carrying pXP732-*lacZ* (CEN/ARS; dark grey bar) and pXP832-*lacZ* (2 μ; light grey bar). Cells were cultured in 1% SDC(A) with 1 mM $CuSO_4$. The activities are averages (with SDs) of triplicate experiments

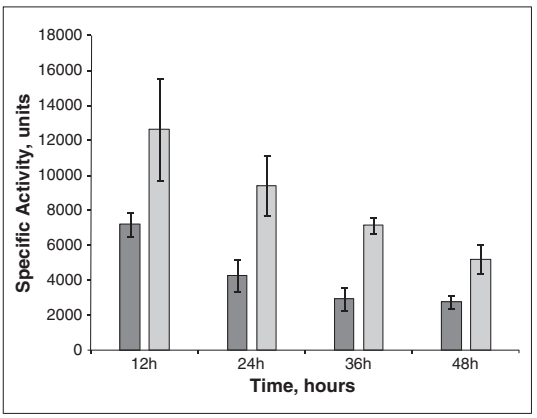

Figure 5. Expression from *GAL1* promoter. Specific β-galactosidase activity for strains carrying pXP722-*lacZ* (CEN/ARS; dark grey bar) and pXP822-*lacZ* (2 μ; light grey bar). Cells were cultured in 1% SGC(A). The activities are averages (with SDs) of triplicate experiments

Comparison of promoter and plasmid performance

The expression of the *lacZ* reporter gene under the four promoters showed very different activity profiles during batch culture. Similar profiles were observed for both the CEN/ARS (Figure 6a) and 2 μ-based plasmids (Figure 6b). It should be noted that the *GAL1* and *CUP1* promoters were induced from time zero; different profiles would result, depending on the induction timing used.

At 12 h, P_{GAL1} yielded the highest specific β-galactosidase activity among the four promoters; however, activity declined as the inducer galactose was consumed as a carbon source. With P_{PGK1}, activity levels were relatively steady between 12 and 48 h. With P_{CUP1}, activities increased continuously throughout the experiment, resulting in the highest β-galactosidase activity in SDC(A) medium at 48 h relative to the other promoters. The promoter P_{ADH2} was strongly repressed at 12 h, as glucose

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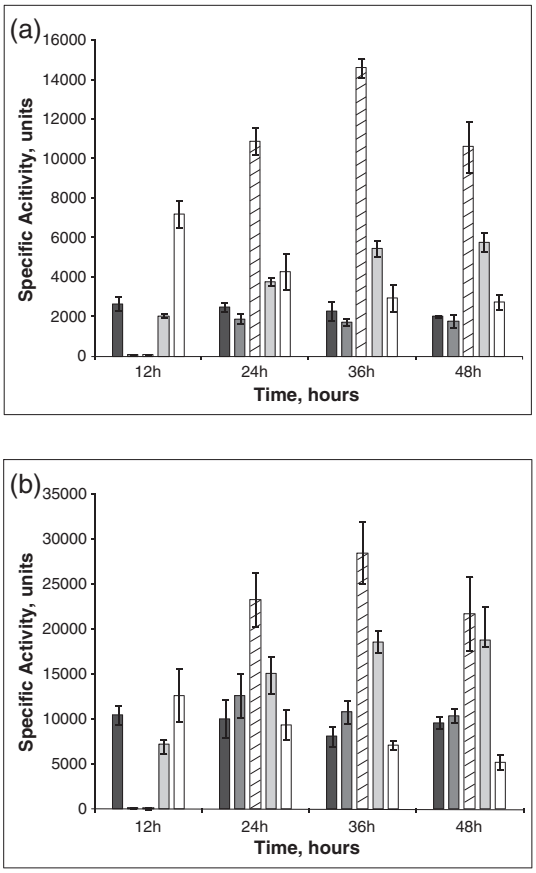

Figure 6. Comparison of expression profiles for *PGK1* promoter (black bar), *ADH2* promoter [dark grey bar for SDC (A), striped bar for YPD], *CUP1* promoter (light grey bar), and *GAL1* promoter (white bar) promoters. (a) Specific β-galactosidase activity for strains carrying CEN/ARS plasmids. (b) Specific β-galactosidase activity for strains carrying 2 μ-based plasmids. The activities are averages (with SDs) of triplicate experiments

was at the highest level. However, P_{ADH2} was derepressed by 24 h and gave the highest activity levels of all promoters when cultivated in rich complex medium.

Expression in strains with an integrated cassette

Similar to our original pXP series (Fang *et al.* 2011), the new vectors were designed to enable chromosomal insertion using the plasmids as templates for PCR-generated integration cassettes. Utilizing primers with sequences complementary to upstream and downstream chromosomal elements allows targeting and replacement of specific loci in the *S. cerevisiae* genome. In our previous work, we identified a group of 12 integration loci that result in similar expression levels (Fang *et al.*, 2011).

To evaluate β-galactosidase expression from a stable copy of the reporter gene under each of the four different promoters (P_{PGK1}, P_{ADH2}, P_{CUP1} and P_{GAL1}), integrant strains were constructed using PCR-amplified promoter-*lacZ* fragments (using the low-copy vectors as templates). The expression cassettes were integrated by replacing the Ty1 element *BLW1-1* in strain BY4741. The use of exactly one integrated copy into identical locations allowed the most accurate comparison of the three promoters; context and copy number are set and plasmid stability is not an issue. Complex media could also be used for all strains.

For each promoter, three independent integrant strains were grown in selective medium for 18 h, and then inoculated into complex YPD (with 1 mM $CuSO_4$ for the P_{CUP1} strain) or YPG (for the P_{GAL1} strain). Samples were taken at 12, 24, 36 and 48 h for β-galactosidase analysis. The specific β-galactosidase activities for the integrant strains are shown in Figure 7. Expression profiles for the yeast strains with one integrated copy of *lacZ* were generally similar to those observed for the plasmid-bearing strains (Figure 6). For the integrated P_{GAL1} cassette, the highest specific β-galactosidase activity was at 12 h, with a gradual 80% decline over the subsequent 36 h. Specific

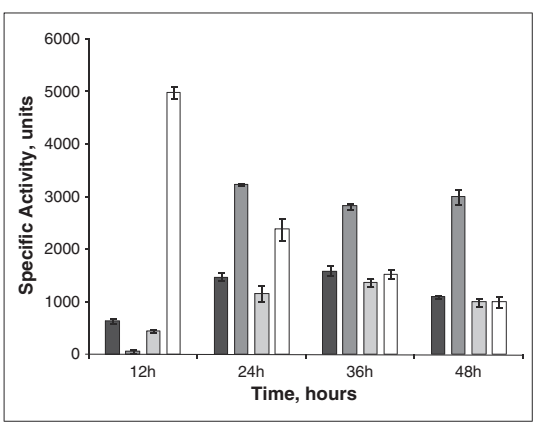

Figure 7. Comparison of expression profiles for one integrated copy of *lacZ* under the control of the *PGK1* promoter (black bar), *ADH2* promoter (dark grey bar), *CUP1* promoter (light grey bar) and *GAL1* promoter (white bar). Specific β-galactosidase activities are averages (with SDs) of triplicate experiments

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activities for strains with integrated P_{PGK1} and P_{CUP1} cassettes were similar in overall level and profile, with a consistent level of activity throughout. The P_{ADH2}-*lacZ* integrants produced the highest specific β-galactosidase levels in the later stages of the batch culture, between 24 and 48 h. In general, the specific β-galactosidase activities in the strains carrying CEN/ARS plasmids were 1.5–3.0-fold higher than the strains with one integrated copy, depending on the promoters and sample time.

The above integrants were constructed using a PCR-generated cassette containing the promoter, reporter gene and *URA3* selection marker. Vectors carrying the *LEU2-d8* marker have also been constructed to provide an alternative (Table 1). To utilize additional selection markers, it is straightforward to use two separate PCR fragments, where the fragment containing promoter and reporter gene is generated independently of the fragment containing the selection marker (e.g. PCR-amplified from the pXP100-400 vectors) (Fang *et al*., 2011), with compatible inside terminal sequences for recombination of the two fragments. Therefore, multiple copies of a gene or genes can be integrated and selected with a group of different markers, and the markers can be removed and recycled simultaneously using *CreA* (Sauer, 1987).

Conclusions

In yeast metabolic engineering, it is advantageous to control the initiation and level of expression of pathway genes. In the present study, we have incorporated three useful inducible promoters, P_{ADH2}, P_{GAL1} and P_{CUP1}, into a set of pXP vectors, thus extending our previous (P_{PGK1}, P_{TEF1} and $P_{HXT3\text{-}391}$) vector set. The vectors can be used as autonomous plasmids or as convenient templates for the integration of genes into the genome. Using the *lacZ* reporter, expression profiles were characterized using both plasmid-based and chromosomally-integrated cassettes. Specific β-galactosidase activities were determined at four time points incorporating exponential and stationary phases. These studies facilitate the application of the vectors for protein expression and metabolic pathway engineering in yeast, and the vectors provide significant additional choices for the successful coordination and modulation of gene expression.

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Supporting information on the internet

The following supporting information may be found in the online version of this article:

Table S1. Primers used for vector and strain construction
Coordinates of the promoters
Growth curves (Figures S1-S6):
Figure S1. Growth curves of *S. cerevisiae* during expression of *lacZ* from the *PGK1* promoter in SDC(A) containing 1% glucose
Figure S2. Growth curves of *S. cerevisiae* during expression of *lacZ* from the *ADH2* promoter in SDC(A) containing 1% glucose
Figure S3. Growth curves of *S. cerevisiae* during expression of *lacZ* from the *ADH2* promoter in YPD containing 1% glucose
Figure S4. Growth curves of *S. cerevisiae* during expression of *lacZ* from the *CUP1* promoter in SDC(A) containing 1% glucose and 1 mM CuSO4
Figure S5. Growth curves of *S. cerevisiae* during expression of *lacZ* from the *GAL1* promoter in SGC(A) containing 1% galactose
Figure S6. Growth curves for the *S. cerevisiae* one-copy integrant strains during expression of *lacZ*

References

Butt TR, Sternberg EJ, Gorman JA, *et al.* 1984. Copper metallothionein of yeast, structure of the gene, and regulation of expression. *Proc Natl Acad Sci USA* **81**: 3332–3336.

Da Silva NA, Bailey JE. 1989. Effects of inducer concentration on *GAL* regulated cloned gene expression in recombinant *Saccharomyces cerevisiae*. *J Biotech* **10**: 253–265.

Etcheverry T. 1990. Induced expression using yeast copper metallothionein promoter. *Methods Enzy* **185**: 319–329.

Fang F, Salmon K, Shen MWY, *et al*. 2011. A vector set for systematic metabolic engineering in *Saccharomyces cerevisiae*. *Yeast* **28**: 123–136.

Ito H, Fukada Y, Murata K, Kimura A. 1983. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**: 163–168.

Johnston M. 1987. A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. *Microbiol Rev* **51**: 458–476.

Lee F. 1996. Amplification and Expression of Heterologous Genes in *Saccharomyces cerevisiae*. PhD Thesis, University of California, Irvine, CA, USA.

Lee F, Da Silva NA. 1997. Sequential δ-integration for the regulated insertion of cloned genes in *Saccharomyces cerevisiae*. *Biotechnol Prog* **13**: 368–373.

Lee KM, Da Silva NA. 2005. Evaluation of the *Saccharomyces cerevisiae ADH2* promoter for protein synthesis. *Yeast* **22**: 431–440.

Price V, Taylor W, Clevenger W, *et al*. 1990. Expression of heterologous proteins in *Saccharomyces cerevisiae* using the *ADH2* promoter. *Methods Enzymol* **185**: 308–318.

Sambrook J, Russell D. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbour Laboratory Press: Cold Spring Harbour, NY.

Sauer B. 1987. Functional expression of the cre–lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol* **7**: 2087–2096.

Srienc F, Campbell J, Bailey J. 1983. Detection of bacterial β-galactosidase activity in individual *Saccharomyces cerevisiae* cells by flow cytometry. *Biotechnol Lett* **5**: 43–48.

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