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Research Article

Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*

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Abstract

The widely used pESC vector series (Stratagene, La Jolla, CA, USA) with the bidirectional *GAL1/GAL10* promoter provides the possibility of simultaneously expressing two different genes from a single vector in *Saccharomyces cerevisiae*. This system can be induced by galactose and is repressed by glucose. Since *S. cerevisiae* prefers glucose as a carbon source, and since its growth rate is higher in glucose than in galactose-containing media, we compared and evaluated seven different promoters expressed during growth on glucose (p*TEF1*, p*ADH1*, p*TPI1*, p*HXT7*, p*TDH3*, p*PGK1* and p*PYK1*) with two strong galactose-induced promoters (p*GAL1* and p*GAL10*), using *lacZ* as a reporter gene and measuring LacZ activity in batch and continuous cultivation. *TEF1* and *PGK1* promoters showed the most constant activity pattern at different glucose concentrations. Based on these results, we designed and constructed two new expression vectors which contain the two constitutive promoters, *TEF1* and *PGK1*, in opposite orientation to each other. These new vectors retain all the features from the pESC–URA plasmid except that gene expression is mediated by constitutive promoters.

Keywords: **promoter activity; expression vector; bidirectional promoter; yeast**

Introduction

Saccharomyces cerevisiae has been widely used as a host organism for the efficient expression of heterologous proteins. To reach this goal, different expression systems, such as yeast integrative plasmids (YIps) for integration of the desired gene into the yeast genome or yeast episomal plasmids (YEps) for high copy number expression, have been designed and developed. Based on these systems, different plasmids harbouring promoters with different regulation profiles, strengths and various additional features have been constructed (Miller *et al*., 1998; Li *et al*., 2008; Hermann *et al*., 1992, Mumberg *et al*., 1994, 1995).

Most expression plasmids allow the expression of only one gene. For the expression of entire metabolic pathways, for example, it is desirable to be able to express more than one gene per plasmid unit. For example, Miller *et al*. (1998) used a constitutively active bidirectional promoter consisting of the promoter of glyceraldehyde 3-phosphate dehydrogenase (p*GPD* = p*TDH3*) and a fragment of the alcohol dehydrogenase 1 promoter (p*ADH1*). The bidirectional expression vectors constructed by Li *et al*. (2008) carry a modified inducible *GAL1* or *GAL10* promoter in one direction and a constitutive *GPD* promoter in the reverse direction.

One widely used expression vector set in *S. cerevisiae* is the pESC series from Stratagene, with the bidirectional *GAL1/GAL10* promoter cassette providing the possibility of expressing two different genes at the same time from a single vector, and its successful applicability has previously been published (e.g. Maury *et al*., 2008; Asadollahi *et al*., 2007). Expression systems based on the

GAL1/GAL10 promoters are among the strongest ones (Schneider *et al*., 1991). However, expression from the *GAL1* and *GAL10* promoters are subject to both galactose induction and glucose repression (Zhang and Rathod, 2002; Lohr *et al*., 1995; West *et al*., 1987). This is a major disadvantage, as the preferred carbon sources for yeast are glucose and fructose; in addition, the inducer galactose can be seen as too costly when scaling up synthesis of commercially valuable products is in focus (Haufa *et al*., 2000). Furthermore, the shift from glucose to galactose causes major metabolic changes (Quintero *et al*., 2007). In order to develop a glucose-based experimental system analogous to the *GAL1/GAL10* system of the pESC vectors, we initiated this study, aiming at comparing the strength of those two strong galactose-based promoters with different glucose-based promoters. Several strong glucose promoters have previously been described and have been shown to be useful for expression of heterologous genes in yeast. In this study, we compared the strength of seven different constitutive or glucose-based promoters derived from the following genes [*TEF1*, encoding transcriptional elongation factor EF-1α (Gatignol *et al*., 1990); *PGK1*, encoding phosphoglycerate kinase (Ogden *et al*., 1986; Holland and Holland, 1978); *TPI1*, encoding triose phosphate isomerase; *HXT7*, encoding a hexose transporter (Diderich *et al*., 1999; Reifenberger *et al*., 1997); *PYK1*, encoding pyruvate kinase 1 (Nishizawa *et al*., 1989); *ADH1*, encoding alcohol dehydrogenase 1 (Denis *et al*., 1983); and *TDH3* (*GPD*), encoding triose phosphate dehydrogenase (Bitter and Egan 1984)] with the strength of the *GAL1* and *GAL10* promoters (Adams, 1972; St. John *et al*., 1981; Laughon and Gestland, 1982). Four of these promoters (p*PGK1*, p*TPI1*, p*PYK1* and p*TDH3*) are promoters of key glycolytic genes and in the literature they are generally considered strong promoters. Full-length p*ADH1*, p*TEF1* and p*TDH3* have also been utilized to construct the widely used p4XXprom vector series (Mumberg *et al*., 1996). For this comparison we used *lacZ* as a reporter gene and constructed nine different integrative plasmids, in which *lacZ* expression was controlled by either of these promoters. In all cases the constructed integrative plasmids were integrated into the *URA3* locus.

Based on this analysis, we constructed two new divergent expression cassettes by replacing the *GAL1/GAL10* promoters in pESC–URA with a *TEF1–PGK1* bidirectional promoter cassette in two different orientations. These two new vectors are called pSP-G1 and pSP-G2, respectively.

Materials and methods

Construction of integrative plasmids

The integrative vector pSF011 used in this study was derived from pRS306 (Sikorski and Hieter, 1989). It contains *URA3* as a selectable marker and reporter gene *lacZ* located downstream of a multiple cloning site (MCS) (Figure 1). *lacZ* and the *CYC1* terminator were cloned as described earlier (Flagfeldt *et al*., 2009). All glucose-based promoters were amplified by PCR from the genome of *S. cerevisiae* CEN.PK 113-7D (*MAT***a** *MAL2-8*^{c} *SUC2*; kindly provided by P. Kötter, University of Frankfurt, Germany). The amplicons of each of the seven glucose-based promoters (p*TEF1*, p*PYK1*, p*HXT7*, p*PGK1*, p*TPI1*, p*ADH1* and p*TDH3*) were digested by *Not*I–*Xho*I and cloned into pSF011 upstream of *lacZ*. The *GAL10* promoter was cloned into pSF011 as a *Not*I–*Bam*HI fragment isolated from pESC–URA (Stratagene, La Jolla, CA, USA). The *GAL1* promoter was first amplified by PCR from pESC–URA, digested by *Xho*I–*Bam*HI and cloned into pSF011. Table 1 shows all the primers used for amplifying the promoters.

Transformation of *S. cerevisiae*

The integrative plasmids were linearized by *Nco*I and transformed into *S. cerevisiae* CEN.PK 113-5D (*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*; kindly provided by P. Kötter) using a standard transformation procedure (Gietz and Woods, 2002). Transformants were selected on plates containing

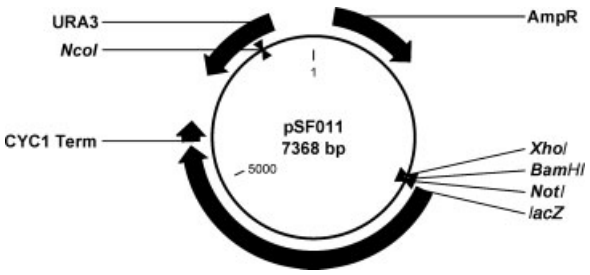

Figure 1. pSF011, integrative plasmid

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Table 1. Oligonucleotide primers used in this study

Primer name	Sequence (5′–3′)
pADH1-top	GTTGTT**CTCGAG**AGGGGGATCGAAGAAATGATG
pADH1-bot	GTTGTT**GCGGCCGC**TGTATATGAGATAGTTGATTG
pHXT7-top	GTTGTT**CTCGAG**CCGTGGAAATGAGGGGTATG
pHXT7-bot	GTTGTT**GCGGCCGC**TTTTTGATTAAAATTAAAAAAAC
pPGK1-top	GTTGTT**CTCGAG**GGAAGTACCTTCAAAGAATG
pPGK1-bot	GTTGTT**GCGGCCGC**TTGTTTTATATTTGTTGTAAAAAG
pPYK1-top	GTTGTT**CTCGAG**GAAAGTTTTTCCGGCAAGCT
pPYK1-bot	GTTGTT**GCGGCCGC**TGTGATGATGTTTTATTTGT
pTEF1-top	GTTGTT**CTCGAG**GCACACACCATAGCTTCAAA
pTEF1-bot	GTTGTT**GCGGCCGC**TTGTAATTAAAACTTAGATTAG
pTPI1-top	GTTGTT**CTCGAG**CTACGTATGGTCATTTCTTC
pTPI1-bot	GTTGTT**GCGGCCGC**TTTTAGTTTATGTATGTG
pTDH3-top	GTTGTT**CTCGAG**CAGTTTATCATTATCAATACTCGCC
pTDH3-bot	GTTGTT**GCGGCCGC**GAATCCGTCGAAACTAAGTTCTGGTG
pGAL1-FW	GTTGTT**CTCGAG**CGTCGTCATCCTTGTAATCC
pGAL1-RE	ATCAACTTCTGTTCCATGTCG
Pgk-fw	GGAAGTACCTTCAAAGAATGG
Tef-fw	CCATTCTTTGAAGGTACTTCC**GGCCGGCC**GCACACACCATAGCTTCAAA
Tef-BamHI	GTTGTT**GGATCC**TTGTAATTAAAACTTAGATTAGATTGC
Tef-NotI	GTTGTT**GCGGCCGC**TTGTAATTAAAACTTAGATTAGATTGC
Pgk-BamHI	GTTGTT**GGATCC**TTGTTTTATATTTGTTGTAAAAAGTAG
Pgk-NotI	GTTGTT**GCGGCCGC**TTGTTTTATATTTGTTGTAAAAAGTAG

Restriction sites are indicated in bold face; the underlined sequence corresponds to the overlapping nucleotides.

1.7 g/l yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories, Detroit, MI, USA), 5 g/l ammonium sulphate, 0.77 g/l complete supplement mixture (CSM without uracil; MP Biomedicals, Solon, OH, USA), 20 g/l glucose and 20 g/l agar.

Shake-flask cultivation

Baffled, cotton-stopped, 500 ml Erlenmeyer flasks were used for precultures and also for promoter evaluations. The shake flasks contained 100 ml medium with the following composition: 7.5 g/l $(NH_4)_2SO_4$, 14.4 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 2 ml/l trace metal solution, 1 ml/l vitamin solution (Verduyn *et al*., 1993) and 50 µl/l synperonic antifoam (Sigma, St. Louis, MO, USA). The pH of the medium was adjusted to 6.5 by adding 2 M NaOH and it was autoclaved separately from the 20% glucose (galactose) solution that was used as a carbon source solution in a final concentration of 2%. Vitamin solution was filter sterilized and aseptically added to the medium after autoclaving. The shake flasks were run in triplicates and incubated at 30 °C and 150 rpm.

Continuous cultivation

Continuous cultivations were carried out in duplicates in well-controlled 2.5 l Braun Biostat manufactured glass bioreactors with a working volume of 2 l. The fermentors were inoculated to initial $OD_{600} = 0.01$ from the liquid precultures. The medium was identical to that used for shake-flask cultivations. Depending on the promoter being tested, either glucose or galactose was added as carbon source at a concentration of 2%. The pH was maintained at 5 by automatic addition of 2 M KOH. The temperature was kept constant at 30 °C. The airflow was 4 l/min (2 vvm) and was sterilized by filtration and the off-gas passed through a condenser. Agitation was adjusted to maintain the dissolved oxygen tension above 20% of air saturation. The dilution rate was set to 0.1/h during operation of the chemostat and steady state was assumed to be obtained after about 50 h of cultivation.

β-Galactosidase assay

β-Galactosidase activity was assayed as described by Miller (1972). 1 ml *S. cerevisiae* cell culture was spun down and the cell pellet was resuspended

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in 1 ml chilled Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$, pH 7) and the OD_{600} was determined. 0.1 ml cell solution was diluted in 0.9 ml Z buffer containing 2.7 ml/l β-mercaptoethanol (ME). 100 µl chloroform and 50 µl 0.1% SDS were added and the sample was vortexed for 15 s. The reaction was started by addition of 0.2 ml prewarmed (30 °C) ONPG (*o*-nitrophenyl-β-galactoside) solution (80 mg ONPG in 20 ml Z buffer plus ME) and after the yellow colour had developed the reaction was stopped by adding 0.5 ml 1 M Na_2CO_3. The reaction mix was spun down at maximum speed and the OD_{420} was determined. *LacZ* activity was expressed in Miller units according to the following equation:

$$\text{Miller units} = 1000 \times OD_{420}/(T \times V \times OD_{600})$$

where T is the time of reaction and V is the volume (ml) of culture used for the assay.

Construction of divergent promoters

The *TEF1* and *PGK1* promoters were fused to each other to construct divergent promoters *TEF1–PGK1* by means of fusion PCR. The PCR was performed with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) in two steps. In the first step, each promoter, p*TEF1* and p*PGK1*, was amplified using the primers shown in Table 1 (Tef-fw/Tef-*Bam*HI and Tef-fw/Tef-*Not*I for amplification of the *TEF1* promoter and Pgk-fw/Pgk-*Bam*HI and Pgk-fw/Pgk-*Not*I primers for amplification of the *PGK1* promoter). Tef-fw primer carries an overhang at the 5′ end which is complementary to the 5′ end of the *PGK1* promoter. The PCR products of the first step were used in a second PCR. The second PCR reaction was started without primers so that the *TEF1* and *PGK1* promoters were fused to each other via the overlapping parts. After 15 cycles, primers were added and the programme was run for 30 additional cycles. The *TEF1–PGK1* cassette was digested by *Bam*HI/*Not*I and cloned in both orientations into pSF011 and pESC–*URA*. The two new plasmids generated by replacing the *GAL1/GAL10* promoter in pESC–*URA* were called pSP-G1 and pSP-G2, respectively (Figure 2A, B).

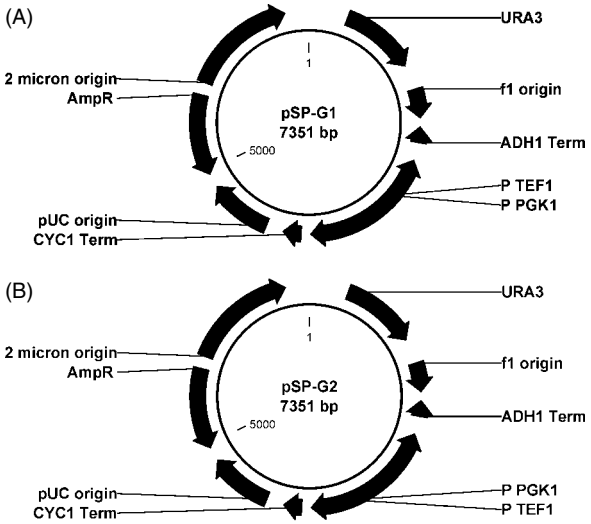

Figure 2. (A) pSP–G1 vector; contains *TEF1–PGK1* bidirectional promoter. (B) pSP–G2 vector; contains *TEF1–PGK1* bidirectional promoter

Results and discussion

Promoter comparison

Several *lacZ* fusions were constructed for comparison of the activity of different promoters, including *TPI1, ADH1, TEF1, PGK1, TDH3, PYK1* and *HXT7* promoters. These fusions were stably integrated in single copy into the genome of *S. cerevisiae* CEN.PK 113-5D, at the *ura3-52* locus. Although in the last decade different reporter systems have been developed and used for promoter analysis in *S. cerevisiae*, such as green fluorescent protein (Li *et al*., 2000; Niedenthal *et al*., 1996), β-lactamase (Cartwright *et al*., 1994) and β-D-glucuronidase (Nacken *et al*., 1996), β-galactosidase encoded by the *lacZ* gene of *Escherichia coli* is the most commonly employed reporter of gene expression in *S. cerevisiae* and is widely used for different purposes (Yocum *et al*., 1984; Flick and Johnston, 1990; Hermann *et al*., 1992). It was shown that *lacZ* as a reporter marker is not compatible with a high copy number vector but suitable for expression monitoring in monocopy (Purvis *et al*., 1987). As we only wanted to compare the strength of different promoters and avoid gene copy number variations, we used *lacZ* on an integrative plasmid pSF011 for this comparison.

First we compared the glucose-based promoters with each other. The expression of *lacZ* controlled

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Table 2. Activities (%) of the promoters used in this study

Time (hours)	p*ADH1*	p*HXT7*	p*PGK1*	p*PYK1*	p*TPI1*	p*TDH3*	p*TEF1*	Glucose (g/l)	Ethanol (g/l)
8	20	10	100	60	60	100	100	12.88	0.44
24	27	109	52	27	31	31	156	nd	0.96
48	14	150	45	14	27	27	136	nd	0.42

The activities were normalized by setting p*TEF1* activity at 8 h to 100%.
nd, not detected.

by these promoters was assayed 8, 24 and 48 h after inoculation in shake flasks with 2% glucose. The results are shown in Table 2. Since the *TEF1* promoter is one of the strongest constitutive promoters (Gatignol *et al*., 1990), and since it showed the most stable and highest activity at different time points, we chose to set the p*TEF1* activity at 8 h as 100% and compared the activity of the other promoters relative to p*TEF1* activity at this time point. The results showed that after 8 h p*PGK1* and p*TDH3* had the same activity as p*TEF1*. p*TPI1* and p*PYK1* showed 60% of the activity of p*TEF1*. The activity of p*ADH1* and p*HXT7* was 20% and 10% of the *pTEF1* activity, respectively.

After 24 h, *ADH1, TPI1, PYK1* and *TDH3* promoters showed a decrease in expression and their activities were in the range 27–31% of the p*TEF1* activity at 8 h. Hauf *et al*. (2000) compared *TPI1, PGK1, ENO1, PYK1, PDC1* and *ADH1* promoters with each other and also showed that in ethanol medium p*PYK1* was the weakest, and that p*TPI1* and p*PGK1* were of similar activity in ethanol medium. Adh1p is responsible for ethanol production during growth on glucose (Young *et al*., 1982) and *ADH1* expression was shown to be reduced when cells enter the ethanol growth phase or during growth on non-fermentable carbon sources (Denis *et al*., 1983). However, Ruohonen *et al*. (1995) showed that short and middle-sized fragments of the *ADH1* promoter kept their activity during the ethanol phase. The activity of p*PGK1* also decreased, whereas p*HXT7* activity increased continuously until 48 h, when it reached 150% of the initial TEF1 activity (Table 2). Our results obtained for the *HXT7* promoter can be explained by previous investigations defining Hxt7p as a high-affinity hexose transporter which is highly expressed at low glucose concentration (<4.4 mM) (Reifenberger *et al*., 1995; Sedlak and Ho, 2004). At the last time point, after 48 h, the activity of most promoters did not change significantly compared with the previous measurement, except that p*ADH1* and p*PYK1* activities decreased and both promoters were considered as the weakest promoters in this study.

In conclusion, we observed that the promoter activity varied with the glucose concentration and whether the cells were growing on glucose or ethanol. Taken together, the promoter activities, with the exception of p*HXT7* and p*TEF1*, decreased during shake-flask cultivation and an overall ranking of the promoters is as follows:

When cells are in glucose consuming phase:

$$\mathrm{p}TEF1 \sim \mathrm{p}PGK1 \sim \mathrm{p}TDH3 > \mathrm{p}TPI1 \sim \mathrm{p}PYK1 > \mathrm{p}ADH1 > \mathrm{p}HXT7$$

When glucose is exhausted and ethanol is being consumed:

$$\mathrm{p}TEF1 \sim \mathrm{p}HXT7 > \mathrm{p}PGK1 > \mathrm{p}TPI1 \sim \mathrm{p}TDH3 > \mathrm{p}PYK1 \sim \mathrm{p}ADH1$$

Comparison in different types of cultivation

In order to evaluate the strength of the studied promoters with two strong and well-characterized promoters, i.e. the two galactose inducible promoters, p*GAL1* and p*GAL10*, four promoters with different activity according to the shake-flask results, p*TEF1*, p*TPI1*, p*ADH1* and p*HXT7*, were chosen and compared in batch and continuous cultures.

Comparison in batch cultivation

p*TEF1*, p*TPI1*, p*ADH1*, p*GAL1* and p*GAL10* activities could be detected and measured from the beginning of the exponential phase, whereas the activity of the p*HXT7* promoter at this step

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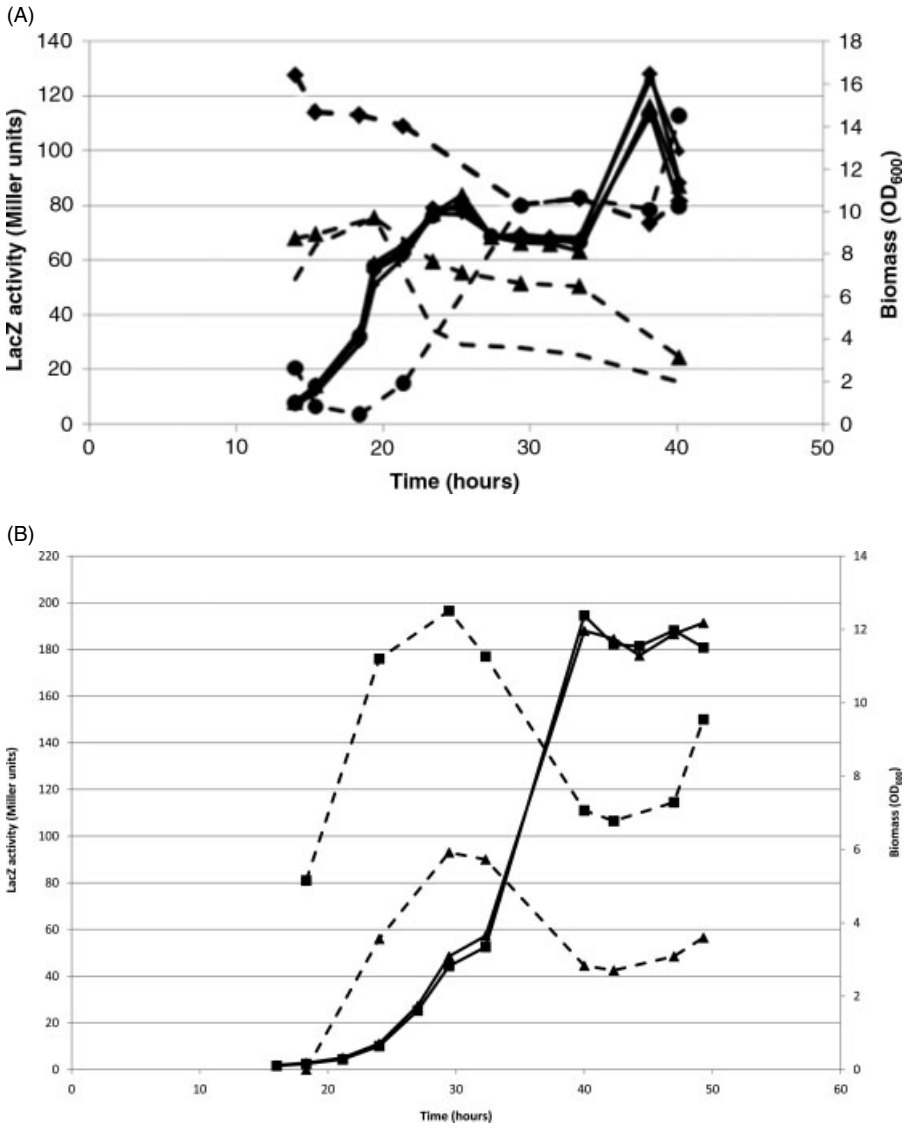

Figure 3. (A) LacZ activity and biomass profile in batch cultivation with glucose based promoters. Dashed lines, β-galactosidase activity; solid lines, biomass (OD_{600}); diamonds, p*TEF1*; circles, p*HXT7*; triangles, p*TPI1*; no symbols, p*ADH1*. (B) LacZ activity and biomass profile in batch cultivation with *GAL1* and *GAL10* promoters. Dashed lines, β-galactosidase activity; solid lines, biomass (OD_{600}); triangles, p*GAL1*; squares, p*GAL10*

was not detectable or very low and then increased. At the end of the exponential phase and in the ethanol phase, this promoter had a higher activity than p*TEF1*, p*TPI1*, p*ADH1* and p*GAL1* (Figure 3A, B). As in the results obtained from comparison of all the glucose-based promoters (Table 2), p*TEF1* showed the most stable activity during the exponential and ethanol phases. This promoter also showed a high and stable activity in galactose-containing medium (data not shown). Therefore, it seems that the activity of p*TEF1* is not affected tremendously by glucose concentration or by changes in the carbon source, i.e. to galactose or ethanol, and it can therefore be characterized as a truly constitutive promoter. The activities of p*GAL1* and p*GAL10* decreased at the end of the exponential phase, when galactose concentration is low and in the ethanol consumption phase. A similar trend could be seen for both p*TPI1* and p*ADH1* (Figure 3A, B).

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Comparison in chemostat

Growth rates of *S. cerevisiae* in glucose- and galactose-containing media are significantly different in batch cultivation. In order to avoid any effect of the growth rate on the promoter strength, the four promoters p*TEF1*, p*HXT7*, p*GAL1* and p*GAL10* were compared in continuous cultures at a fixed dilution rate (0.1/h in both glucose and galactose media) and the β-galactosidase enzyme assay was performed when the cultures were in steady state. The results are shown in Figure 4.

As expected from the shake-flask results, p*HXT7* showed the highest activity among all four promoters in the chemostat (Figure 4). p*TEF1* and p*GAL10* come in second and showed similar activity levels, whereas the *GAL1* promoter was the weakest (it exhibited half of the average activity of the *GAL10* and *TEF1* promoters). The high activity of p*HXT7* in the glucose-limited chemostat is consistent with its role as a high-affinity hexose transporter (Reifenberger *et al*., 1995; Sedlak and Ho, 2004). Expression from p*HXT7* is maximized when maintaining very low glucose concentrations in the chemostat (Figure 4). This high expression level was not observed in the shake flasks, as glucose is only present in low concentration for a short period before being exhausted (Figure 3A). As observed in the shake flasks (Figure 3B), p*GAL10* is also stronger than p*GAL1* in the chemostat and this is in contrast to previous investigations. Yocum *et al*. (1984) cloned a 914 bp fragment containing the *GAL1/GAL10* divergent promoters in front of *lacZ* into single, multicopy and integrative plasmids and evaluated the promoter activity in different carbon sources. They showed for all conditions that *GAL1* had a two- to four-fold higher activity than *GAL10*. In another approach, West *et al*. (1987) constructed different chimeric promoter cassettes, including the upstream activating sequence (UAS) from the *CYC1* promoter and fragments of the *GAL1* or *GAL10* promoter, and they used *lacZ* as a reporter gene. Evaluating the efficiency of different regulative elements on *GAL1/GAL10* promoters, they also observed a generally much higher activity for p*GAL1* than p*GAL10*. In the last example, Cartwright *et al*. (1994) used β-lactamase as a secreted reporter in single and multicopy vectors to compare *PGK1, GAL1, GAL10, PHO5* and *CUP1* promoters under varying nutritional conditions. Again, the results showed that the *GAL1* promoter was more active than the *GAL10* promoter. To ensure that there was only a single integration of p*GAL10*, we tested the strains by Southern blot analysis, and this showed a single integration for both the p*GAL1* and the p*GAL10* strain (data not shown).

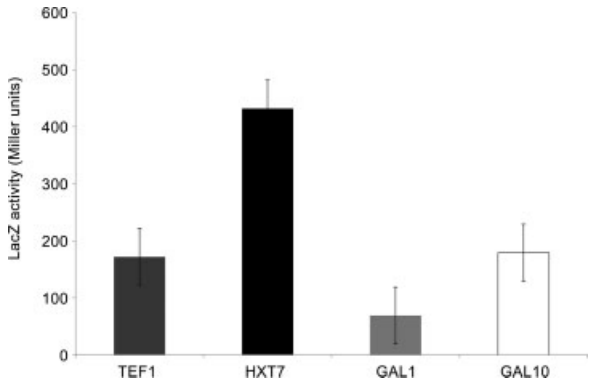

Figure 4. LacZ activity in chemostat. p*TEF1*, grey column; p*HXT7*, black column; p*GAL1*, light grey column; p*GAL10*, white column. Error bars represent SEM

However, as these two promoters were cloned using different restriction enzymes (p*GAL1* cloned using *Xho*I/*Bam*HI and p*GAL10* cloned using *Not*I/*Bam*HI), the distance between the promoter and the *lacZ* gene was different in both cases. Since our cloning strategy also differed from those used in the other studies, this may have some effect on the translation efficiency.

Since the aim of this investigation was to construct a dual glucose-based expression system to replace the *GAL1/GAL10* promoters in pESC–*URA*, we needed two promoters with a similar expression profile. As the results of the first comparison (Table 2), the *PGK1* and *TDH3* promoters represent options for a promoter that can be combined with p*TEF1*. Although both of them start with the same activity as p*TEF1* after 8 h, their activities decline. After 24 h this loss of activity for the *TDH3* promoter is higher than for the *PGK1* promoter. Previous investigations by Mellor *et al*. (1985) showed that when the *PGK1* gene was cloned into a multicopy plasmid and expressed in yeast, Pgk1p accumulated to up to approximately 50% of total cell protein. Furthermore, different powerful expression vectors were constructed, based on the promoter region of the *PGK1* gene, and these vectors have been used to

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study the expression of a number and heterologous genes (Dernyk *et al*., 1983; Tuite *et al*., 1982; Masuda *et al*., 1994). We therefore chose the *TEF1* and *PGK1* promoters as the basis for construction of a new vector containing two bidirectional promoters.

Comparison of p*TEF1* and p*PGK1* in different contexts

A PCR fusion fragment consists of two PCR fragments which are fused together by using a pair of matched adaptamers, which contain a complementary sequence at their 5′ ends (Erdeniz *et al*., 1997). We used fusion PCR to fuse *TEF1* and *PGK1* promoters in opposite orientation to each other, and thereby constructed a nucleotide sequence containing bidirectional *TEF1–PGK1* promoter. These bidirectional promoter were cloned into pSF011 in front of the *lacZ* gene in both orientations and the resulting integrative vectors were integrated into the genome of *S. cerevisiae* CEN.PK 113-5D at the *ura3-52* locus. To evaluate the activity of p*TEF1* and p*PGK1* in the newly bidirectional promoter, we compared their activities with individual p*TEF1* and p*PGK1*, respectively, in shake flasks using the same conditions as described above. The results show that the activity of the *PGK1* promoter after fusion to p*TEF1* was not significantly different when compared with that of p*PGK1* alone (Figure 5A). p*TEF1* does not show significant change in activity after the fusion with p*PGK1* either (Figure 5B).

Final constructions

After comparison of the different promoters, we constructed four different expression vectors with bidirectional strong promoter, p*TEF1*– p*PGK1*. Two of these constructs, pSP-G1 and pSP-G2 (Figure 2A, B), are useful for evaluating and expressing two different genes at the same time. The two different promoter orientations in pSP-G1 and pSP-G2 allow for a greater variety of cloning strategies due to the different promoter–multicloning site (MCS) combinations. Constitutive enzyme (α-amylase) expression with the help of these vectors has been verified (data not shown).

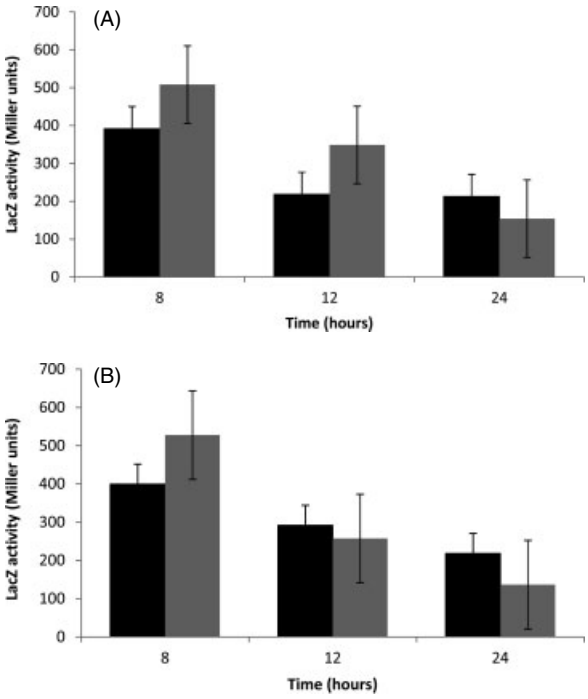

Figure 5. (A) Activity of p*PGK1* in different context. Black columns correspond to the activity of p*PGK1* alone; grey columns correspond to the activity of p*PGK1* fused to p*TEF1*. Error bars represent SEM. (B) Activity of p*TEF1* in different context. Black columns correspond to the activity of p*TEF1* alone; grey columns correspond to the activity of p*TEF1* fused to p*PGK1*. Error bars represent SEM

Conclusion

Here, the activities of seven different constitutive and glucose-based promoters, p*TEF1*, p*TPI1*, p*TDH3*, p*ADH1*, p*PGK1*, p*HXT7* and p*PYK1*, were compared with each other and also compared with the two strong galactose-inducible promoters, p*GAL1* and p*GAL10*. We used *lacZ* as reporter and the integrative plasmid pSF011 for this comparison. We further constructed a bidirectional promoter cassette consisting of p*TEF1*– p*PGK1* and showed that the two promoters, in this context, have expression profiles similar to the corresponding isolated promoters, and can therefore support high level gene expression. We then integrated this bidirectional promoter based on p*TEF1* and p*PGK1* into an expression vector that retains all the features of the pESC–*URA* plasmid, except that gene expression is mediated by constitutive promoters. Two vectors were constructed with opposite orientation of the bidirectional promoter. Both vectors are very

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useful for metabolic engineering projects that aim at high level production of valuable products using yeast as a production platform.

Our results showed varying profiles of activity for each promoter; for example, the *TEF1* promoter showed the most constant activity during fermentation and p*HXT7* represented the strongest one in continuous culture limited by glucose. We used p*PGK1* and p*TEF1* for constructing the new vector but, depending on the purpose, one can use different promoter pairs with comparable or a different expression pattern. For example, the *HXT7* promoter is suggested for fed-batch or continuous cultivation in glucose-limited conditions to reach very high gene expression levels. On the other hand, the full-length *ADH1* promoter would be suitable for conditional expression of genes at high glucose concentrations. It may therefore be a good idea to use different promoter combinations for different experiments, since it is simple to exchange the present promoters with any other promoter used in this study to change the expression level rate of the cloned genes.

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