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Research Article

The *CUP1* promoter of *Saccharomyces cerevisiae* is inducible by copper in *Pichia pastoris*

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Abstract

We report the construction of a *Pichia pastoris* integrating vector which contains the inducible *CUP1* promoter from *Saccharomyces cerevisiae*. We show that the promoter is indeed inducible by copper when used in *P. pastoris* and that the level of induction is dependent on the amount of copper in the medium. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: ***Pichia pastoris*; *CUP1* promoter; *Saccharomyces cerevisiae*; *lacZ***

Introduction

The methylotrophic yeast *P. pastoris* is widely used as a eukaryotic expression system to produce heterologous proteins. In most cases, the alcohol oxidase 1 promoter (P_{AOX1}) is being used for expression. This promoter is tightly regulated: it is completely repressed when cells are grown on glucose and maximally induced when grown on methanol (Tschopp *et al.*, 1987). Other promoters which have been cloned and can be used for the production of foreign proteins include the promoters of the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) and the glutathione-dependent formaldehyde dehydrogenase (*FLD1*) genes (Waterham *et al.*, 1997; Shen *et al.*, 1998). The *GAP* promoter (P_{GAP}) is constitutively expressed, with its strength varying depending on the carbon source. It can, however, yield expression levels in glucose that are higher than the commonly used P_{AOX1} on methanol (Waterham *et al.*, 1997). The *FLD1* promoter (P_{FLD1}) can be induced by either methanol as a sole carbon source (with ammonium sulphate as nitrogen source) or methylamine as sole nitrogen source (with glucose as carbon source). The levels of induction in both these media are comparable to that seen with the methanol-induced P_{AOX1} (Shen *et al.*, 1998).

S. cerevisiae is also being used for the production of foreign proteins. Several constitutive and inducible promoters have been employed. Some of the constitutive promoters are derived from the phosphoglycerate kinase (*PGK*), alcohol dehydrogenase (*ADH*), and glyceraldehyde-3-phosphate dehydrogenase (*GAP*) genes. The galactose-inducible promoters are derived from the galactose-regulated genes, *GAL1*, *GAL7* and *GAL10*. Other inducible promoters are the *MET3* promoter, derepressed by the depletion of methionine (Cherest *et al.*, 1985) and the promoter of the phosphate-regulated gene *PHO5*, which is regulated by phosphate depletion (for reviews, see Mendoza-Vega *et al.*, 1994; Schneider and Guarente, 1991). Furthermore, several inducible promoters are in use which can be activated by adding a substance to the growth medium: the *CUP1* promoter (P_{CUP1}), inducible by adding copper (Macreadie *et al.*, 1991); promoters which are inducible by steroids [deoxicorticosterone (Picard *et al.*, 1990) or dihydrotestosterone (Purvis *et al.*, 1991)]; and an activator/repressor system utilizing the antibiotic tetracycline (Nagahashi *et al.*, 1997).

P. pastoris has also been employed in cell biology research in such fields as peroxisome biogenesis and degradation (Gould *et al.*, 1992; Tuttle *et al.*, 1993; Sakai *et al.*, 1998) and the study of the organization of the transitional endoplasmic reticulum and Golgi apparatus (Rossanese *et al.*, 1999). For certain

experiments, it is not always desirable to change the nitrogen or glucose source to induce the production of a protein. We therefore undertook experiments to test if we could use the *CUP1* system from *S. cerevisiae* in *P. pastoris*.

Materials and methods

Strains and growth conditions

The *E. coli* strain JM109 (Invitrogen, Carlsbad, CA) was used in cloning experiments. *P. pastoris* strains used were: the wild-type strain PPY12 (*arg4, his4*), strain STK10 (PPY12, Δ*pex22::Zeocin*) (Koller *et al*., 1999) and strain SSH4 (PPY12, Δ*pex10::Zeocin*) (Snyder *et al*., 1999). Yeast strains were grown in either YPD (1% yeast extract, 2% peptone, 2% glucose), SD (0.67% synthetic media, 2% glucose) and SM (0.67% synthetic media, 0.5% methanol), supplemented with amino acids, $SDCu^{2+}$ (SD + copper) and $SMCu^{2+}$ (SM + copper), supplemented with amino acids and the indicated amount of cupric sulphate.

Cloning of plasmids

Techniques used to clone the plasmids were essentially as described (Sambrook *et al*., 1989). The *CUP1* promoter (P_{CUP1}) was amplified from plasmid YEp105 (Ellison and Hochstrasser, 1991) by the polymerase chain reaction (PCR) with primers TK141 (5′-AAGATCTCCCATTACCGACATTTGGGC-3′) and TK142 (5′-AGGATCCAGTTTGTTTTTCTTAATATCTATTTCG-3′). The resulting fragment was cloned into the vector pCRblunt (Invitrogen, Carlsbad, CA). This plasmid was then cut with BamHI (inside primer TK142) and NotI and cloned into pBluescriptIIKS (Stratagene, La Jolla, CA) cut with the same enzymes. The fragment containing P_{CUP1} was then cut from this plasmid using BglII and EcoRI and cloned into the plasmid pPIC3K (Invitrogen, Carlsbad, CA) cut with EcoRI and partially with BglII (to remove the 5′AOX1 promoter). The resulting plasmid, containing P_{CUP1} instead of P_{AOX1} in plasmid pPIC3K, was named pJV4. Plasmid pJV5, expressing GFP–SKL from P_{CUP1}, was cloned by ligating an EcoRI fragment from pTW51 (Wiemer *et al*., 1996) into EcoRI-cut pJV4. Plasmid pJV6, expressing *PpPEX22* from P_{CUP1} was cloned by ligating a BamHI–EcoRI fragment from pTK10 (Koller *et al*., 1999) into pJV4 cut with BamHI–EcoRI. Plasmid pTK52, containing the *lacZ* gene downstream of P_{CUP1}, was cloned by introducing a blunt-ended *lacZ* fragment (a gift from W. Snyder, University of California, San Diego) into the blunt-ended BamHI site of pJV4. Plasmid pTK54, expressing *PpPEX10* from P_{CUP1} was cloned by ligating a BamHI–EcoRI PCR fragment of *PpPEX10* created with primers TK167 (5′-AGGATCCATGCCCCCATCTGAAGAGATC-3′) and TK164 (5′-AAAGCTTATTCACACCACATGGTTGGGGG-3′) from genomic DNA into pJV4 cut with BamHI–EcoRI.

β-galactosidase assay

To measure the activity of P_{CUP1}, strain PPY12 transformed with plasmid pTK52 was grown on SD medium to mid-log phase. At time 0, copper was added to the final concentrations of 0–200 μM. After 2 h of incubation, cells were harvested and analysed for β-galactosidase activity according to Miller (1972).

Miscellaneous

Fluorescence microscopy and TCA lysates of strains were done as described (Koller *et al*., 1999). Western blotting was performed according to standard procedures. The Pex22p antibody was used at a dilution of 1 : 2000, the glucose-6-phosphake dehydrogenase (G6PDH) antibody was used at 1 : 3000.

Results

The inducible promoter systems used in *P. pastoris* depend on a specific carbon or nitrogen source for their activation (Tschopp *et al*., 1987; Waterham *et al*., 1997; Shen *et al*., 1998). Sometimes it is not desirable to switch or to grow cells in specific carbon or nitrogen sources to express a gene. To overcome this, the *S. cerevisiae* P_{CUP1} was cloned into pPIC3K (Invitrogen, Carlsbad, CA) replacing P_{AOX1} to create pJV4 (see Materials and methods). The plasmid retains all other properties of the parent plasmid pPIC3K (see Figure 1). Furthermore, the plasmid can be cut with NcoI or SalI to be integrated into the *HIS4* locus of *P. pastoris*. In addition, the *P. pastoris HIS4* and the kanamycin resistance genes can be used to screen transformants for HIS prototrophy or G418 resistance, respec-

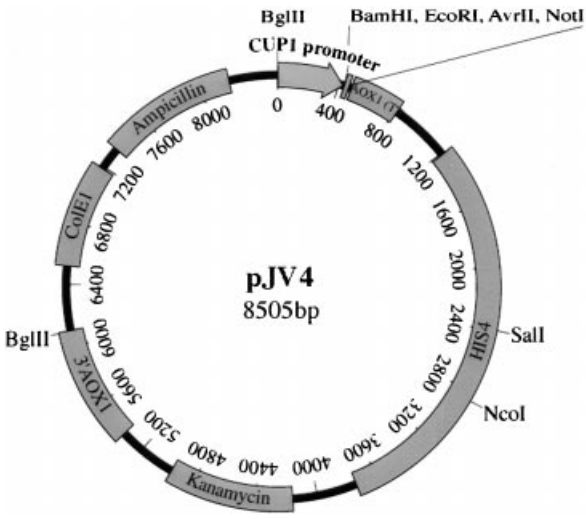

Figure 1. Map of pJV4. Multiple cloning site is shown as BamHI, EcoRI, AvrII, NotI. Shaded boxes show the localization of the various elements of the plasmid. *CUP1* promoter; AOX1 (T), transcription terminator from *P. pastoris AOX1*; HIS4, *P. pastoris HIS4*; Kanamycin, Kanamycin-resistance gene; 3′ AOX1, 3′ AOX1 fragment; ColE1, origin of replication; Ampicillin, Ampicillin-resistance gene. Restriction sites for NcoI and SalI for use in the integration of the plasmid into the *HIS4* locus are indicated

tively. 90% of transformants that pass both tests exhibit, in our experience, correct integration of the desired plasmid.

To test the inducibility of P_{CUP1} in *P. pastoris*, a fragment encoding GFP–SKL was placed downstream of P_{CUP1} in plasmid pJV4, creating plasmid pJV5. GFP–SKL is a fusion protein of GFP (green fluorescent protein) with the tripeptide peroxisomal

Table 1. β-galactosidase (β-Gal) activity in *P. pastoris* expressing *lacZ* from P_{CUP1} treated with different concentrations of copper

Cu^{2+} conc. (μM)	β-Gal units	Induction
0	28.5	1.0
1	114.6	4.0
2	171.1	6.0
5	322.4	11.3
10	419.0	14.7
20	459.1	16.1
50	573.5	20.1
100	748.8	26.3
200	803.9	28.2

Strain PPY12 transformed with pTK52 was grown in SD and copper at the indicated concentrations.

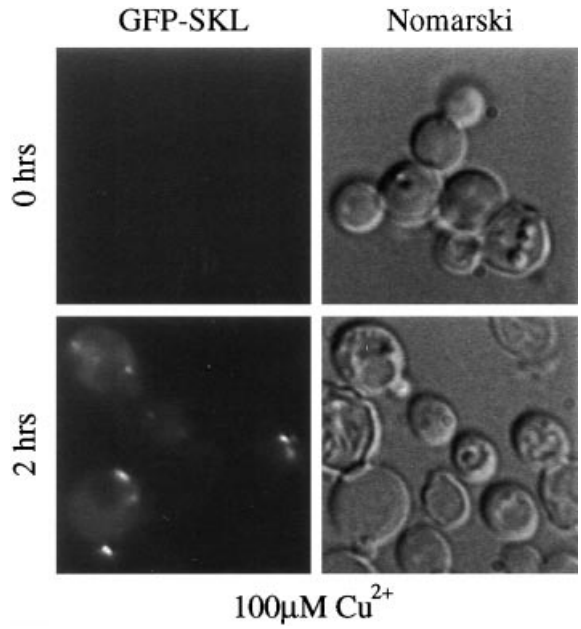

Figure 2. GFP–SKL is induced by copper. Strain PPY12 transformed with pJV5 was grown on glucose containing copper (100 μM). GFP–SKL was detected under a fluorescence microscope at time 0 and 2 h

targeting signal, SKL. This fusion protein is targeted to peroxisomes in wild-type cells. The plasmid pJV5 was integrated into the wild-type strain PPY12, grown to log phase and induction with copper (100 μM) led to the appearance of GFP–SKL in cells after 2 h, whereas at time 0 no GFP–SKL could be observed (Figure 2). As

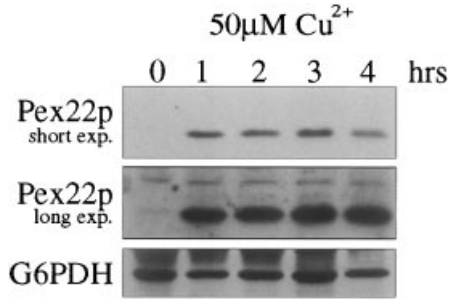

Figure 3. Pex22p is inducible by copper. Strain Δ*pex22* transformed with pJV6 was grown on glucose containing copper (50 μM). Cells at an $OD_{600} = 2$ were taken at the indicated time points and TCA lysates prepared. Equal amounts were loaded on a gel, blotted onto nitrocellulose and tested for the presence of Pex22p and G6PDH with specific antibodies

expected, the GFP–SKL could be seen accumulating in small dots, characteristic of peroxisomes.

To measure the degree of induction, we cloned the *E. coli lacZ* gene downstream of P_{CUP1} and transformed this construct into PPY12. Cells were grown and induced with different concentrations of copper (0, 1, 2, 5, 10, 20, 50, 100, 200 μM). 200 μM was chosen as the highest concentration because PPY12 incubated with higher concentrations of copper did have a decreased growth rate (data not

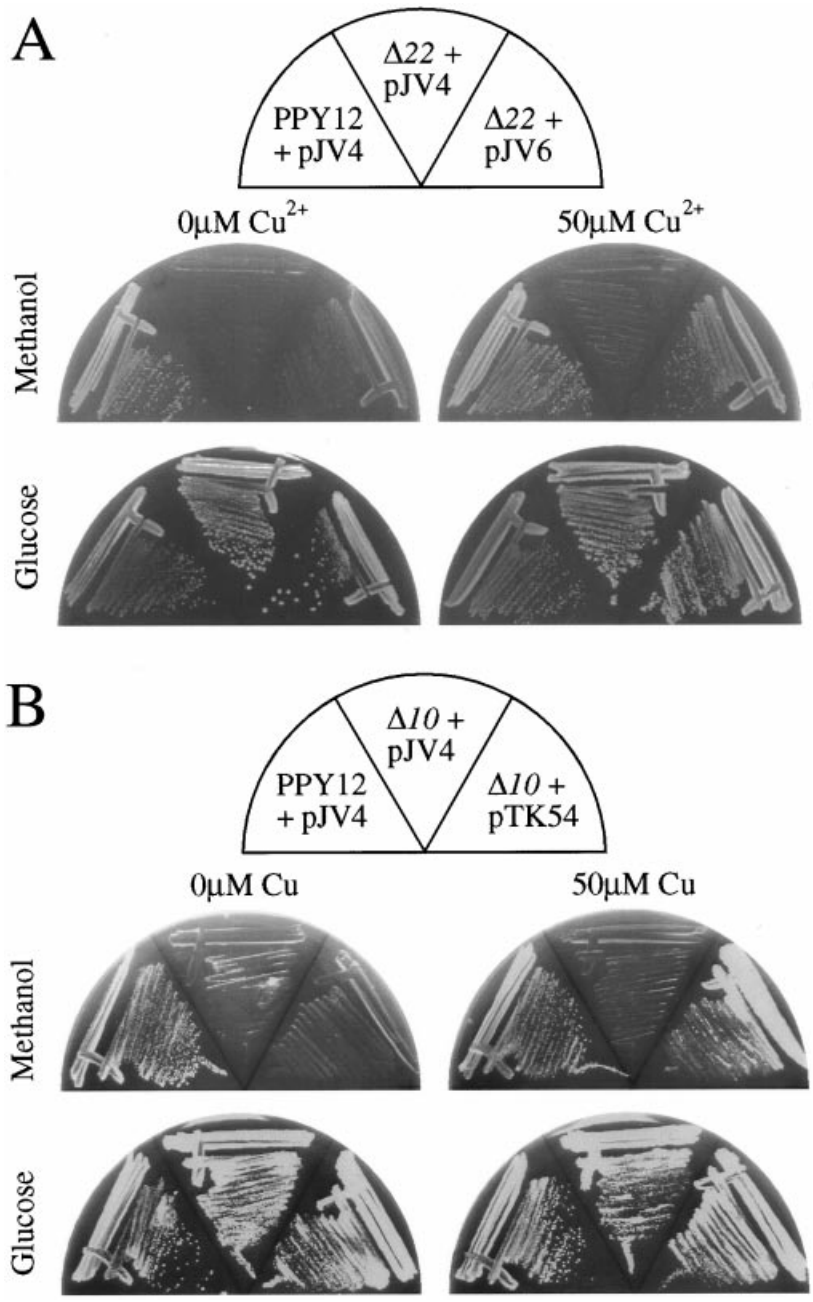

Figure 4. Complementation of Δ*pex22* (A) and Δ*pex10* (B) by a copper-inducible *PEX22* or *PEX10*, respectively. Strains transformed with pJV4 (as negative control) and with pJV6 (A) or pTK54 (B), respectively, as well as PPY12 transformed with pJV4 (as positive control), were streaked on glucose or methanol plates supplemented with the indicated amount of copper

shown). Increasing the amount of copper led to an increase of β-galactosidase activity following a 2 h induction (Table 1). Furthermore, even when no copper was added, there was a small amount of β-galactosidase activity.

Next, we determined the possibility of using a P_{CUP1}-driven gene to complement its deletion. *PpPEX22* was chosen, as its deletion prevents growth on methanol-containing medium (Koller *et al*., 1999). *PpPEX22* was cloned downstream of P_{CUP1} in pJV4, creating plasmid pJV6, and transformed into STK10 (Δ*pex22*). The strain was grown in SD medium and the *PpPEX22* gene induced with copper (50 μM). At intervals of 0, 1, 2, 3 and 4 h, a sample was taken and analysed for the appearance of Pex22p. As can be seen in Figure 3, Pex22p was induced with copper. No Pex22p could be seen at time 0. Overexposure of the film, however, revealed a faint band of Pex22p at time 0. The strain was also streaked on methanol-containing plates (SM) containing 0 or 50 μM copper. Although it did not grow like wild-type on plates containing no copper, there was some growth of this strain. Adding 50 μM copper to the plate did confer growth which was indistinguishable from wild-type (Figure 4A). To check if the complementation of deleted strains on media without any added copper is a general phenomenon or specific for *PEX22*, we cloned *PpPEX10* into pJV4, transformed it into Δ*pex10* (Kalish *et al*., 1995), and checked for complementation on different plates. We could only observe complementation on plates which had 50 μM copper added but not on plates with 0 μM copper (Figure 4B). The presence of Pex22p without any copper in the medium, as well as the small activity of β-galactosidase found above, explains the result that even in the absence of copper, the Δ*pex22* strain is partially complemented by a P_{CUP1}-driven *PEX22*. These results show that the level of expression from P_{CUP1} is not zero in the uninduced state. However, depending on the gene in question, this low level of expression from the uninduced promoter may (e.g. *PpPEX22*) or may not (e.g. *PpPEX10*) be sufficient for complementation of the appropriate deletion strain.

Discussion

We have clearly demonstrated that P_{CUP1} is inducible in *P. pastoris* as a function of increasing copper concentration. GFP–SKL is produced in sufficient amounts from the induced promoter to be detected by fluorescence microscopy. The induction rates in *P. pastoris* are comparable to those found in *S. cerevisiae* (Mascorro-Gallardo *et al*., 1996). Furthermore, β-galactosidase activity produced from P_{AOX1} on methanol (254 β-galactosidase units) are in the same range as the ones found with the P_{CUP1}, but the induction ratio of P_{AOX1} is much higher due to the tight regulation of this promoter on glucose (0.03 β-galactosidase units; 8457x induction) (A. Koller, unpublished results). Using P_{CUP1} with *PpPEX22* however shows that, for this gene, the level of expression in a medium without any Cu^{2+} leads to the production of PpPex22p, which is enough to partially complement the disruption. This partial complementation was not seen when the same experiment was performed with *PpPEX10*. The Δ*pex10* strain could only be complemented by a P_{CUP1}-driven *PpPEX10* by adding 50 μM copper to the medium. Therefore, the *S. cerevisiae* P_{CUP1} can be used in *P. pastoris* as a promoter to induce genes, e.g. for complementation of their disruption or for expression of foreign proteins.

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