



Yeast Functional Analysis Report

An efficiently regulated promoter system for *Cryptococcus neoformans* utilizing the *CTR4* promoter

Jeramia J. Ory, Cara L. Griffith and Tamara L. Doering*

Washington University School of Medicine, Department of Molecular Microbiology, 660 S. Euclid Avenue, Campus Box 8230, Saint Louis, MO 63110, USA

*Correspondence to:

Tamara L. Doering, Washington University School of Medicine, Department of Molecular Microbiology, 660 S. Euclid Avenue, Campus Box 8230, Saint Louis, MO 63110, USA.
E-mail: doering@borcim.wustl.edu

Abstract

Cryptococcus neoformans is an opportunistic fungal pathogen responsible for serious meningitis. Although many useful molecular tools have been developed for its study, there are currently few inducible promoters available for general use. To address this need, we have constructed expression plasmids incorporating upstream elements of the *C. neoformans* copper transporter gene *CTR4*, and tested them in *C. neoformans* serotypes A and D. In response to copper deprivation, these plasmids mediate high-level expression of a reporter protein. This expression can be completely repressed using physiologically low concentrations of copper. Notably, this new family of copper-sensing promoters demonstrates excellent expression in serotype A, contrasting with other available promoters. These plasmids therefore offer efficient and regulated expression for both serotypes A and D, and should be valuable tools for the *C. neoformans* research community. Copyright © 2004 John Wiley & Sons, Ltd.

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Introduction

Cryptococcus neoformans is a ubiquitous soil fungus and an opportunistic pathogen. The global rise in the number of individuals who are immunocompromised due to HIV has caused a corresponding increase in reported cases of cryptococcal meningitis. Although this trend has been attenuated by the introduction of highly active antiretroviral therapy (HAART) in developed countries (for reviews, see Mitchell and Perfect, 1995; Casadevall and Perfect, 1998), cryptococcosis continues to be a problem in developing countries, accounting for up to half of reported neurological disease in HIV patients (Banerjee *et al.*, 2001). Current treatment of cryptococcosis requires suppressive therapy for as long as patients remain immunocompromised (Powderly, 2000).

Along with the increased incidence of disease caused by *C. neoformans*, study of this organism's

biology and pathogenesis has greatly expanded in the last 15 years. With the sequencing of serotypes D and A now complete (www.tigr.org, sequence.stanford.edu, www.broad.mit.edu, cneo.genetics.duke.edu), a new stage in cryptococcal research has arrived. In light of the wealth of genome information now in the public domain, it is appropriate to review the molecular tools available to the cryptococcal research community. Two reverse genetic approaches have been described in the literature. Gene disruption or specific mutation by homologous recombination, using either biolistic transformation or electroporation, has been available for some time (Edman and Kwon-Chung, 1990; Lodge *et al.*, 1994; Toffaletti *et al.*, 1993). More recently, we have utilized double-stranded RNA interference for specific gene silencing (Cottrell and Doering, 2003; Liu *et al.*, 2002). Forward genetic studies to date have been based

on mutagenesis using UV irradiation or random insertions (Edman and Kwon-Chung, 1990; Janbon *et al.*, 2001; Moyrand *et al.*, 2002; Nelson *et al.*, 2001; Still and Jacobson, 1983; Wickes and Edman, 1995).

Although a variety of auxotrophic and drug resistance markers are available, there are only a few promoters that have been used in *C. neoformans*. For constitutive gene expression, the upstream regions of *ACT1* (Cox *et al.*, 1995) and *GPD* (Varma and Kwon-Chung, 1999) have been successfully used. For inducible expression, two systems have been described. One is homologous to the galactose-inducible system frequently used in *Saccharomyces cerevisiae*. In *C. neoformans*, expression of *GAL7* is repressed when cells are grown on glucose, and greatly enhanced when galactose is the carbon source (Wickes and Edman, 1995). The promoter region of this gene has been used to drive gene expression by several laboratories. The promoter of the mating pheromone gene *MFa1* induces gene expression when cells are grown in V-8 medium (del Poeta *et al.*, 1999), conditions which induce production of Mf α 1p. However, this medium also greatly reduces growth rate and initiates sporulation in *C. neoformans*, limiting its use as a general-purpose inducible promoter.

Our laboratory is interested in the synthesis and regulation of the polysaccharide capsule produced by *C. neoformans*, which is its major virulence factor (reviewed recently in Bose *et al.*, 2003). As different concentrations of glucose have been shown to alter formation of a key capsule component (Cleare and Casadevall, 1999), we have been hesitant to utilize the *GAL7* system in our studies. This dilemma and the general need for additional inducible systems in *C. neoformans* encouraged us to investigate other promoters.

The *CTR4* gene encodes a high-affinity copper transporter in *Schizosaccharomyces pombe*. Promoter elements upstream of this gene have been used to construct an expression system for heterologous proteins in that organism (Bellemare *et al.*, 2001). As shown in Figure 1, *CTR4* transcription is regulated in *Sz. pombe* by the DNA binding protein Cuf1p (Beaudoin and Labbe, 2001). In conditions of low copper, Cuf1p interacts with copper-sensing elements (CuSE) upstream of *CTR4*, greatly enhancing transcription. Under copper-replete conditions, Cuf1p binds copper and adopts a conformation with little to no

affinity for the upstream CuSEs. When this occurs, transcription of *CTR4* is effectively repressed. Below we describe the development of a copper-regulated expression system in *C. neoformans*.

Materials and methods

Strains and growth conditions

C. neoformans was grown with continuous shaking at 30 °C in minimal medium containing 2% (w/v) glucose and lacking uracil (Ura⁻ medium; Ausubel *et al.*, 2001), supplemented as indicated below. The wild-type serotype D strain JEC21 and the serotype D *ura5* strain JEC43 were from Dr Joseph Heitman (Duke University Medical Center), and the serotype A *ura5* strain H99R was from Dr Gary Cox (Duke University Medical Center).

Genomic searching

Tblastn (Zhang *et al.*, 1998) was used to compare amino acid sequences for *Sz. pombe* Ctr4p (Genebank Accession No. O94722) and Cuf1p (Q09728) to both the *C. neoformans* strain JEC21 EST database (www.tigr.org) and a TWINSCAN-derived database of ORFs predicted from the JEC21 genomic sequence (Flicek *et al.*, 2003; Korf *et al.*, 2001). Hits with an Expect score less than 1e⁻⁵ were analysed.

Construction of pCTR4 plasmids

The CIP3-GUST plasmid (Wickes and Edman, 1995) was obtained from Dr Brian Wickes (University of Texas Health Science Center) and used as a base for all constructions (Figure 2). For clarity, this plasmid will be referred to below as pGAL7/Gust. pGAL7/Gust contains a gene for ampicillin resistance, the *C. neoformans* *URA5* gene, and the *E. coli* reporter gene *gusA* flanked by the *C. neoformans* *GAL7* promoter and terminator. The *GAL7* promoter was released from this plasmid by *Nde*I and *Cla*I digestion, and directly replaced with promoters CTR4, CTR4-0 and CTR4-1 (Figure 3) to create pCTR4/Gust, pCTR4-0/Gust and pCTR4-1/Gust. The replacement promoters were generated by PCR amplification of genomic DNA from *C. neoformans* strain JEC21, using the primers shown in Table 1. To

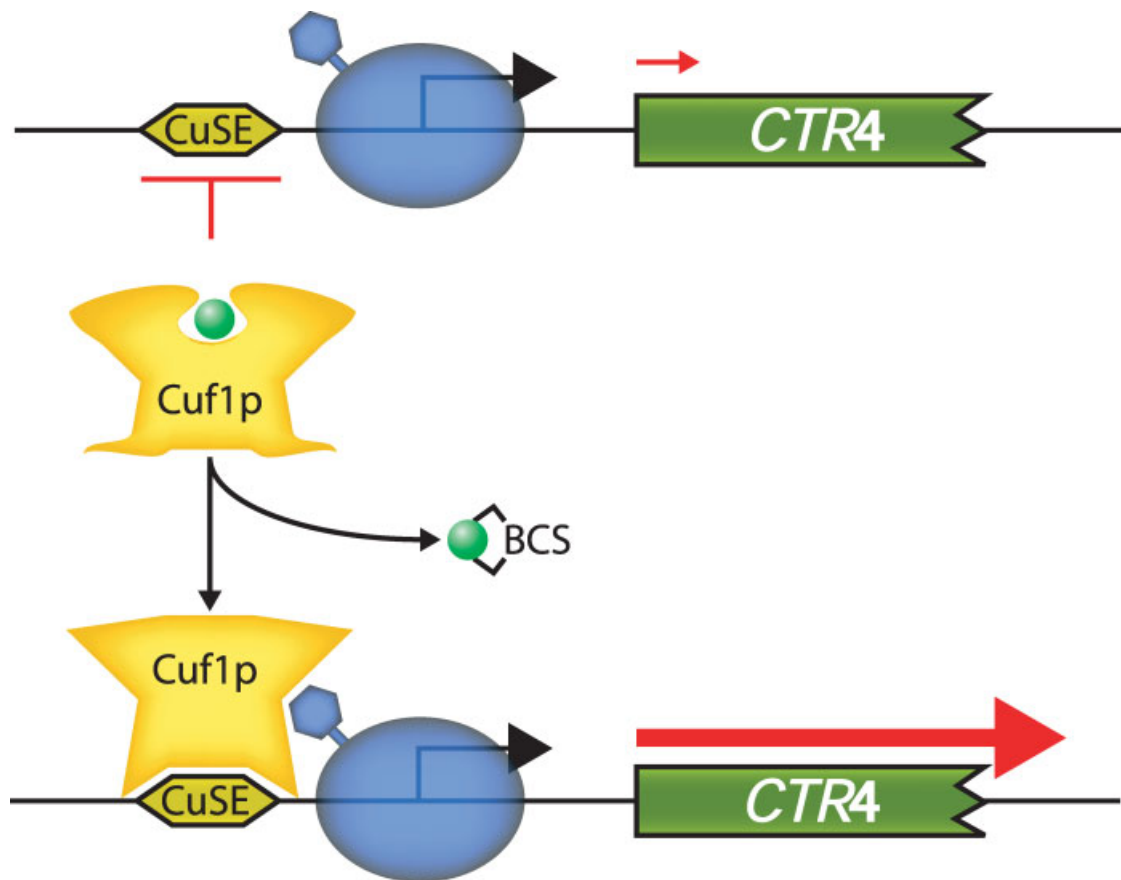


Figure 1. Schematic of Cuf1p-mediated transcription. The small green sphere associated with Cuf1p represents copper, and the blue shape represents transcription machinery. BCS, bathocuproinedisulphonic acid. See text for explanation

construct pCTR4-2/Gust, a region of the pCTR4-1/Gust plasmid equivalent to -236 to -431 of the *CTR4* promoter was amplified by PCR and cloned into the pCTR4-1/Gust plasmid at the *Cla*I site in the forward orientation. To generate pACT1/Gust, the *ACT1* promoter was removed from pCAP59i (Liu *et al.*, 2002) with *Nde*I and *Cla*I and ligated into the corresponding sites of pGAL7/Gust. All promoter replacement constructs were confirmed by sequencing (Nucleic Acid Chemistry Laboratory, Washington University School of Medicine).

Transformation and assay

C. neoformans ura5 cells (either JEC43 or H99R) were grown in minimal medium containing $20 \mu\text{g/ml}$ uracil for 16 h. They were then transformed by electroporation with *Not*I linearized

plasmids (Wickes and Edman, 1994) and transformants were selected on Ura^- plates.

For testing, individual transformants were grown for 16 h from a single colony in 5 ml Ura^- medium with appropriate supplementation. To test cells containing pCTR4-derived plasmids, cultures were supplemented with either $25 \mu\text{M}$ cupric sulphate (Sigma, St. Louis, MO) or $200 \mu\text{M}$ bathocuproinedisulphonic acid (BCS) (Sigma, St. Louis, MO). pGAL7/Gust transformants were grown in Ura^- medium or in the same medium with 2% (w/v) galactose as the carbon source instead of glucose. pACT1/Gust transformants were grown in Ura^- medium with no supplementation.

To assay activity, cells were sedimented (5 min, $6000 \times g$, 4°C) and assayed as previously described (Wickes and Edman, 1995) with minor changes. Briefly, cells were washed once each with distilled water and assay buffer (50 mM NaPO_4 ,

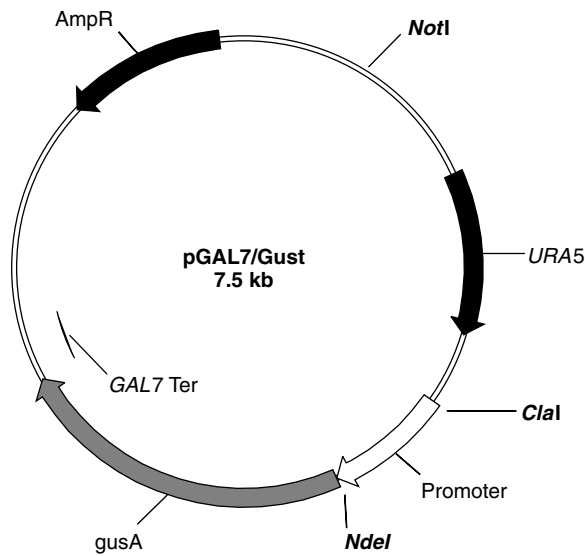


Figure 2. Map of pGAL7/Gust with coding regions and restriction sites used in cloning indicated. The open arrow indicates where promoters of interest were inserted. *gusA*, *E. coli* β -glucuronidase gene *gusA*; AmpR, *E. coli* *bla* gene; URA5, *C. neoformans* URA5 gene; GAL7 Ter, terminator region of *C. neoformans* GAL7 gene

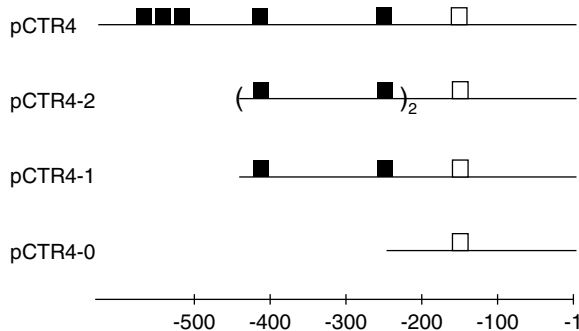


Figure 3. Summary of promoter constructs tested. The scale bar indicates approximate position, with -1 indicating one base pair before the predicted starting ATG of *CTR4*. See text for exact positions. Closed boxes, potential metal regulatory elements; open box, TATA box

pH 7.0, 5 mM DTT, 1 mM EDTA, 0.1% (v/v) Triton X-100). They were then resuspended in 500 μ l assay buffer, mixed with 200 μ l 0.5 mm glass beads (BioSpec Products, Inc., Bartlesville, OK), and vortexed three times for 3 min intervals, alternating with 3 min on ice. The cells were visually inspected after vortexing to ensure efficient breakage. Unbroken cells and cell debris were pelleted by centrifugation, and the supernatant fraction was removed and assayed for protein concentration (BioRad, Hercules, CA). 200 μ g total protein was adjusted to a final volume of 400 μ l with assay buffer, mixed with 100 μ l of 5 mM 4-methylumbelliferyl- β -D-glucuronide (MUG) (Sigma, St. Louis, MO), and incubated for 1 h at 37°C. The reaction was stopped by 10-fold dilution with 0.2 M Na₂CO₃, pH 9.5. Fluorescence of the liberated methylumbelliferone (MU) product was detected using a Beacon 2000 Variable Temperature Fluorescence Polarization System (PanVera, Madison, WI) with excitation at 388 nm and emission at 480 nm. Concentration of MU produced was calculated from a standard curve (data not shown) and final activity was reported as pmol MU/ μ g/min. Four transformants of each plasmid were tested and the one with the highest induced activity was used for additional studies. In these experiments, cells were grown for 24 h in Ura⁻ medium, diluted to 0.1 OD₆₀₀/ml in appropriate inducing or repressing medium, and grown for an additional 24 h. The cells were then harvested and assayed in triplicate for β -glucuronidase activity.

Results and discussion

A putative copper transport system in *C. neoformans*

To investigate copper regulation in *C. neoformans*, we first compared the 289 amino acid *Sz. pombe*

Table 1. Amplification primers for promoter construction

Promoter	Sense primer	Antisense primer
pCTR4-0	CCATCGATGCATAGCCCTCGGGAAGG ^a	GGAATTCCATATGGATTGGTGAAGTCGTTG
pCTR4-1	CCATCGATGGATATTGCTGTTTCTAC	GGAATTCCATATGGATTGGTGAAGTCGTTG
pCTR4-2	CCATCGATGGTCCATATGGACCAATT GGATATTGCTGTTTCTAC	CCATCGATGATGAAAGCAGCGTAACGG
pCTR4	CCATCGATGGCACAGGCTTTACGAAGGC	GGAATTCCATATGGATTGGTGAAGTCGTTG

^a All sequences are listed 5' to 3'.

Ctr4p sequence to predicted ORFs in *C. neoformans*. A significant match (Expect = $1e^{-14}$) was found to the C-terminal 169 amino acids of Ctr4p, which corresponded to a predicted ORF of 209 amino acids. A search against the expressed sequence tag (EST) database (www.tigr.org) confirmed that this gene was actively transcribed.

We next examined the genome sequence upstream of the predicted cryptococcal *CTR4* gene for promoter elements. At position -159 we noted the sequence 5'-TATATA-3', which is a canonical TATA box (Benoist and Chambon, 1981; Mathis and Chambon, 1981). We also noted the sequence 5'-ATATTGCTGT-3' at position -431. This sequence matches the consensus copper-sensing element (CuSE) established for *Sz. pombe*, 5'-D(T/A)DDHGCTGD-3' (D = A, G or T; H = A, C or T) (Beaudoin and Labbe, 2001). To search for other potential regulatory sites, the core sequence for metal regulatory elements (MRE) (Koch and Thiele, 1996), 5'-GCTG-3', was compared to the region upstream of *C. neoformans CTR4*. This sequence was present at positions -532, -520, -506, -434 and -256 (Figure 3); no other potential CuSEs were found within 1.5 kb upstream of the *CTR4* gene.

The sequence motifs we found upstream of *C. neoformans CTR4* suggested that this gene is regulated similarly to *Sz. pombe CTR4*. If this is true, its genome should encode a transcription factor similar to *Sz. pombe* Cuf1p. Cuf1p and the 15 other copper sensing transcription factors reported in the NCBI database are highly similar in their N-terminal 50–60 amino acids, but diverge in their C-terminal domains. True to this pattern, we found a *C. neoformans* ORF with significant sequence similarity (Expect = $1e^{-11}$) to the N-terminal 50 amino acids of *Sz. pombe* Cuf1p, which was also present in the EST database. The existence and transcription of *CTR4* and *CUF1* indicate that *C. neoformans* expresses the proteins necessary to regulate Cuf1p-mediated transcription of a high-affinity copper transporter (Ctr4p) in response to copper.

A widely used inducible promoter for *S. cerevisiae* is based on the promoter region of *CUP1*, a copper-inducible metallothionein (Macreadie *et al.*, 1989). When the *S. cerevisiae* Cup1p amino acid sequence was searched against a database of predicted proteins in the *C. neoformans* genome, a weak homology hit was found (Expect = 0.024).

The potential promoter for this gene was PCR-amplified and tested for activity in a reporter construct. This construct failed to exhibit any response to copper starvation or copper-replete conditions (data not shown).

Expression studies in serotype D

Encouraged by the presence of copper-regulated transcription machinery in *C. neoformans*, we next tested whether *CTR4* promoter regions could drive expression of the *E. coli* β -glucuronidase gene, *gusA*. To induce expression, medium was supplemented with 200 μ M bathocuproinedisulphonic acid (BCS), a copper-specific chelator. This concentration achieved the highest expression level without adversely affecting cell growth rate (data not shown). Maximal repression of *gusA* expression occurred in medium supplemented with 25 μ M CuSO₄ and 1 mM ascorbic acid (data not shown). The ascorbic acid acts as a reductant to increase the concentration of Cu⁺ in solution; this is the electronic form of copper transported by the CTR family of proteins (Dancis *et al.*, 1994) and sensed by the Cuf1p class of transcription factors (Jensen and Winge, 1998). Cells grown under these conditions were then assayed for β -glucuronidase activity as described in the methods.

We first tested construct pCTR4-1/Gust, which contains two potential MREs, one of which matches the *Sz. pombe* consensus CuSE (Figure 3). In the presence of copper, minimal *gusA* activity was observed for pCTR4-1/Gust, comparable to pGAL7/Gust repressed in glucose (Table 2). pCTR4-1/Gust transformants grown in the presence of 200 μ M BCS showed a 22-fold increase in activity, a substantial induction but less than that seen with galactose induction in this serotype.

In an effort to increase transcriptional efficiency, we PCR-amplified the region containing potential MREs, and ligated it in tandem upstream of the two original MRE's to create pCTR4-2/Gust (Figure 3). pCTR4-2/Gust transformants exhibited remarkable levels of *gusA* activity in the presence of 200 μ M BCS, approximately 10-fold higher than expression driven by the *ACT1* promoter (Table 2; Figure 4A). This extremely high activity meant that, despite a greater than 160-fold difference in activity between inducing (BCS) and repressing (copper) conditions, twice that of the *GAL7* system, activity driven by this promoter could not be completely repressed.

We also tested a larger portion of the *CTR4* promoter, pCTR4/Gust (Figure 3), which encompasses all potential CuSEs in the region. pCTR4/Gust transformants exhibited both complete repression in the presence of copper and high levels of induction when BCS was added. Although induction was not as high on an absolute scale as that driven by pCTR4-2/Gust, the fold induction of *gusA* activity was almost 300. As a negative control we used pCTR4-0/Gust, which contains the TATATA element upstream of *CTR4*, but no potential CuSEs. This construct demonstrated no ability to regulate *gusA* activity in response to copper, and no basal *gusA* expression. Activity driven by all of these promoters is shown in Figure 4A and Table 2.

Expression studies in serotype A

Although the genome sequences of serotypes A and D are quite similar, we have noted differences in promoter efficacy. We therefore tested whether the promoters we had developed in serotype D functioned similarly in serotype A. To do this we transformed pACT1/Gust, pGAL7/Gust and the four pCTR4-derived constructs into serotype A strain H99R, and assayed for *gusA* activity. As shown in Figure 4B, the pattern of expression for the different transformants is similar to that in serotype D (Figure 4A), but the magnitude of the overall response is greatly muted in serotype A. Notably, the pGAL7/Gust transformants display low *gusA* activity in the presence of galactose, with only a three-fold induction over background in serotype A compared to 83-fold in serotype D. In contrast, the pCTR4-2/Gust transformants

demonstrate an approximately 80-fold increase in activity in the presence of 200 μM BCS, with no measurable activity in the presence of CuSO_4 . This absence of residual activity is in contrast to what we observed with this promoter in serotype D.

The total amount of *gusA* induced by pCTR4-2 in serotype A approaches the activity seen for pACT1/Gust transformants in serotype D (Table 2). The *ACT1* promoter has been used successfully in serotype D by our lab and others, including for effective double-stranded RNA interference (RNAi) (Liu *et al.*, 2002). This suggests that pCTR4-2 will be of great utility. Activity of the other constructs is shown in Figure 4B and Table 2.

Judging by sequence similarity between predicted *C. neoformans* polypeptides and known proteins, we believe that both the high-affinity copper transporter *CTR4* and its copper-responsive transcription factor *CUF1* exist in this organism. Utilizing elements of the upstream region of *CTR4*, we have created a series of plasmids that can express heterologous proteins in response to copper depletion. In serotype D, these plasmids can be used for either tight regulation (pCTR4) or for extremely high expression (pCTR4-2). In serotype A, the pCTR4-2 plasmid provides both tight regulation and levels of expression higher than previously achieved in this serotype. We are currently investigating use of these plasmids for inducible RNAi in serotypes D and A and for protein expression/purification in serotype D. The plasmids are freely available, and it is our hope that they will be useful molecular tools for the cryptococcal research community.

Table 2. Glucuronidase activity by promoter and serotype

	Serotype D			Serotype A		
	Off	On	Fold induction	Off	On	Fold induction
pACT1		2.7 ± 0.29^a	n.a.		0.15 ± 0.0047	n.a.
pGAL7	0.047 ± 0.013	3.9 ± 0.22	83	0.024 ± 0.0015	0.06 ± 0	3
pCTR4-0	0.028 ± 0.00023	0.028 ± 0.00078	0	0.027 ± 0.0015	0.03 ± 0	0
pCTR4-1	0.027 ± 0.002	0.60 ± 0.03	22	0.025 ± 0.0043	0.32 ± 0.2	13
pCTR4-2	0.17 ± 0.011	27 ± 1.7	159	0.027 ± 0.00089	2.1 ± 0.045	78
pCTR4	0.034 ± 0.0014	9.4 ± 0.76	276	0.027 ± 0.0013	0.66 ± 0.048	24

^a Activity reported in pmol MU produced/ $\mu\text{g}/\text{min}$. pACT transformants were grown in Ura^- medium with no supplementation (On). pGAL7 transformants were grown in Ura^- medium with either glucose (Off) or galactose (On) as the carbon source. pCTR4 transformants were grown in Ura^- medium supplemented with either 25 μM CuSO_4 and 1 mM ascorbic acid (Off) or 200 μM bathocuproinedisulphonic acid (On). Assays were performed in triplicate as detailed in Methods, and standard deviations are reported. These data are representative of four independent experiments.

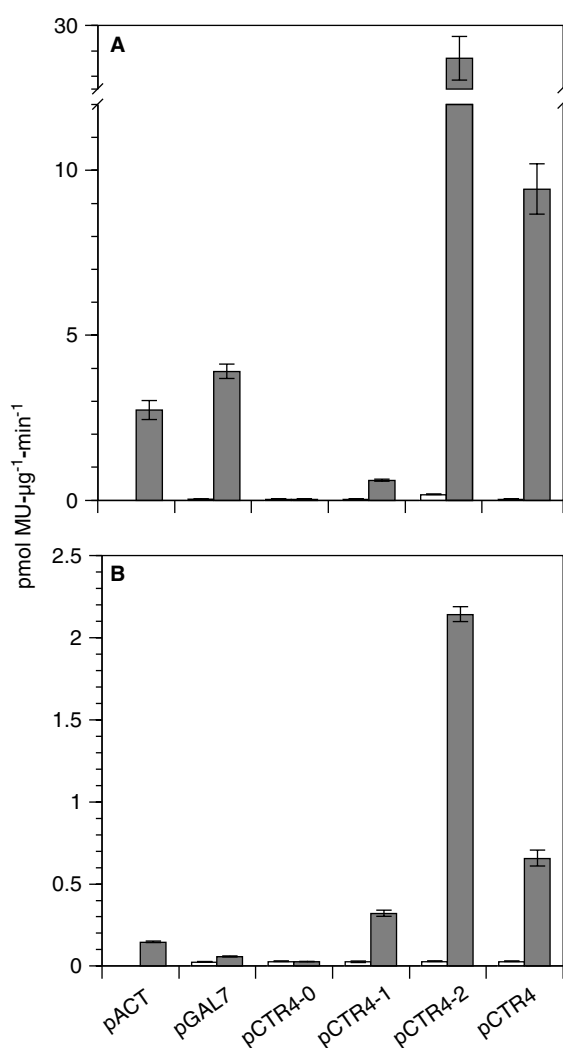


Figure 4. Expression under 'on' (shaded bar) and 'off' (open bar) conditions for each promoter (defined in Table 2) is shown. Error bars represent standard deviation of three replicates. (A) Serotype D. (B) serotype A. Note the discontinuity in scale on the ordinate in (A) and the scale difference between (A) and (B)

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