Expression Plasmids for Use in Candida glabrata

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ABSTRACT We describe a series of CEN/ARS episomal plasmids containing different Candida glabrata promoters, allowing for a range of constitutive or regulated expression of proteins in C. glabrata. The set of promoters includes three constitutive promoters (EGD2pr, HHT2pr, PDC1pr), two macrophage/phagocytosis-induced promoters (ACO2pr, LYS21pr), and one nutritionally regulated promoter (MET3pr). Each promoter was cloned into two plasmid backbones that differ in their selectable marker, URA3, or the dominant-selectable NAT1 gene, which confers resistance to the drug nourseothricin. Expression from the 12 resulting plasmids was assessed using GFP as a reporter and flow cytometry or quantitative reverse-transcription polymerase chain reaction to assess expression levels. Together this set of plasmids expands the toolkit of expression vectors available for use with C. glabrata.

Candida glabrata is an important fungal pathogen, causing both superficial and deep infections. C. glabrata has several traits that have been linked to virulence, including a repertoire of adhesins, the ability to adapt to and modify the macrophage phagolysosomal environment, and an inherent resistance to azole antifungals (Pfaller 2012). The organism is genetically tractable, and both transposon mutagenesis and reverse genetic approaches have been used to generate mutants (Petter et al. 1995; Chen 1996; Brachmann et al. 2004; Mumberg et al. 1992; Gould et al. 1992; Mumbreg et al. 1995; Chen 1996; Brachmann et al. 1998; Adams et al. 2005; Chee and Haase 2012).

This series of cloning vectors contains a pMB1 (ColE1 family) origin of replication and Ap8 marker for propagation in E. coli. A C. glabrata CEN sequence and an ARS element permit propagation and stable maintenance of the plasmid. A polylinker positioned between the C. glabrata–specific promoter and a transcriptional terminator facilitate cloning of genes of interest under control of a given promoter. We constructed this series of plasmids with a range of C. glabrata promoters; there are three constitutively active promoters, two macrophage-induced promoters, and one nutritionally regulated promoter. These options allow researchers to vary the level of expression for a target gene, and because the plasmids use the same polylinker, target genes can be easily shuttled between backbones with different promoters. Additionally, there is a choice of two selectable markers for use in C. glabrata, the URA3 auxotrophic marker or the dominant NAT drug-resistance cassette.

We describe the construction of these plasmids and quantify the expression level driven by each promoter by monitoring GFP expression by flow cytometry or by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). This set of vectors will facilitate regulated or constitutive expression of genes in C. glabrata and expands the genetic toolbox available for C. glabrata.

MATERIALS AND METHODS

Growth media

C. glabrata was routinely grown on YPD media (10 g/liter yeast extract, 20 g/liter peptone, 2% dextrose) at 30°C. All solid media contained 2% agar. Nourseothricin (NAT; clonNAT; Werner BioAgents) was supplemented to liquid YPD media at 50 μg/ml and to solid YPD media at 100 μg/ml to select for C. glabrata strains containing pCN vectors. Strains containing URA3-marked plasmids (pCU series) were grown in SD-Ura (1.7 g/liter yeast nitrogen base without amino acids or ammonium sulfate, 5 g/liter ammonium sulfate, 6 g/liter casamino acids, 2% dextrose).

The MET3 promoter is controlled by the presence of methionine and cysteine in the media. Media lacking methionine, cysteine, and uracil was used to induce expression, whereas addition of Met and Cys (2 mM each) was used to repress the MET3 promoter. SD−Met−Cys−Ura and SD+Met+Cys−Ura media were made using +Met+Cys−Ura or −Met−Cys−Ura amino acid mixtures, respectively. For the pCN-MET3 vectors, SED media (1.7 g/liter yeast nitrogen base without amino acids or ammonium sulfate, 1 g/liter monosodium glutamate, 2% dextrose) is used instead of standard SD, because NAT is not inhibitory in the presence of ammonium sulfate (Cheng et al. 2000). The resulting SD−Met−Cys−Ura and
pCN plasmids were transformed into strain BG2 and transformants formed into strain BG14 and transformants selected on SD described previously (Castano et al. 2003). pCU plasmids were transformed into strain BG14 and transformants selected on SD—Ura plates. pCN plasmids were transformed into strain BG2 and transformants were selected in the presence of NAT (100 μg/ml).

Plasmid construction

All plasmids used in this study are listed in Table 2. TheURA3-marked and NAT-marked plasmid backbones are derived from the pGRB2.1 and pBM16 plasmids, respectively (Friedman et al. 2002; Ma et al. 2009). We include a more complete description of their design here.

The pGRB vectors were created by cloning a chimeric C. glabrata CEN/ARS sequence into pRS406 (Sikorski and Hieter 1989), which was linearized via AatII restriction digestion, creating pGRB2.0. The CEN sequence was isolated from C. glabrata strain BG2 centromere H, based on previously identified C. glabrata centromeric sequences (Kitada et al. 1996). The ARS sequence was functionally isolated from C. glabrata strain BG2 and corresponds to nucleotides 286060-286210 of chromosome F in the published C. glabrata CBS138 sequence. The HIS3 3’ untranslated region was amplified from C. glabrata strain BG2 using polymerase chain reaction (PCR) and cloned into pGRB2.0 as a XhoI-KpnI fragment, creating pGRB2.1. The S. cerevisiae PGK1 promoter was amplified from S. cerevisiae using PCR and cloned into pGRB2.1 as a SacI-Xhol fragment, creating pGRB2.2 (Domergue et al. 2005). The plasmid pGRB2.3 contains yEGFP3 (Cormack et al. 1997) downstream of the PGK1 promoter, cloned as an EcoRI and Sall fragment.

The basic NAT8 backbone for use in C. glabrata is pBM16 (Ma et al. 2009). The NAT8 cassette was generated by PCR-amplifying ScTEF1p and the NAT1 open reading frame and subcloning them into pRS416 (Sikorski and Hieter 1989); the resulting ScTEF1p-NAT1-ScCYC1 3’ UTR cassette was isolated as a SalI/KpnI fragment, blunt-ended, and subcloned into the Ndel site in the pUC19 backbone, which itself had been blunt-ended. The C. glabrata CEN/ARS sequence was PCR-amplified from pGRB2.0 using primers that contained AatII restriction sites and then subcloned into the unique AatII site in the pUC19 backbone, thus creating pBM16. pBM16.1 is the same as pBM16, except its multiple cloning site (MCS) was replaced with an oligonucleotide containing SacI and KpnI sites, to facilitate cloning of SacI/KpnI fragments from pGRB2.0-derived vectors.

Primers used to amplify each C. glabrata promoter during plasmid construction are listed in Table 3. They contain restriction sites to facilitate cloning; all forward primers contain a SacI site and reverse primers contain SpeI (for LYS21pr) or Xhol sites (all others). The MET3 promoter was amplified from BG2 genomic DNA; all other promoters were amplified from CBS138 genomic DNA. The PCR products were TOP10-cloned (Invitrogen) and sequenced. Empty CEN/ARS URA3-marked vectors (pCU series; Figure 1A) were created by subcloning each promoter using SacI/SpeI (for LYS21) or SacI/Xhol (all others) and ligating them into pGRB2.1 cut with SacI/Xhol. The LYS21 promoter contains an internal Xhol site, so SpeI was used for subcloning; this eliminates Xhol from the MCS of the LYS21 vectors. The NAT-marked versions of the empty vectors (pCN series; Figure 1) were created by cloning each pCU series plasmid with SacI/KpnI to release the promoter-MCS-terminator fragment and ligating this into pBM16.1. These NAT-marked empty vectors are the pCN series of plasmids (Figure 1B). pCU-Aco2 was subcloned to the pBM16.1 vector using a SacI/partial KpnI digestion, because the ACO2 promoter contains a KpnI site.

Each promoter also was subcloned into pGRB2.3 using SacI/Xhol or SacI/SpeI restriction digestions, positioning the promoter upstream of the MCS and GFP open reading frame, creating a GFP reporter plasmid. To construct NAT-marked versions of the GFP reporter strains, the promoter-MCS-GFP-terminator fragment was cut from the pGRB backbone using SacI/Acc65I restriction digestions.
### Table 1. \textit{C. glabrata} strains used in this study

| Strain | Description | Genotype | Parent Strain | Source |
|--------|-------------|----------|---------------|--------|
| **General use** | | | | |
| BG2 | Clinical isolate | Wild-type clinical isolate | NA | Cormack and Falkow 1999 |
| BG14 | Ura\textsuperscript{-} version of BG2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-EGD2 | BG2 | Cormack and Falkow 1999 |
| CBS138 | Clinical isolate | Wild-type clinical isolate (ATCC#2001) | NA | ATCC |
| BG2389 | qPCR control | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, sla2\textsuperscript{A::HygR (URA3, Amp\textsuperscript{R})} | BG14 | S. Pan, unpublished |
| **Constitutive promoters (URA3-marked)** | | | | |
| BG3316 | pCU-EGD2 \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-EGD2 | BG14 | This work |
| BG3317 | pCU-EGD2 \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-EGD2 | BG14 | This work |
| BG2988 | pCU-EGD2-GFP \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-EGD2-GFP | BG14 | This work |
| BG3318 | pCU-HHT2 \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-HHT2 | BG14 | This work |
| BG3319 | pCU-HHT2 \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-HHT2 | BG14 | This work |
| BG2989 | pCU-HHT2-GFP \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-HHT2-GFP | BG14 | This work |
| BG3320 | pCU-PDC1 \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-PDC1 | BG14 | This work |
| BG3321 | pCU-PDC1 \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-PDC1 | BG14 | This work |
| BG2990 | pCU-PDC1-GFP \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-PDC1-GFP | BG14 | This work |
| BG3149 | pCU-PDC1-GFP \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-PDC1-GFP | BG14 | This work |
| **Constitutive promoters (NAT\textsuperscript{R}-marked)** | | | | |
| BG3328 | pCN-EGD2 \#1 | pCN-EGD2 | BG2 | This work |
| BG3329 | pCN-EGD2 \#2 | pCN-EGD2 | BG2 | This work |
| BG3342 | pCN-EGD2-GFP \#1 | pCN-EGD2-GFP | BG2 | This work |
| BG3343 | pCN-EGD2-GFP \#2 | pCN-EGD2-GFP | BG2 | This work |
| BG3330 | pCN-HHT2 \#1 | pCN-HHT2 | BG2 | This work |
| BG3331 | pCN-HHT2 \#2 | pCN-HHT2 | BG2 | This work |
| BG3344 | pCN-HHT2-GFP \#1 | pCN-HHT2-GFP | BG2 | This work |
| BG3345 | pCN-HHT2-GFP \#2 | pCN-HHT2-GFP | BG2 | This work |
| BG3332 | pCN-PDC1 \#1 | pCN-PDC1 | BG2 | This work |
| BG3333 | pCN-PDC1 \#2 | pCN-PDC1 | BG2 | This work |
| BG3346 | pCN-PDC1-GFP \#1 | pCN-PDC1-GFP | BG2 | This work |
| BG3347 | pCN-PDC1-GFP \#2 | pCN-PDC1-GFP | BG2 | This work |
| **Phagocytosis-induced promoters (URA3-marked)** | | | | |
| BG3322 | pCU-ACO2 \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-ACO2 | BG14 | This work |
| BG3323 | pCU-ACO2 \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-ACO2 | BG14 | This work |
| BG2978 | pCU-ACO2-GFP \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-ACO2-GFP | BG14 | This work |
| BG2979 | pCU-ACO2-GFP \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-ACO2-GFP | BG14 | This work |
| BG3324 | pCU-LYS21 \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-LYS21 | BG14 | This work |
| BG3325 | pCU-LYS21 \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-LYS21 | BG14 | This work |
| BG2982 | pCU-LYS21-GFP \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-LYS21-GFP | BG14 | This work |
| BG2983 | pCU-LYS21-GFP \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-LYS21-GFP | BG14 | This work |
| **Phagocytosis-induced promoters (NAT\textsuperscript{R}-marked)** | | | | |
| BG3334 | pCN-ACO2 \#1 | pCN-ACO2 | BG2 | This work |
| BG3335 | pCN-ACO2 \#2 | pCN-ACO2 | BG2 | This work |
| BG3348 | pCN-ACO2-GFP \#1 | pCN-ACO2-GFP | BG2 | This work |
| BG3349 | pCN-ACO2-GFP \#2 | pCN-ACO2-GFP | BG2 | This work |
| BG3336 | pCN-LYS21 \#1 | pCN-LYS21 | BG2 | This work |
| BG3337 | pCN-LYS21 \#2 | pCN-LYS21 | BG2 | This work |
| BG3350 | pCN-LYS21-GFP \#1 | pCN-LYS21-GFP | BG2 | This work |
| BG3351 | pCN-LYS21-GFP \#2 | pCN-LYS21-GFP | BG2 | This work |
| **Nutritionally regulated promoters (URA3-marked)** | | | | |
| BG3326 | pCU-MET3 \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-MET3 | BG14 | This work |
| BG3327 | pCU-MET3 \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-MET3 | BG14 | This work |
| BG2512 | pCU-MET3-GFP \#1 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-MET3-GFP | BG14 | This work |
| BG3340 | pCU-MET3-GFP \#2 | ura\textsuperscript{3A}:Tn903 G418\textsuperscript{R}, pCU-MET3-GFP | BG14 | This work |
| **Nutritionally regulated promoters (NAT\textsuperscript{R}-marked)** | | | | |
| BG3338 | pCN-MET3 \#1 | pCN-MET3 | BG2 | This work |
| BG3339 | pCN-MET3 \#2 | pCN-MET3 | BG2 | This work |
| BG3352 | pCN-MET3-GFP \#1 | pCN-MET3-GFP | BG2 | This work |
| BG3353 | pCN-MET3-GFP \#2 | pCN-MET3-GFP | BG2 | This work |

NA, not applicable; NAT\textsuperscript{R}, nourseothricin-resistant.
Table 2 Plasmids used in this study

| Plasmid Name | Description | Parent Vector | Bacterial Stock | Source | Genbank Accession | Addgene ID |
|--------------|-------------|---------------|----------------|--------|------------------|------------|
| General use  |             |               |                |        |                  |            |
| pGRB2.0      | CEN/ARS plasmid [Ap<sup>R</sup>, URA3] | pRS406 b65  | G. Rotano, unpublished |        | KF040394 45340   |            |
| pGRB2.1      | CEN/ARS plasmid containing HIS3 3' UTR [Ap<sup>R</sup>, URA3] | pGRB2.0 b54  | Frieman et al. 2002 |        | KF040395 45341   |            |
| pGRB2.2      | PGK1pr on CEN/ARS plasmid also containing HIS3 3' UTR [Ap<sup>R</sup>, URA3] | pGRB2.1 b162  | Frieman et al. 2002 |        | KF040396 45342   |            |
| pGRB2.3      | GFP driven by PGK1pr on CEN/ARS plasmid [Ap<sup>R</sup>, URA3] | pGRB2.2 b164  | This work |        | KF040397 45343   |            |
| pBM16        | CEN/ARS plasmid containing NAT<sup>R</sup> cassette [Ap<sup>R</sup>, NAT<sup>R</sup>] | pUC19 b1564  | Ma et al. 2009 |        | KF040398 45344   |            |
| pBM16.1      | CEN/ARS plasmid containing NAT<sup>R</sup> cassette; same backbone as pBM16, with more limited MCS. [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16 b1879  | This work |        | KF040399 45345   |            |
| Constutive promoters |             |               |                |        |                  |            |
| pCU-EGD2     | EGD2pr empty vector [Ap<sup>R</sup>, URA3] | pGRB2.1 b2448  | This work |        | KF040370 45315   |            |
| pCU-EGD2-GFP | EGD2pr-GFP [Ap<sup>R</sup>, URA3] | pGRB2.3 b2037  | This work |        | KF040371 45316   |            |
| pCN-EGD2     | EGD2pr empty vector [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16.1 b2454  | This work |        | KF040372 45317   |            |
| pCN-EGD2-GFP | EGD2pr-GFP [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16 b2236  | This work |        | KF040373 45318   |            |
| pCU-HHT2     | HHT2pr empty vector [Ap<sup>R</sup>, URA3] | pGRB2.1 b2449  | This work |        | KF040374 45319   |            |
| pCU-HHT2-GFP | HHT2pr-GFP [Ap<sup>R</sup>, URA3] | pGRB2.3 b2038  | This work |        | KF040375 45320   |            |
| pCN-HHT2     | HHT2pr empty vector [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16.1 b2455  | This work |        | KF040376 45321   |            |
| pCN-HHT2-GFP | HHT2pr-GFP [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16 b2238  | This work |        | KF040377 45322   |            |
| pCU-PDC1     | PDC1pr empty vector [Ap<sup>R</sup>, URA3] | pGRB2.1 b2450  | This work |        | KF040378 45323   |            |
| pCU-PDC1-GFP | PDC1pr-GFP [Ap<sup>R</sup>, URA3] | pGRB2.3 b2039  | This work |        | KF040379 45324   |            |
| pCN-PDC1     | PDC1pr empty vector [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16.1 b2456  | This work |        | KF040380 45325   |            |
| pCN-PDC1-GFP | PDC1pr-GFP [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16 b2240  | This work |        | KF040381 45326   |            |
| Macrophage-induced promoters |             |               |                |        |                  |            |
| pCU-ACO2     | ACO2pr empty vector [Ap<sup>R</sup>, URA3] | pGRB2.1 b2451  | This work |        | KF040382 45327   |            |
| pCU-ACO2-GFP | ACO2pr-GFP [Ap<sup>R</sup>, URA3] | pGRB2.3 b2230  | This work |        | KF040383 45328   |            |
| pCN-ACO2     | ACO2pr empty vector [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16.1 b2457  | This work |        | KF040384 45329   |            |
| pCN-ACO2-GFP | ACO2pr-GFP [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16 b2235  | This work |        | KF040385 45330   |            |
| pCU-LYS21    | LYS21pr empty vector [Ap<sup>R</sup>, URA3] | pGRB2.1 b2452  | This work |        | KF040386 45331   |            |
| pCU-LYS21-GFP | LYS21pr-GFP [Ap<sup>R</sup>, URA3] | pGRB2.3 b2232  | This work |        | KF040387 45332   |            |
| pCN-LYS21    | LYS21pr empty vector [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16.1 b2458  | This work |        | KF040388 45334   |            |
| pCN-LYS21-GFP | LYS21pr-GFP [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16 b2239  | This work |        | KF040389 45335   |            |
| Nutrionally regulated promoters |             |               |                |        |                  |            |
| pCU-MET3     | MET3pr empty vector [Ap<sup>R</sup>, URA3] | pGRB2.1 b2453  | This work |        | KF040390 45336   |            |
| pCU-MET3-GFP | MET3pr-GFP [Ap<sup>R</sup>, URA3] | pGRB2.3 b1971  | This work |        | KF040391 45337   |            |
| pCN-MET3     | MET3pr empty vector [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16.1 b2459  | This work |        | KF040392 45338   |            |
| pCN-MET3-GFP | MET3pr-GFP [Ap<sup>R</sup>, NAT<sup>R</sup>] | pBM16 b2410  | This work |        | KF040393 45339   |            |

**UTR, untranslated region; NAT<sup>R</sup>, nourseothricin-resistant.**

and ligated into pBM16. pBM16 has E. coli origin of replication and selectable markers, a C. glabrata CEN/ARS, and a NAT resistance (NAT<sup>R</sup>) cassette.

All plasmids have been deposited with corresponding sequences at Addgene. Addgene and GenBank accession numbers are included in Table 2.

**Plasmid copy number**

Total DNA was isolated from C. glabrata strains carrying pCU or pCN series plasmids. Log-phase cultures of C. glabrata strains were pelleted by centrifugation and washed in a solution of 50 mM Tris (pH 8.0), 10 mM EDTA, and 50 mM β-mercaptoethanol. The cells were resuspended in spheroplasting buffer [1.1 M sorbitol, 50 mM Tris (pH 8.0), 10 mM EDTA] supplemented with 1 mM β-mercaptoethanol. Yeast lytic enzyme (#152270; ICN Biomedicals) was added and the cells were incubated at 30°C for 10 min. Spheroplasts were collected by centrifugation and washed twice in spheroplasting buffer. DNA was isolated from the washed spheroplasts using the MasterPure Yeast DNA Purification Kit (#MPY80200; Epicentre). Total DNA was treated with RNase and cleaned by phenol/chloroform extraction and ethanol precipitation. Total DNA was then used in a quantitative PCR (qPCR) reaction to assess relative amounts of plasmid and genomic DNA. Plasmid DNA was monitored by amplifying a portion of the Ap<sup>R</sup> gene (primers 6596 and 6598); genomic DNA was monitored by amplifying a portion of the TUB1 gene (primers 5883 and 6426) (Table 3 shows primer sequences). Average cycle threshold values for experimental samples were compared with a standard curve of genomic DNA. Strain BG2389 has a single copy of the Ap<sup>R</sup> gene integrated into the genome and is expected to have Ap<sup>R</sup> and TUB1 in equal copy number. Quantitative PCR was performed in technical triplicate on each sample with each primer set. The average Ap<sup>R</sup> and
plasmid loss per generation was calculated as:

\[
\text{gen} = \log_2\left(\frac{\text{OD}_{t2}}{\text{OD}_{t1}}\right)
\]

t = 10, using the formula 

The rate of plasmid loss between time points was the sum of the number of times the culture had doubled, and then culture was diluted (OD) of OD600 = 0.02 in YPD (nonselective) media (t = 0) and grown were washed twice in sterile water and diluted to an optical density or YPD+NAT media for pCU or pCN plasmids, respectively. Cultures Yeast strains were grown to mid-log phase under selection in SD-Ura (Ura- or NATS) or having retained the plasmid (any portion of the population that contained the plasmid on the original YPD plates were scored for colonies having lost the plasmid (any portion of the colony is Ura+ or NATR). At each time point, the fraction of the population that contained the plasmid on the original YPD plates was calculated from the selective plates as CFU_{growth}/CFU_{total}. The number of generations between time points was the sum of the number of generations from t = 0 to t = 5, and then from t = 5 to t = 10, using the formula \[\#\text{gen} = \log_2(\text{OD}_{t2}/\text{OD}_{t1})\]. The rate of plasmid loss per generation was calculated as:

\[
1-\left\{\left[\frac{\text{fraction of the population with plasmid at } t = 10h}{\text{fraction of population with plasmid at } t = 0h}\right]\right\}^{(1/\text{generations})}
\]

**Table 3 Primers used in this study**

| Oligo Number | Description | Sequence (5’–3’) |
|--------------|-------------|------------------|
| NAT\(^R\) cassette | ScTEF1p, for | caaggaagtctCACGATTCACACCTACCTCC |
| 2540 | ScTEF1p, rev | cgccggatcccAACCTGATAGATTGC |
| 2541 | NAT1 orf, for | ctatagatcAAATGGCCACACCCTGACAG |
| 2542 | NAT1 orf, rev | acgcgctgactTTAGGGCAGGGCATACTCATG |
| pBM16.1 MCS | MCS, for | aattggagctgcatcaagggtactcctgca |
| | MCS, rev | aggtgacctttgatgctgagctc |
| Constitutive promoters | EGD2p, for | aaaaagagctcTGTCACCTCCTACCCAG |
| 4634 | EGD2p, rev | aaaaaagagctcTGTTATTGATATTATATTG |
| 4636 | HHT2p, for | aaaaaagagctcAGCATTTTATACCGTTTAC |
| 4637 | HHT2p, rev | aaaaaagagctcAGCATTTTATACCGTTTAC |
| 4638 | PDC1p, for | aaaaagagctcAGCATTTTATACCGTTTAC |
| 4639 | PDC1p, rev | aaaaagagctcAGCATTTTATACCGTTTAC |
| Macrophage-induced promoters | ACO2p, for | attagagctcTGTCAGTGTCGCCCTT |
| 5114 | ACO2p, rev | attactagatcCTTTAGATATTATTTG |
| 5115 | LYS2p, for | attagagctcAGAAGAACAGAATATATC |
| 5120 | LYS2p, rev | attaactagtCTTTAATATTCTTTGC |
| 5121 | Nutritional regulated promoters | MET3p, for | ctttagagctcATTACCGTTACATTTATATTTAGTTTAC |
| 4036 | MET3p, rev | gctctgaATTCCGTTAACATTTATTAGTTTAC |
| 4035 | qRT-PCR primers | CCACTAATCTGCTTATC |
| 5980 | GFP, for | ATCCATACCTGCTTATC |
| 5981 | GFP, rev | GGTGATGTGGTAACAAGAGATG |
| 5982 | TUB1, for | GGTGATGTGGTAACAAGAGATG |
| 5883 | TUB1, rev | GGTGATGTGGTAACAAGAGATG |
| 5884 | TUB1, rev 2 | GGTGATGTGGTAACAAGAGATG |
| 4626 | Ap\(^R\) gene, for | AAGCCATACCAAACGACAG |
| 6597 | Ap\(^R\) gene, rev | TTGCCGGAAGCAGTGG |

The DNA sequences of primers used in this article are listed. Capital letters in the DNA sequence hybridize to the target sequence; linkers and restrictions sites added are written in lowercase letters. NAT\(^R\), nourseothricin-resistant; for, forward; rev, reverse; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

**Rate of plasmid loss**

Yeast strains were grown to mid-log phase under selection in SD-Ura or YPD+NAT media for pCU or pCN plasmids, respectively. Cultures were washed twice in sterile water and diluted to an optical density (OD) of OD_{600} = 0.02 in YPD (nonselective) media (t = 0) and grown at 30°C. After 5 hr, an OD_{600} measurement was taken to calculate the number of times the culture had doubled, and then culture was diluted back to OD_{600} = 0.02 to keep it in log phase at 30°C. At t = 10 hr, the OD_{600} of the culture was again measured and cells were plated onto YPD (3 plates/media/strain) at approximately 200 cells/plate. Plates were grown at 30°C for 1–2 days and then were replica-plated onto selective media (SD–Ura or YPD+NAT). After 1 day of growth at 30°C, the selective plates were scored for colonies having lost the plasmid (Ura\(^R\) or NAT\(^R\)) or having retained the plasmid (any portion of the colony is Ura\(^R\) or NAT\(^R\)). At each time point, the fraction of the population which contained the plasmid on the original YPD plates was calculated from the selective plates as CFU_{growth}/CFU_{total}. The number of generations between time points was the sum of the number of generations from t = 0 to t = 5, and then from t = 5 to t = 10, using the formula \[\#\text{gen} = \log_2(\text{OD}_{t2}/\text{OD}_{t1})\]. The rate of plasmid loss per generation was calculated as:

\[
1-\left\{\left[\frac{\text{fraction of the population with plasmid at } t = 10h}{\text{fraction of population with plasmid at } t = 0h}\right]\right\}^{(1/\text{generations})}
\]

**Plasmid integration into genome**

Two C. glabrata yeast strains carrying pCU-PDC1 were grown to saturation in liquid SD–Ura media. Cultures were plated onto 10 SD–Ura plates each, at a density of approximately 100 colonies per plate and grown 2 days at 30°C. These colonies were then replica-plated onto 5-FOA media and grown overnight at 30°C. Each colony on the original SD–Ura plate was monitored to determine if it was sensitive or resistant to 5-FOA. If the pCU-PDC1 plasmid had integrated into the genome, we would have anticipated a 5-FOA\(^R\) phenotype (Castano et al. 2003), whereas cells in which the pCU plasmid is maintained as an episome show a 5-FOA\(^S\) phenotype, reflecting plasmid loss.

**Flow cytometry**

Cells from liquid cultures were washed twice in sterile PBS and resuspended in 0.5 ml PBS. A FACSalibar flow cytometer (BD Biosciences) was used to measure GFP fluorescence. Detector levels were adjusted such that a nonfluorescent strain had a median fluorescence of approximately 10. Populations also were gated based on forward and side scatter of the nonfluorescent strain. For each sample, data for 10⁴ gated cells were collected using CellQuest Pro software (BD Biosciences). Further statistical analysis and graphical representation were performed using FloJo software. Fluorescence was
a placeholder to designate the name of the promoter present in a particular vector. (C) The lengths of the promoters cloned into the pCU and pCN vectors, measured from two independent strains carrying each construct. "Stationary" samples were taken from cultures grown overnight in appropriate media. The overnight cultures were diluted 1:20 and grown at 30°C for 4 hr, to OD600 of approximately 0.3, and used as the "exponential" samples.

**Phagocytosis assays and C. glabrata isolation**

Phagocytosis assays were performed as previously described, with some alterations (Kaur et al. 2007). Murine macrophage-like cell line J774A.1 cells were seeded into 15-cm-diameter tissue culture (TC) dishes (BD353025; BD Bioscience) and grown to confluence in DMEM plus fetal bovine serum (FBS) plus P/S (DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin; 120-095-72; Quality Biologicals). Log-phase C. glabrata cells were added to the J774A.1 cells at a multiplicity of infection of 3 and incubated at 37°C plus 5% CO2 for 1 hr to allow the yeast to settle onto the J774A.1 cells. After incubating for 1 hr, the non-cell-associated yeast were removed by aspiration and washing in DMEM. The J774A.1 and C. glabrata were coincubated for an additional 2 hr. After 2 hr, media was decanted and the macrophages were scraped from the TC dishes and lysed in 10 ml cold DEPC dH2O supplemented with RNaseA (Fermentas EN0531) and RNaseA/T1 (Fermentas EN0551) to digest mammalian RNA. C. glabrata released from the J774A.1 cells were collected by centrifugation (4000 rpm, 4°C, 10 min). The C. glabrata cells were then washed twice in cold DEPC dH2O containing RNase inhibitors (Protect RNA, Sigma R7397) and spun as described previously. Finally, the C. glabrata cells were resuspended in 1 ml DEPC dH2O plus Protect RNA and transferred to 2-ml screw-cap tubes and lysed in 10 ml cold DEPC dH2O supplemented with RNaseA/T1 and RNaseA (Fermentas EN0551) to digest mammalian RNA. C. glabrata released from the J774A.1 cells were collected by centrifugation (4000 rpm, 4°C, 10 min). The supernatant accessible above the glass beads was aspirated and the yeast were frozen in liquid nitrogen and stored at −80°C until RNA isolation commenced.

To control for growth in DMEM and TC conditions, C. glabrata cells also were inoculated into TC dishes with DMEM supplemented with FBS and P/S without J774A.1 and grown for a total of 3 hr at

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**Table 4** Restriction sites found in the multiple cloning sites of the pCU and pCN plasmids

| Promoter | MCS | Other Sites |
|----------|-----|-------------|
| **pCU series** | | |
| pCU | EGD2 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | HHT2 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | PDC1 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | ACO2 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | LYS21 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | MET3 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| **pCN series** | | |
| pCN | EGD2 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | HHT2 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | PDC1 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | ACO2 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | LYS21 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |
| | MET3 | XbaI | SpeI | BamHI | Smal | PstI | EcoRI | EcoRV | HindIII | Clal | Sall | Xhol | Sacl | KpnI |

Bold lettering indicates the sequence is unique in that particular plasmid. Sites listed under the MCS heading are found in the multiple cloning site of the plasmid; Sall and KpnI are located at the beginning of the promoter and end of the His3 terminator, respectively. MCS, multiple cloning site.
37°C plus 5% CO₂. The contents of the dish were transferred to a 50-ml conical tube and centrifuged (4000 rpm, 4°C, 10 min). The pelleted yeast were washed in DEPC-dH₂O supplemented with Protect RNA, transferred to a screw-cap tube, and pelleted with glass beads as described.

Quantitative reverse-transcription PCR to monitor promoter strength
RNA was isolated from *C. glabrata* (phagocytosed or media-grown controls) using a guanidine thiocyanate mixture (4 M guanidine thiocyanate, 2% β-ME) lysis and acid phenol extraction; 0.4 ml guanidine thiocyanate mixture was added to each frozen pellet and cells were disrupted using a Fast-Prep (Bio101 Thermo Savant). Lysates were transferred to a new nuclease-free tube and RNA was recovered by performing two acid phenol/chloroform extractions. The extracted RNA was then precipitated and resuspended in DEPC-dH₂O. The RNA was treated with DNase (NEB B0303S) to remove any residual genomic DNA, and then the acid phenol extraction and ethanol precipitation were performed again. Reverse-transcription of purified RNA was performed linearly with Superscript III (Invitrogen). Quantitative PCR was performed in triplicate, monitoring EvaGreen fluorescence in a BioRad Thermocycler. Relative GFP expression for each strain is calculated as the average GFP signal normalized to the average TUB1 values (primers 5883 and 5884). Figure 4 shows the relative expression for each strain, averaged across two biological replicates of the macrophage infection.

Phagocytosis assays and microscopic imaging
*C. glabrata* strains carrying pCU-ACO2-GFP or pCU-LYS21-GFP plasmids were grown to OD of 7.8–9.4 in SD-Ura media. The cells were washed in PBS and resuspended at a density of 4×10⁶ cells/ml in DMEM supplemented with FBS and P/S. J774A.1 cells were seeded

Figure 2: Fluorescence of exponential phase *C. glabrata* strains carrying pCU series plasmids. Flow cytometry was used to measure fluorescence levels in *C. glabrata* strains carrying pCU series plasmids containing EGD2 (A), HHT2 (B), PDC1 (C), ACO2 (D), and LYS21 (E) promoters. The strains were diluted from a saturated overnight culture and grown to OD₆₀₀ of approximately 0.3 before fluorescence was measured. Each panel depicts histograms of fluorescence for two *C. glabrata* strains containing the empty vectors (red and blue lines) and two *C. glabrata* strains containing GFP reporter vectors (green and orange lines) for a given promoter. The median fluorescence value for each strain population is shown.
into 24-well tissue culture plates (Beckman Dickinson 353047) with or without sterile glass coverslips in each well. Once the J774A.1 had grown to confluency, they were washed with fresh DMEM, which was then aspirated from the wells. Each well was then filled with 100 μl yeast cells in DMEM supplemented with FBS and P/S, which is effectively a multiplicity of infection of 1 (estimating 4×10⁵ macrophages/well). After a 1-hr incubation at 37°C plus 5% CO₂, during which the yeast settled onto the macrophage and were engulfed, the media and unbound yeast were aspirated from the wells. The J774A.1 cells were washed twice with DMEM to remove any unbound yeast and 500 μl DMEM supplemented with FBS and P/S was added to each well. The yeast and J774A.1 cells were coincubated for an additional 2 hr. To stop the infection, the media was aspirated from the wells with coverslips and the J774A.1 and C. glabrata were fixed in a 1% formaldehyde solution for 5 min. The coverslips were then washed in PBS and mounted onto glass slides with Vectashield. In parallel, 100 μl yeast cells were seeded into 24-well plates without J774A.1 cells to control for growth conditions and were allowed to grow for a total of 3 hr. These yeast were pelleted in microfuge tubes, media was aspirated, and the cells were resuspended and fixed in 1% formaldehyde solution for 5 min. The fixed unphagocytosed yeast were mounted onto glass slides without Vectashield. All samples were imaged on a Zeiss Axioskop microscope at 100x objective and bright field differential image contrast and fluorescent images were captured.

RESULTS

Plasmid design and construction

The plasmids described here were designed as tools for molecular genetic experiments in C. glabrata. Promoters of six genes were chosen and cloned into URA3-marked or NAT5-marked episomal plasmids. In addition to making “empty” vectors suitable for cloning any desired gene under the control of each promoter, reporter constructs were created where GFP was cloned into each set of plasmids so that the relative strength of each promoter could be measured by monitoring GFP abundance.

Three “constitutive” promoters were chosen from unpublished data analyzing transcriptional profiles of C. glabrata cells growing in

Figure 3 Fluorescence of stationary phase C. glabrata strains carrying pCU series plasmids. Flow cytometry was used to measure fluorescence levels in C. glabrata strains carrying pCU series plasmids containing EGD2 (A), HHT2 (B), PDC1 (C), ACO2 (D), and LYS21 (E) promoters. Fluorescence of saturated overnight cultures was measured. Each panel depicts histograms of fluorescence for two C. glabrata strains containing the empty vectors (red and blue lines) and two C. glabrata strains containing GFP reporter vectors (green and orange lines) for a given promoter. The median fluorescence value for each strain population is shown.
ARS and a NATR cassette for selection and maintenance in E. coli grown in laboratory media (B. Green, unpublished data). From this starvation, we selected genes that were not strongly induced in the stationary phase, as compared with the exponential phase, when grown in laboratory media (B. Green, unpublished data). From this analysis, we chose AC02 (CAGL002431g) and LYS21 (CAGL009240g) as phagocytosis-induced genes. Finally, the MET3 (CAGL003833g) promoter was developed as a regulatable promoter based on similar constructs used in S. cerevisiae and Candida albicans (Stark 1998; Care et al. 1999).

For most genes, we used PCR amplification to isolate and clone the promoter (the complete intergenic region upstream of the designated gene). For the MET3 promoter, we amplified 1026 bp immediately upstream of the MET3 open reading frame. The length of each cloned promoter is indicated in Figure 1C.

Ultimately, each promoter was cloned into four plasmid backbones for use in C. glabrata. The backbones contain a URA3 auxotrophic marker or a dominant NATR cassette, as well as a multiple cloning site or GFP downstream of the promoters. Plasmids containing the C. glabrata CEN/ARS and the URA3 auxotrophic marker are designated as the pCU series (Figure 1A). They are derived from the pGEM plasmid backbone, which contains a S. cerevisiae URA3 cassette to serve as an auxotrophic marker and functions for selection in C. glabrata. The pCU plasmids also contain an ApR gene and F1, M13, and pMB1 (ColE1 family) origins for growth and selection in E. coli. The pCN series of plasmids have the C. glabrata CEN/ARS and a NATR cassette for selection and maintenance in C. glabrata (Figure 1B). The pCN plasmids are derived from pBM16.1, which has an ApR gene and a pMB1 (ColE1 family) origin for growth and selection in E. coli. Plasmid names and descriptions are indicated in Table 3.

The “empty” versions of each pCU and pCN series plasmid contain an MCS immediately downstream of the promoter, followed by a HIS3 terminator. These vectors are suitable for further subcloning, placing any gene of interest under the control of the given promoter. Table 4 lists the restriction enzyme recognition sites found in the MCS for each plasmid and indicates which sites are unique for each construct. Additionally, we made versions of each plasmid that have GFP placed under the control of each promoter. These reporter plasmids were used to assess the strength of each promoter through monitoring GFP expression levels.

**Plasmid maintenance**

Three aspects of these plasmids were monitored to characterize plasmid maintenance in C. glabrata: the copy number, the rate of plasmid loss, and the rate at which the plasmids integrate into the genome. Copy number was measured using qPCR to amplify the ApR gene (from the plasmid backbone) and TUB1 (from the genome) from total DNA isolated from select yeast strains. C. glabrata strains carrying pCU-EGD2, pCN-EGD2, pCU-ACO2, or pCN-ACO2 were chosen because these constructs contain the shortest (EGD2) and longest (ACO2) promoters in both backbones. As shown in Table S2, the pCU vectors are present at approximately 2 copies per cell. The pCN vectors are present at 0.6 to 0.8 copies per cell.

Plasmid stability was measured by growing four C. glabrata strains carrying pCU-PDC1 or pCN-PDC1 (2 strains per construct) non-selectively for 10 hr and determining how many cells retain the plasmid by plating on selective media. We estimate pCU-PDC1 plasmids were lost at a rate of 4.6% per generation, and pCN-PDC1 plasmids were lost at a rate of 6.53% per generation (Table S3).

Finally, we wanted to ensure that the pCU and pCN plasmids do not integrate into the genome. Two C. glabrata strains carrying pCU-PDC1 were grown selectively in SD–Ura media and then plated to SD–Ura. The SD–Ura plates were replica-plated to plates containing 5-FOA to counterselect any colonies that contained the URA3 gene. Any 5-FOA-resistant colonies would represent cells that had integrated the URA3 gene (and presumably the entire plasmid) into the genome. Conversely, 5-FOA-resistant cells represent those with URA3 on an episomal plasmid, which may be lost and permit growth on 5-FOA. No 5-FOA-resistant colonies were detected in either strain tested, suggesting rates of integration into the genome are <0.04% (Table S4).

**Expression from C. glabrata promoters**

The relative strength of each promoter was analyzed by monitoring GFP expression in C. glabrata strains carrying each plasmid construct. Fluorescence in strains carrying the pCU series of plasmids growing exponentially in SD–Ura was measured by flow cytometry (Figure 2). For each promoter construct, two C. glabrata strains carrying the empty vector and two strains carrying the GFP reporter plasmid were tested. Comparing the median fluorescence for the populations in Figure 2, A–C, the flow cytometry indicates expression from the pCU-EGD2-GFP is the lowest (median = 20), followed by HHT2 (median = 89), and strains carrying pCU-PDC1-GFP had the highest level of fluorescence (median = 202). This relationship of increasing strength of promoters (EGD2 < HHT2 < PDC1) is also true in stationary cultures, but expression from all strains is lower than what is observed in the corresponding log-phase cultures (Figure 3). In Figure 2, the histograms for strains carrying pCU-HHT2-GFP and pCU-PDC1-GFP have shoulders, possibly reflecting cells that have lost the plasmid or alternatively were delayed in exiting stationary phase.

**Figure 4** Quantitative reverse-transcription PCR (qRT-PCR) results of GFP expression during phagocytosis. qRT-PCR assessment of relative expression of GFP (normalized to TUB1) in C. glabrata grown in media or after phagocytosis by J774A.1 macrophage-like cells. The data are averages of two biological replicates (qPCR performed in triplicate) for each strain; error bars indicate the SD between the averages. The numbers above the bars indicate the average fold-change in GFP expression (normalized to TUB1) in C. glabrata that have been phagocytosed by J774A.1 (macrophage) vs. growth in tissue culture (TC) media (media). Data are shown for one C. glabrata strain carrying pCU-ACO2-GFP and two independent strains carrying pCU-lys21-GFP.
The expression driven by the ACO2 and LYS21 phagocytosis-induced promoters also was assessed in standard laboratory media to assess the basal levels of expression from the ACO2 and LYS21 promoters during laboratory culture. To this end, C. glabrata strains carrying the pCU-ACO2-GFP and pCU-LYS21-GFP plasmids were grown in SD2Ura media and fluorescence was assessed using flow cytometry (Figure 2, D and E). As seen in Figure 2D, the ACO2 promoter has extremely low basal expression in exponentially growing C. glabrata cells in SD2Ura media. The LYS21 promoter has low basal expression in laboratory media, on par with the levels seen using the constitutive promoter EGD2 (Figure 2, E and A).

Similar results were seen for C. glabrata strains carrying the pCN series of plasmids when grown in YPD+NAT media (data not shown).

**Expression after phagocytosis**

The C. glabrata strains carrying plasmids with the phagocytosis-induced ACO2 and LYS21 promoters were used to infect J774A.1 cells and expression from the promoters was assessed by measuring GFP transcript levels using qRT-PCR. C. glabrata cells were isolated from J774A.1 cells after 2 hr of infection and GFP expression was measured (as normalized to TUB1). To control for growth in tissue culture media and conditions, C. glabrata also were grown in DMEM supplemented with FBS and P/S for an equivalent amount of time and GFP expression was monitored. As seen in Figure 4, expression of GFP from the pCU-ACO2-GFP or pCU-LYS21-GFP plasmids increased markedly in C. glabrata strains that had been phagocytosed by J774A.1 macrophage-like cells. Increased GFP expression also can be observed by fluorescence microscopy of phagocytosed C. glabrata cells (Figure 5).

**Nutritional regulation of the MET3 promoter in C. glabrata**

The MET3 promoter has been used successfully as a regulated promoter in C. albicans, and thus the plasmids pCU-MET3 and pCN-MET3 were constructed to allow for control of target gene expression in C. glabrata based on the presence or absence of Met and Cys in the media. The control of the MET3 promoter in C. glabrata was monitored by flow cytometry on strains carrying the pCU-MET3 plasmid. In media containing excess Met and Cys (2 mM each), expression is repressed and the fluorescence profiles of pCU-MET3-GFP strains closely match the fluorescence profiles of strains carrying the pCU-MET3 empty vectors (Figure 6, A and B, “OFF”). In media lacking Met and Cys, the expression of GFP was greatly increased in strains carrying the pCU-MET3-GFP plasmids (Figure 6, “ON”). This tight regulation is seen in both log- and stationary-phase cultures of strains carrying pCU-MET3-GFP (compare Figure 6A and 6B). Similar results were seen for strains carrying the pCN-MET-GFP plasmids when grown in SED+Met+Cys2Ura or SED2Met2Cys2Ura media (data not shown).

**DISCUSSION**

We chose to make a set of “constitutive” plasmids to facilitate cloning and expression of genes in C. glabrata. As evidenced by the
flow cytometry data monitoring GFP expression in *C. glabrata* strains (Figure 2), the constitutive promoters are of three different strengths, with the rank order being *EGD2pr*< *HHT2pr*< *PDC1pr*.

We also designed versions of our centromeric plasmids with the *MET3* promoter. As in *C. albicans*, this promoter was tightly regulated by the presence or absence of methionine and cysteine in the media (Figure 6). In particular, cells growing exponentially in media supplemented with Met and Cys to repress the *MET3* promoter appear to have shut-off all expression; the curves of the *pCU-MET3-GFP* curves are super-imposed on empty vector controls in the far left panel of Figure 6. We note that repression of the *MET3* promoter seems a bit leakier in stationary phase cells. Vectors with the *MET3*pr will allow for tightly controlled expression of a target gene and may be appropriate to use for conditional control of essential genes.

Vectors also were constructed with the phagocytosis-induced promoters *ACO2* and *LYS21*. Expression from these promoters is low when cells are grown in laboratory media (Figure 2, Figure 3, Figure 4). However, 2 hr after engulfment within J774.A1 cultured macrophage cells, expression from the promoters increased. This increase expression is observed with both qRT-PCR and fluorescence microscopy (Figure 4 and Figure 5), and we estimate a 24-fold to 59-fold increase of expression from these promoters after phagocytosis (Figure 4). Increased expression from the *ACO2pr* and *LYS21pr* after phagocytosis likely reflects a response to carbon and amino acid limitation in the phagolysosome (Lorenz et al. 2004; Kaur et al. 2007). The leaky expression from the *LYS21pr* in stationary phase laboratory media (Figure 3, panel E) may reflect nutrient depletion in a saturated culture. Vectors containing *ACO2pr* or *LYS21pr* may prove useful to express a target gene in yeast specifically within the phagolysosome. Potentially, expression from the *ACO2-GFP* or *LYS21-GFP* vectors may serve as indirect indicators of phagocytosis. In the future, it may be possible to develop other plasmids that allow for variable expression within the macrophage or are specific to particular intra-cellular environments.

The “constitutive” promoters can be used to express proteins at different levels in standard laboratory media in log phase and stationary phase. We noticed that expression from the “constitutive” *PDC1* promoter decreased after phagocytosis by *J774A.1* macrophage-like cells (data not shown). Although this is expected based on the transcriptional microarray data comparing phagocytosed and unphagocytosed *C. glabrata* (Kaur et al. 2007), it serves to emphasize that expression from the three “constitutive” promoters described in this work should be tested empirically when cells are grown under conditions other than the standard laboratory conditions monitored here.

The plasmids are stably maintained episomally, with no detectable integration into the genome and approximately 5–8% plasmid loss/generation as measured by a plating assay. The *pCU* vectors maintained at approximately 2 copies per cell; this is comparable with the plasmid copy numbers measured in haploid *S. cerevisiae* cells (Karim et al. 2013). The *pCN* vectors were measured at 0.6–0.8 copies per cell, suggesting that a portion of the yeast cells grown under selection might have lost the *pCN* plasmid. This may reflect the ability of cells that have lost *pCN* plasmids to survive and replicate a small number of generations before dying. This is consistent with flow cytometry experiments measuring GFP expression showing that strains carrying *pCN-XXX-GFP* vectors have a fraction of the population that do not express GFP (data not shown). We conclude that though NAT selection allows overall maintenance of the plasmid, a small fraction of the cells in the culture no longer contain a plasmid, likely resulting from growth of cells that have lost the plasmid for a small number of divisions.

The standardized structure of the plasmids allow for easy modification. A gene of interest cloned into the MCS of one vector can be easily moved to another vector, which could allow researchers to adjust the strength of expression (by choosing a different promoter) or the selectable marker (by choosing the *pCU* or *pCN* backbones). For ease, the characteristics of the promoters described in this article are summarized in Table S5. Additionally, the plasmids can be customized by introducing any promoter of choice into either the *pCU* or the *pCN* backbone, using a *SacI*-XbaI restriction digest. This flexibility should allow investigators to introduce their favorite gene under control of its native promoter. The set of expression vectors described here can be used in many applications in *C. glabrata* and will expand the cloning tools available to the community.

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