DOI: 10.1534/genetics.110.120824

Novel Acid Phosphatase in *Candida glabrata* Suggests Selective Pressure and Niche Specialization in the Phosphate Signal Transduction Pathway

Brianne R. Orkwis,* Danielle L. Davies,* Christine L. Kerwin,* Dominique Sanglard† and Dennis D. Wykoff*,[1]

**Department of Biology, Villanova University, Villanova, Pennsylvania 19085 and †Institute of Microbiology, University of Lausanne and University Hospital Center, CH-1011 Lausanne, Switzerland*

Manuscript received July 12, 2010
Accepted for publication August 15, 2010

ABSTRACT

Evolution through natural selection suggests unnecessary genes are lost. We observed that the yeast *Candida glabrata* lost the gene encoding a phosphate-repressible acid phosphatase (*PHO5*) present in many yeasts including *Saccharomyces cerevisiae*. However, *C. glabrata* still had phosphate starvation-inducible phosphatase activity. Screening a *C. glabrata* genomic library, we identified *CgPMU2*, a member of a three-gene family that contains a phosphomutase-like domain. This small-scale gene duplication event could allow for sub- or neofunctionalization. On the basis of phylogenetic and biochemical characterizations, *CgPMU2* has neofunctionalized to become a broad range, phosphate starvation-regulated acid phosphatase, which functionally replaces *PHO5* in this pathogenic yeast. We determined that *Cg*Pmu2, unlike *Sc*Pho5, is not able to hydrolyze phytic acid (inositol hexakisphosphate). Phytic acid is present in fruits and seeds where *S. cerevisiae* grows, but is not abundant in mammalian tissues where *C. glabrata* grows. We demonstrated that *C. glabrata* is limited from an environment where phytic acid is the only source of phosphate. Our work suggests that during evolutionary time, the selection for the ancestral *PHO5* was lost and that *C. glabrata* neofunctionalized a weak phosphatase to replace *PHO5*. Convergent evolution of a phosphate starvation-inducible acid phosphatase in *C. glabrata* relative to most yeast species provides an example of how small changes in signal transduction pathways can mediate genetic isolation and uncovers a potential speciation gene.

THE phosphate signal transduction (PHO) pathway in *Candida glabrata* is similar to the PHO pathway in *Saccharomyces cerevisiae* and serves as a model to examine species-specific changes to a signal transduction pathway (Kerwin and Wykoff 2009). *C. glabrata* is a mammalian pathogen that can cause candidiasis and is relatively closely related to budding yeast, *S. cerevisiae* (Redondo-Lopez *et al.* 1990; Cormack and Falkow 1999; Domergue *et al.* 2005). Thus, identifying differences in the PHO pathway between the two species may shed light on their environmental niche. In both species, the transcription factor Pho4 is critical to the phosphate starvation response, and it appears to be regulated similarly in both species—through phosphorylation by the Pho80/Pho85/Pho81 complex (Schneider *et al.* 1994; O'Neill *et al.* 1996; Kerwin and Wykoff 2009). However, another transcription factor, Pho2, which is essential for the starvation response in budding yeast, appears to be unimportant in *C. glabrata* (Barbaric *et al.* 1998; Springer *et al.* 2003; Kerwin and Wykoff 2009).

Activation of the PHO pathway in *S. cerevisiae* often is measured by the transcription of *PHO5*, which encodes a phosphate starvation-inducible acid phosphatase (Huang and O'Shea 2005). Assays can quantitatively or qualitatively measure the extent of Pho5 activity and serve as a proxy for Pho4 activity. In studying *C. glabrata*, we observe strong acid phosphatase activity that is regulated by Pho4; however, the sequenced genome does not appear to contain a *PHO5* homolog (Kerwin and Wykoff 2009). Through complementation of a *Scpho5*Δ strain with a *C. glabrata* genomic library (Sanglard *et al.* 1999), we identified a three-gene cluster (which we have named *CgPMU1*, *CgPMU2*, and *CgPMU3*) that contains the functional analog of *PHO5*. This cluster, present in only *C. glabrata* and in none of the other sequenced yeast genomes, provides an interesting model system to understand how gene duplication impacts the species-specific features of the PHO pathway in *C. glabrata*.

Gene duplication is a major evolutionary force through which new functions can appear (He and Zhang 2005; Conant and Wolfe 2008). Duplication

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.110.120824/DC1.

[1]*Corresponding author:* Department of Biology, Villanova University, 800 Lancaster Ave., Villanova, PA 19085.
E-mail: dennis.wykoff@villanova.edu

of genes can allow one gene to maintain its ancestral function while the duplicate is relieved from stringent selection (Zhang 2003; Beisswanger and Stephan 2008). The duplicated gene can be lost through pseudogenization, can partition functions between the paralogs (subfunctionalize), or can acquire new, but often related functions through neofunctionalization (Conant and Wolfe 2008). A new function can generate a species-specific feature that might isolate one species from another (Ting *et al.* 2004). Generation of a new function in one pathway is unlikely to be sufficient for genetic isolation; however, many small changes to signal transduction pathways through duplication and drift or selection may reinforce isolated genetic populations and drive speciation.

We studied the function of three homologs of the *ScPMU1* gene in *C. glabrata* to establish which paralog is regulated by phosphate starvation and Pho4. Upon identifying *CgPMU2* as the *ScPHO5* analog in *C. glabrata*, we determined that *CgPMU2* has neofunctionalized to hydrolyze phosphate from organic phosphate sources that *CgPMU1* and *CgPMU3* are unable to hydrolyze. Finally, we determined that loss of *PHO5* and its replacement with the analog *PMU2* has restricted *C. glabrata* from an environmental niche that contains phytic acid as the sole phosphate source.

MATERIALS AND METHODS

Strains and media: Yeast strains (Table 1) were grown in synthetic dextrose (SD) media with complete supplement mixture (CSM) (Sunrise Science Products, San Diego, CA) with or without histidine. For all experiments, cells were grown at 30° until logarithmic growth phase (OD_{600} ~0.5). For phosphate starvation experiments, cells grown to logarithmic phase were harvested by centrifugation, washed three times in media without phosphate, and then transferred to media without phosphate (no-phosphate conditions) or to media with 10 mm KH_2PO_4 added (high-phosphate conditions) and grown at 30° for 3 hr (Kerwin and Wykoff 2009).

C. glabrata mutants were generated using the antibiotic resistance genes *KANMX6* or *NATMX6* (Kerwin and Wykoff 2009) and homologous recombination to inactivate *PMU1*, *PMU2*, or *PMU3* in a *C. glabrata his3*$^-$ background strain (Cormack and Falkow 1999; Hentges *et al.* 2005; Kerwin and Wykoff 2009). Refer to supporting information, Table S1 for a list of primers used to inactivate genes. Deletion of genes was confirmed by PCR and a phosphatase plate assay of multiple isolates.

Identification of *PMU2* genomic clone: To screen for the *C. glabrata* phosphatase gene, we transformed by electroporation a *C. glabrata* genomic DNA library (Sanglard *et al.* 1999) into a *Scpho5*Δ strain. Cells were plated on SD−Ura plates, at a density of ~1000 colonies per plate. Approximately 38,000 transformants were replica plated onto SD −Ura plates lacking phosphate. After ~16 hr, the colonies were assayed for the presence of a secreted acid phosphatase using the semiquantitative phosphatase assay described previously (Wykoff and O'Shea 2001). Red colonies were reassayed in high-phosphate and no-phosphate conditions for phosphatase activity. From yeast genomic DNA preparations of the selected colonies, plasmids were isolated by chemical transformation of *Escherichia coli* cells, and ~500 nucleotides were sequenced on each side of the plasmid insert.

Assays for phosphatase activity: For the semiquantitative assay, agar plates were overlaid with Fast Blue Salt B stain, 1-naphthyl phosphate (1-NP), and 0.1 m sodium acetate (pH 4.2) (Wykoff *et al.* 2007). For quantitative measurements of hydrolysis of *p*-nitrophenylphosphate phosphate (PNPP), 1 ml of cells (OD_{600} ~0.5) was pelleted by centrifugation and resuspended in sterile water. Because PNPP does not cross the plasma membrane and <5% of total phosphatase activity can be washed away from the cells (data not shown), we conclude that we are measuring periplasmic phosphatase activity. Cells were incubated with 10 mm PNPP at pH 4.2 at 25° for 10 min or 20 min. The reaction was quenched with saturated Na_2CO_3. Phosphatase activity was measured in units expressed as OD_{400}/OD_{600} (Huang and O'Shea 2005).

Quantitative reverse-transcription PCR: RNA was extracted using a phenol–chloroform protocol (Huang and O'Shea 2005) and converted to cDNA by a reverse-transcription reaction (Bio-Rad iScript cDNA synthesis kit). Quantitative PCR was performed with a Chromo-4 PCR machine (Bio-Rad) using SyberGreen I dye (Sigma-Aldrich, St. Louis, MO) with a 50-μl PCR reaction (Kerwin and Wykoff 2009). Primers (Table S1) were designed for *C. glabrata PMU1*, *PMU2*, and *PMU3* and for *S. cerevisiae PMU1* and *ACT1*, and data were normalized to expression of *ACT1* (Kerwin and Wykoff 2009). Each gene was equally amplified with the designed primers using 10-fold genomic DNA dilutions.

Bacterial expression and purification of Pmu1, Pmu2, and Pmu3: Using primers O218–O223 (Table S1), *PMU1*, *PMU2*, and *PMU3* were amplified with PCR, digested with *Bam*HI and/or *Xho*I and ligated into pET16b vector. Sequence confirmed clones were transformed into C3013H *E. coli* cells (New England Biolabs). Pooled transformants were grown at 30° and induced with 1mm IPTG for 2 hr. Cells were lysed by sonication in 10% glycerol, 50 mm Tris (pH 8), 250 mm NaCl, 0.1% NP-40 (or Tween 20), 10 mm imidazole (pH 8), 1 mm 2-mercaptoethanol, and protease inhibitors (G-Biosciences, St. Louis, MO). IDA resin (Sigma-Aldrich) was charged with cobalt chloride, loaded with cell extract, and washed with the same buffer until no protein was detected in the flow through. The tagged proteins were eluted with 20 mm EDTA and dialyzed overnight in a 500-fold volume lysis buffer. Because additional bands were observed in each extract along with the His_{10}-tagged protein, densitometry was used to estimate the purity of the extract. When assaying against various phosphate substrates, this percentage was multiplied by the total concentration of protein in the extract (determined by measuring the absorbance at 280 nm) to calculate the concentration of pET16b-tagged protein in each reaction.

Detection of phosphatase activity—phosphate released: To ascertain the optimum pH of each enzyme, the His_{10}-purified proteins were incubated with solutions ranging in pH from 3.4 to 8.0, all at 0.1 m final osmolarity. Solutions were made from combining different amounts of 100 mm acetate and 100 mm Tris. Final pH readings were measured using a pH meter. To determine the specificity of each enzyme, the His_{10}-purified proteins were incubated with various phosphate-containing substrates. Using twofold dilutions, the kinetics of each reaction were observed. The substrates glycerol-1 phosphate and glycerol-2-phosphate were tested at concentrations ranging from 100 mm to 0.78 mm. Inosine-5′-monophosphate was tested at concentrations ranging from 25 mm to 0.195 mm, trehalose-6-phosphate from concentrations ranging from 10 mm to 0.078 mm, and PNPP from concentrations ranging from 14.85 mm to 0.116 mm. Reactions were performed with and without enzyme, using the optimum pH buffer for each enzyme and differing concentrations of phosphate substrate.

TABLE 1

Strains used in this study

Strain	Genotype	Reference
	C. glabrata	
BG99	*his3*Δ (1 + 631)	Cormack and Falkow (1999)
DG2	*pho4*Δ*::KANMX6* in BG99	Kerwin and Wykoff (2009)
DG66	*pmu1*Δ*::KANMX6* in BG99	This study
DG29	*pmu2*Δ*::NATMX6* in BG99	This study
DG30	*pmu3*Δ*::NATMX6* in BG99	This study
DG87	*pmu3*Δ*::NATMX6* in BG99	This study
	S. cerevisiae	
EY57	K699 with *MATa*	Wykoff and O'Shea (2001)
EY132	*pho5::TRP1*	Wykoff *et al.* (2007)

The genotype of K699 is *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3.*

The reactions were terminated by heating at 99° for 5 min. The amount of phosphate released was quantified by incubating the reactions with 300 μl Brilliant Green phosphate dye in a final volume of 1 ml at 30° for 30 min (Chen *et al.* 1956). Phosphate released was measured in units of OD_{639}, and reactions with and without enzyme were compared. Data were normalized using a standard curve with known amounts of inorganic phosphate (KH_2PO_4). V_{max} and K_M values were calculated using either Hanes–Woolf or Lineweaver–Burk plots. All calculations were normalized by subtracting a sample with non-His_{10}–tagged protein added. When comparing substrate specificity, we used the same purification batches to eliminate any error in the estimation of protein concentration, which could impact V_{max} measurements. This prevented us from performing three replicates of all of the assays. A linear regression with at least four points ($R^2 > 0.8$) was required for a determination of V_{max} and K_M values, and if we did not observe consistent Michaelis–Menton kinetics then the measurement was considered not detected (ND). We were unable to measure 1-naphthyl phosphatase activity because of interfering levels of inorganic phosphate.

For measuring phytase activity of whole cells, cells were grown overnight in SD + CSM + 10 mm KH_2PO_4, washed three times with no-phosphate media, and starved in no-phosphate media for ~6 hr. Two independent cultures of *S. cerevisiae* wild type, *S. cerevisiae pho5*Δ, and *C. glabrata* wild type were assayed for phytase activity. Aliquots were thawed at 4° with lysis buffer (see above). The cell density was measured by OD_{600} and normalized on the basis of the values that 1 OD of *S. cerevisiae* is equivalent to 5×10^7 cells/ml/OD and that 1 -OD of *C. glabrata* is equivalent to 2.5×10^8 cells/ml/OD. Approximately, the same optical density of cells (5 μl) was added to 100 μl of 0.125 mm potassium phytate pH 3.4 and incubated for 10 min. The concentration of phytate was chosen to minimize the amount of contaminating inorganic phosphate. After 10 min, the cells were centrifuged away from the reaction, and 80 μl of the clarified reaction was boiled at 99° for 5 min. To detect the amount of inorganic phosphate liberated from phytate, 40 μl of the reactions was incubated with Brilliant Green phosphate dye (protocol described above). It is worth noting that whether this reaction is normalized to optical density or to cell density, the results have similar trends.

Generation of regulated expression of the *PMU* genes in a *Cgpmu2*Δ strain: Using primers to place a gene under the control of the *ScPHO5* promoter (Table S1), *ScPMU1*, *CgPMU1*, *CgPMU2*, and *CgPMU3* were amplified using PCR. The template for these PCR reactions was either the pET16b plasmid containing a *PMU* gene or *S. cerevisiae* genomic DNA. A vector (DB146) containing 1000 bp of the *ScPHO5* promoter and YFP separated by a *Pac*I site was digested, combined with each PCR product, and transformed into the *pmu2*Δ strain. When translationally fused to YFP, *Cg*Pmu2 was not functional; however, inclusion of a stop codon at the end of *CgPMU2* allowed for full complementation of the *Cgpmu2*Δ strain. All four constructs included a stop codon, were rescued and subjected to diagnostic digests and PCR to confirm their identity, and retransformed into the *pmu2*Δ strain. We also generated plasmids using the same scheme as above with the *ScADH1* promoter and the *CgPMU2* promoter using primers described in Table S1. The *CgPMU2* promoter contained ~1040 bp upstream of the *CgPMU2* start codon and did not include any portion of the *CgPMU1* ORF. Genomic clones of each *PMU* gene were generated with primers indicated in Table S1 and cloned by homologous recombination into pRS313.

RESULTS

Identification of *PMU* region in *C. glabrata* as containing inducible phosphatase: Whereas the *C. glabrata* genome does not contain a homolog of the *ScPHO5* gene, *C. glabrata* exhibits phosphatase activity during phosphate starvation conditions (Figure 1A), suggesting the presence of a cryptic gene in *C. glabrata* that encodes a phosphate starvation-inducible acid phosphatase. To identify this gene, we transformed a *C. glabrata* genomic DNA library (Sanglard *et al.* 1999) into a *Scpho5*Δ strain. We hypothesized that the library contained genomic clones capable of restoring phosphate starvation-inducible phosphatase activity and some clones would functionally complement the *Scpho5*Δ strain. Five colonies out of ~38,000 colonies exhibited significant phosphatase activity during phosphate starvation and no significant activity in high-phosphate conditions (Figure 1A). When the ends of the genomic clones were sequenced, all 5 contained genes within an ~13-kb region on chromosome 11 in *C. glabrata* (Figure 1B). This region contains three paralogs of *PMU1*, a gene that in *S. cerevisiae* encodes a phosphomutase-like protein (Elliott *et al.* 1996; Rebora *et al.* 2005; Byrne and Wolfe 2006). Because phosphomutase binds to phosphoglycerate and isomerizes carbon phosphate bonds, it seemed plausible that these proteins might bind to and hydrolyze organic phosphate compounds. We named these three paralogs *CgPMU1* (CAGL0K07524g), which has the most similarity to *ScPMU1*, *CgPMU2* (CAGL0K07546g), and *CgPMU3* (CAGL0K07568g). *CgPMU2* and *CgPMU3* appear to have derived from small-scale duplication events as the genes surrounding this cluster have conserved synteny in other Ascomycetes, including *S. cerevisiae* (Figure 1B).

Relatively little is known about *ScPMU1* in *S. cerevisiae.* When *ScPMU1* is overexpressed, it suppresses a histidine

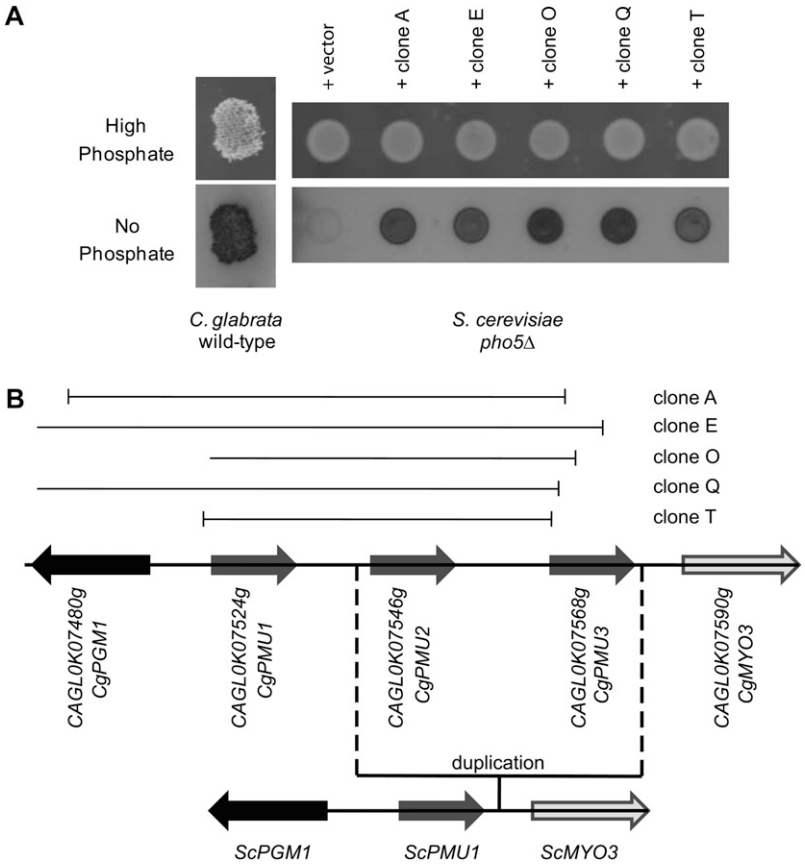

FIGURE 1.—Complementation of *Scpho5*Δ by *C. glabrata* genomic library. (A) A semiquantitative phosphatase plate assay was performed on wild-type *C. glabrata* grown in high-phosphate media and in no-phosphate media with the dark color indicating secreted acid phosphatase activity. Five genomic clones conferred phosphatase activity in no-phosphate conditions and repressed phosphatase activity in high-phosphate conditions. (B) The genomic clones with phosphatase activity were further analyzed. Clone A spans nucleotides 740743–747471, clone E 748278–738994, clone Q 747445–735219, and clone T 743041–747455. Clone O ends at nucleotide 747642 (numbering is based on chromosome K sequence NC_006034). Using *CgPMU1* primers designed to amplify the ORF, it was determined that clone O contains *CgPMU1*; however, one end was not refractory to sequencing. The same region of chromosome 11 from *S. cerevisiae* is below the *C. glabrata* schematic showing the conserved synteny. The direction of the arrows indicates the direction of transcription.

auxotrophy of the *ade3 ade16 ade17* triple mutant (REBORA *et al.* 2005). The histidine requirement is probably suppressed by detoxifying AICAR, a monophosphate nucleotide derivative (REBORA *et al.* 2005). Because of its potential phosphomutase activity, *ScPMU1* might remove the phosphate group of AICAR (REBORA *et al.* 2005). *ScPMU1* has also been isolated as a high-copy suppressor of the temperature sensitivity of a *tps2* mutant that lacks trehalose-6-phosphatase activity (ELLIOTT *et al.* 1996). In this case, *Sc*Pmu1 likely removes the phosphate from trehalose-6-phosphate, preventing the accumulation of trehalose-6-phosphate (ELLIOTT *et al.* 1996). These previous studies, in combination with *PMU1* containing a domain that is related to phosphomutases, suggest that *PMU1* may function to manipulate or hydrolyze phosphate groups from organic compounds (ELLIOTT *et al.* 1996). Because *PMU1* contains a potential phosphomutase domain, it may have some affinity for phosphate; therefore, all three close relatives in *C. glabrata* may also have the ability to bind and hydrolyze organic phosphate compounds.

To determine the similarity and possible evolutionary history between *ScPMU1* and these three paralogs, we performed a ClustalW alignment with the predicted protein sequences (Figure 2A). *Sc*Pmu1 is more similar to *Cg*Pmu1 (51% identical) than to *Cg*Pmu2 (45%) or *Cg*Pmu3 (46% identical). We hypothesize *CgPMU1* is likely an ortholog of *ScPMU1* because *CgPMU1* is most closely related to the ancestral *PMU1* gene in other Ascomycetes. It is noteworthy that there are 22–24 amino acids on the N terminus of all three paralogs in *C. glabrata* that are not present in *S. cerevisiae* or most other Ascomycetes (Figure 2A). This likely is a signal sequence allowing for secretion of all three products into the periplasm, much like *Sc*Pho5 or *Kluyveromyces lactis* Pho5 (FERMINAN and DOMINGUEZ 1997). Our assumption is supported by analysis of the sequences for a putative signal peptide with SignalP (BENDTSEN *et al.* 2004). On the basis of similarity, we hypothesize that the ancestral *PMU1* acquired a signal sequence altering its ancestral function or localization (but not eliminating it) and then duplicated twice, because all three *PMU* genes are located in tandem on chromosome 11 in *C. glabrata*. This tandem triplet of *PMU1*-like genes is only observed in *C. glabrata* and not in the other sequenced Ascomycetes genomes (WAPINSKI *et al.* 2007), suggesting positive selection on this gene family in *C. glabrata* (Figure 2B).

Identification of *CgPMU2* as inducible phosphatase: To determine which of these duplicate genes has phosphate starvation-inducible secreted acid phosphatase activity, each gene was individually inactivated with a *KANMX6* or *NATMX6* cassette (LONGTINE *et al.* 1998; KERWIN and WYKOFF 2009). Each deletion strain was assayed for phosphatase activity with a qualitative phosphatase plate assay (KERWIN and WYKOFF 2009), where

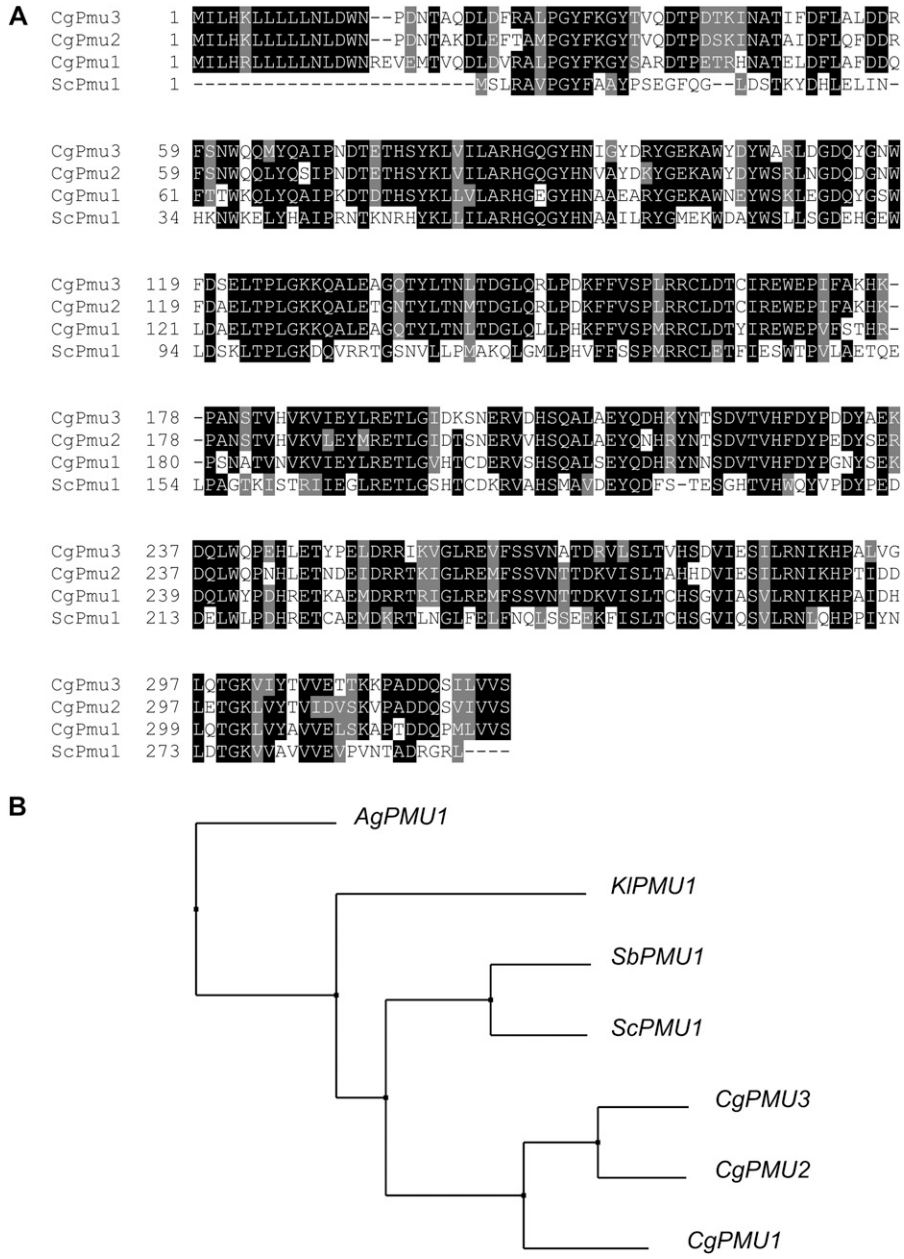

FIGURE 2.—(A) ClustalW alignment of *Cg*Pmu proteins with *Sc*Pmu1. After alignment, the ALN file was entered into BOXSHADE with a cutoff of identity of 0.7. Solid boxes indicate identity and shaded boxes indicate similarity. (B) Tree of relationships generated from JalView 2.4 using a neighbor joining tree with BLOSSUM62. The sequences used were: *Ashbya gossypii* AEL304C, *Kluyveromyces lactis* KLLA0B12628g, *Saccharomyces bayanus* sbayc559-g5.1, and previously identified protein sequences. This tree is representative of trees generated by other methods as well.

colonies remain white if there is little phosphatase activity and turn red through a diazo coupling reaction when phosphate is cleaved from 1-NP. We expected *CgPMU2* to be the phosphate starvation-inducible secreted acid phosphatase in *C. glabrata* because it is the only gene with both the ORF and promoter region present in all five genomic clones (Figure 1B). In a *Cgpmu2*Δ strain, there is essentially no secreted acid phosphatase activity in *C. glabrata* (Figure 3A). However, if either *CgPMU1* or *CgPMU3* is deleted, there is still phosphatase activity in phosphate starvation conditions. Pmu1 and Pmu3 are not critical for phosphatase activity in *C. glabrata* because when either is inactivated, there is still detectable acid phosphatase activity. We quantified phosphatase activity using PNPP during phosphate starvation and confirmed these qualitative results (Figure 3B) (HUANG and O'SHEA 2005). Additionally, we quantified levels of *PMU2* transcript through quantitative (q) PCR on reverse transcribed RNA of each of the strains (KERWIN and WYKOFF 2009) and observed that the levels of *PMU2* were very similar to the levels of phosphatase activity, consistent with *PMU2* encoding the phosphate starvation-inducible phosphatase activity.

It is worth noting that there is a significant decline in phosphatase activity in the *Cgpmu1*Δ strain and this could be explained in one of two ways—either *CgPMU1* is encoding approximately half of phosphatase activity or disruption of *CgPMU1* compromises the function of the *CgPMU2* promoter, preventing full expression of the phosphatase. To differentiate between these two possibilities, we deleted *PMU1* again, but deleted only the promoter and start codon of the *PMU1* ORF, maintain-

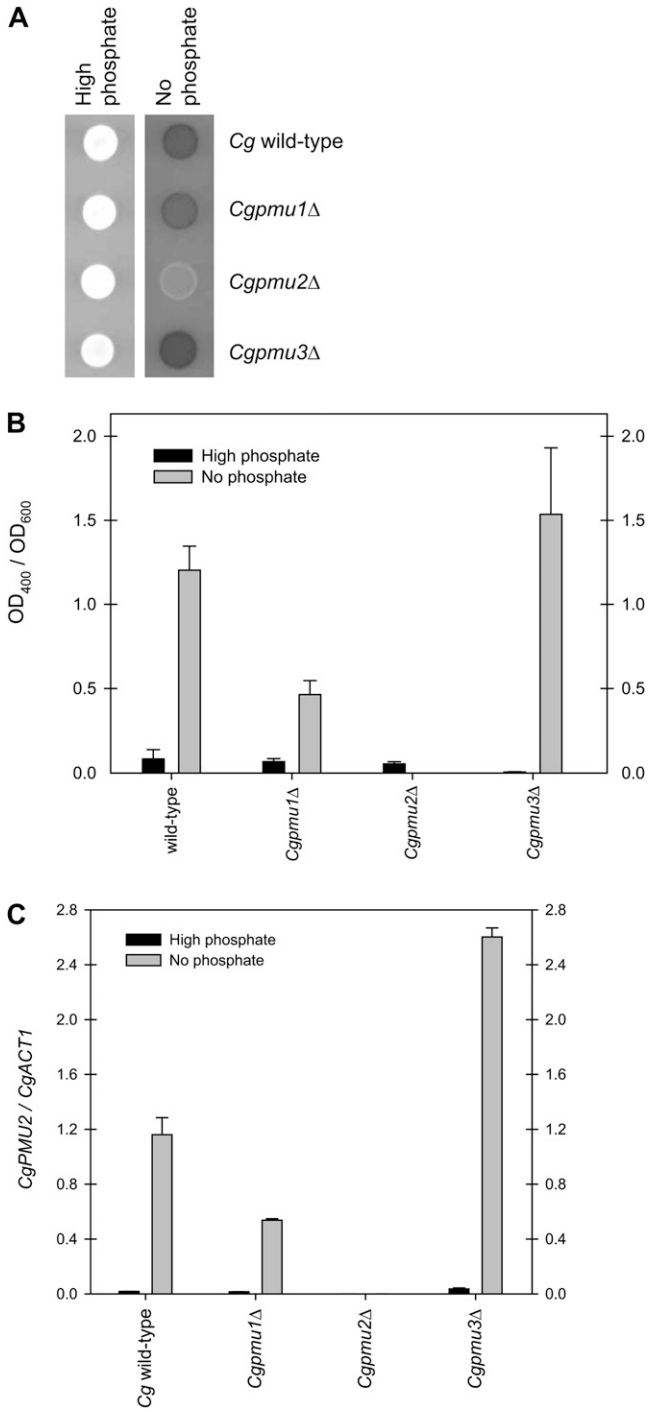

Figure 3.—Pmu2 is phosphate starvation-inducible acid phosphatase in *C. glabrata*. (A) Phosphatase plate assay in high- and no-phosphate conditions with only the *pmu2*Δ strain having a major defect in phosphatase activity. (B) The phosphatase activity (PNPP hydrolysis) of the deletion strains was quantified to determine activity normalized to cell density. Data in this figure and all following figures are expressed as mean ± SEM, $n = 3$ for each strain unless noted. Generation of new *Cgpmu1*Δ strain (Figure S1), which did not disrupt *CgPMU2* promoter, prevented this effect. (C) Quantitative real-time PCR was performed on the same strains and *CgPMU2* was quantified and normalized to the expression level of *CgACT1* because the expression of *CgACT1* does not change in response to phosphate starvation (Kerwin and Wykoff 2009). Deletion of *CgPMU3* appears to lead to increased phosphatase activity and increased levels of *CgPMU2* transcript. This 1.5- to 2-fold increase is consistently observed, but was not tractable enough to pursue in this study.

ing more of the *PMU2* promoter. This strain had higher levels of PNPP hydrolysis and expressed *PMU2* near wild-type levels (Figure S1, A and B), consistent with *PMU1* not being directly required for phosphatase expression. Additionally, we introduced genomic clones of each *PMU* gene (1 kbp of promoter and the ORF) into the *Cgpmu1*Δ, *Cgpmu2*Δ, or *Cgpmu3*Δ strains (Figure S2A). Only when the *PMU2* gene is inserted into a *pmu2*Δ strain, phosphatase activity is restored, as characterized with a quantitative PNPP hydrolysis assay (Figure S2B). Genomic clones of *PMU1* and *PMU3* had no effect on phosphatase activity when measured with 1-NP or PNPP. Therefore, Pmu2 is responsible for the majority of phosphatase activity during phosphate starvation conditions in *C. glabrata* and the defect observed in the *Cgpmu1*Δ strain is likely a consequence of promoter disruption.

Regulation of *CgPMU2* by *Cg*Pho4: To determine whether the transcription of the three paralogs was regulated by phosphate starvation and by the transcription factor *Cg*Pho4, we utilized qPCR on reverse transcribed RNA to measure gene expression levels (Kerwin and Wykoff 2009). Expression levels of each *PMU* gene (Figure 4) were first examined in wild-type *C. glabrata* to determine which genes are upregulated in the absence of phosphate. Primers were verified to be specific to each *PMU* gene by analyzing expression levels in each appropriate mutant (see Figure S3). Assuming equal amplification with primers (which appears to occur with control samples) and normalization to *CgACT1*, *CgPMU1* is an abundant transcript and is induced approximately twofold in response to phosphate starvation; *CgPMU3* is expressed at a low level and does not change abundance in response to starvation (Figure 4). *CgPMU2* is expressed at a low level in high-phosphate conditions and is ~20-fold induced during phosphate starvation. To determine whether this induction is ancestral, we determined that in *S. cerevisiae*, *ScPMU1* is not regulated by phosphate starvation (data not shown), suggesting regulation by phosphate starvation is derived. We confirmed that the upregulation of *CgPMU2* during phosphate starvation was regulated by *Cg*Pho4 because there was not a significant increase in transcript abundance during phosphate starvation in a *Cgpho4*Δ strain (Figure 4). The twofold increase in *CgPMU1* is also Pho4 dependent, but on the basis of Figure S2, it seems unlikely that *CgPMU1* plays a large role in the induction of phosphatase activity during phosphate starvation. It is worth noting that disruption of *CgPMU3* appears to lead to an increase in phosphatase activity; however, the difference is under twofold and often this difference is within the standard error of the experi-

ment. We cannot explain the increase in phosphatase activity in the *pmu3*Δ strain, but increased activity does correlate with an increase in *PMU2* transcript levels. These results may suggest there may be feedback in the regulation of these genes, although we have not explored this further in this study.

What makes *Cg*Pmu2 unique? Because all three paralogs are ~75% identical to one another, we asked whether any of the paralogs were capable of conferring inducible phosphatase activity when regulated by a phosphate starvation-inducible promoter (*ScPHO5*). If *CgPMU1* or *CgPMU3* were capable of conferring inducible phosphatase activity, then it would suggest that any paralog was capable of cleaving 1-NP like *Cg*Pmu2, but that it simply is not highly induced by phosphate starvation. When *CgPMU3* is placed under control of the *ScPHO5* promoter, complementation of the *Cgpmu2*Δ strain was observed on the basis of the semi-quantitative plate assay, suggesting that both the *CgPMU2* and *CgPMU3* ORFs have activity against 1-NP (Figure 5A). Additionally, this is confirmed when we measure PNPP hydrolysis (Figure 5B). *CgPMU1* and *ScPMU1* have a lower degree of similarity to *CgPMU2* and do not have significant phosphatase activity. We confirmed that the *ScPHO5* promoter induced the expression of the *PMU* genes by performing qPCR on reverse-transcribed RNA from cells containing the appropriate plasmids (Figure S4). Furthermore, we confirmed that *CgPMU2* and *CgPMU3* encode significant 1-NP and PNPP phosphatase activity by placing these genes under the control of another phosphate starvation regulated promoter—*CgPMU2* (Figure S5).

To determine the *in vitro* enzymatic differences between the three homologs, we purified from bacteria the three proteins of interest (Pmu1, Pmu2, and Pmu3) tagged with an N-terminal His_{10} tag by immobilized metal affinity chromatography. We determined that all three proteins had detectable phosphatase activity against PNPP (Figure 6), did not require Mg^{2+} or Ca^{2+} for activity (data not shown), and had maximal activity at a pH <4 (Figure S6), similar to *Sc*Pho5. Additionally, we characterized phosphatase activity against different organic phosphate compounds. We hypothesized that *CgPMU1*, because it is most similar to *ScPMU1*, would have significant activity against trehalose-6-phosphate and glycerol phosphate and that *Cg*Pmu2 might have new broader range substrate specificity.

To dissect specificity, we determined the activity of all three proteins against varying concentrations of PNPP (Figure 6). All three exhibited Michaelis–Menten kinetics against PNPP and a K_M of ~1 mM; however, *Cg*Pmu2 had greater than ~27 times the V_{max} of the other two homologs (Table 2 and Figure 6). In contrast, when we measured the K_M and V_{max} of the three enzymes using glycerol-1-phosphate and glycerol-2-phosphate, we observed that *Cg*Pmu2 had a lower V_{max} than the other two paralogs (although *Cg*Pmu2 has a higher affinity for glycerol-1-phosphate than the other two paralogs). Furthermore, only *Cg*Pmu2 was capable of hydrolyzing phosphate from inosine-5′-monophosphate (Table 2). We conclude from these data that, whereas all three have phosphatase activity against a variety of substrates, *Cg*Pmu2 has evolved to have a novel broad-range phosphatase activity relative to the two other paralogs. This process is similar to the paralogous drug efflux pumps in *C. albicans*, where *CaCDR2* is under positive selective pressure and the ancestral copy, *CaCDR1*, is under purifying selection (Holmes *et al.* 2006).

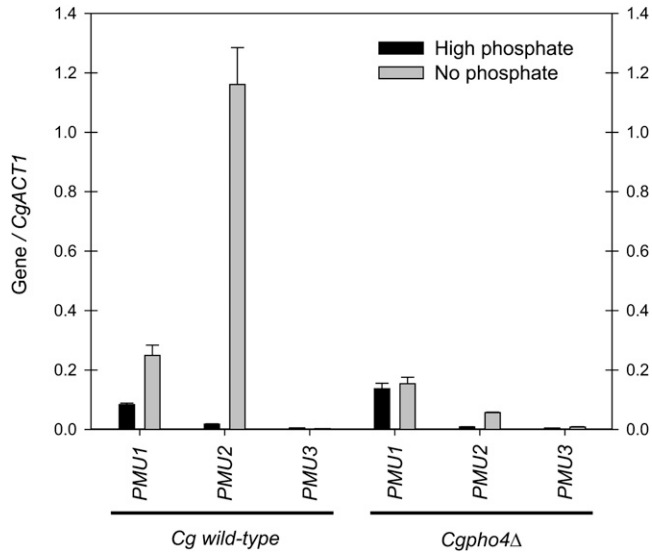

Figure 4.—*CgPMU2* is strongly regulated by phosphate starvation conditions and the transcription factor Pho4. Quantitative real-time PCR was performed on each *PMU* gene and normalized to the expression level of *ACT1*. Each primer set was verified to amplify only the indicated gene by examining qPCR of deletion strains (Figure S3). *CgPMU1* and *CgPMU2* appear regulated by CgPho4, but *CgPMU1* to a much lesser extent. The *CgPMU3*/*CgACT1* ratio is ~0.003 and does not change in response to phosphate condition or *CgPHO4* deletion.

Caution should be used in the interpretation of these *in vitro* results. When we overexpress *CgPMU3* in yeast, we observe dramatic 1-NP and PNPP hydrolysis, demonstrating that *CgPMU3* encodes a broad-range phosphatase, but when purified from bacteria, His_{10}–Pmu3 does not have a high V_{max} like His_{10}–Pmu2. These *in vivo* data suggest that the only reason that *CgPMU3* is not the primary acid phosphatase is that it is not highly expressed during phosphate starvation. Because the bacterially purified version of His_{10}–Pmu3 has higher activity against glycerol-1-phosphate and trehelose-6-phosphate than His_{10}–Pmu1 or His_{10}–Pmu2, we believe that His_{10}–Pmu3 is partially functional, but that our assays do not completely reflect its *in vivo* activity.

Evolutionary requirements for phosphatase activity: In the environment of *S. cerevisiae*, phytic acid (inositol hexakisphosphate) is likely a substantial source of phosphate as phytic acid is relatively abundant in plant

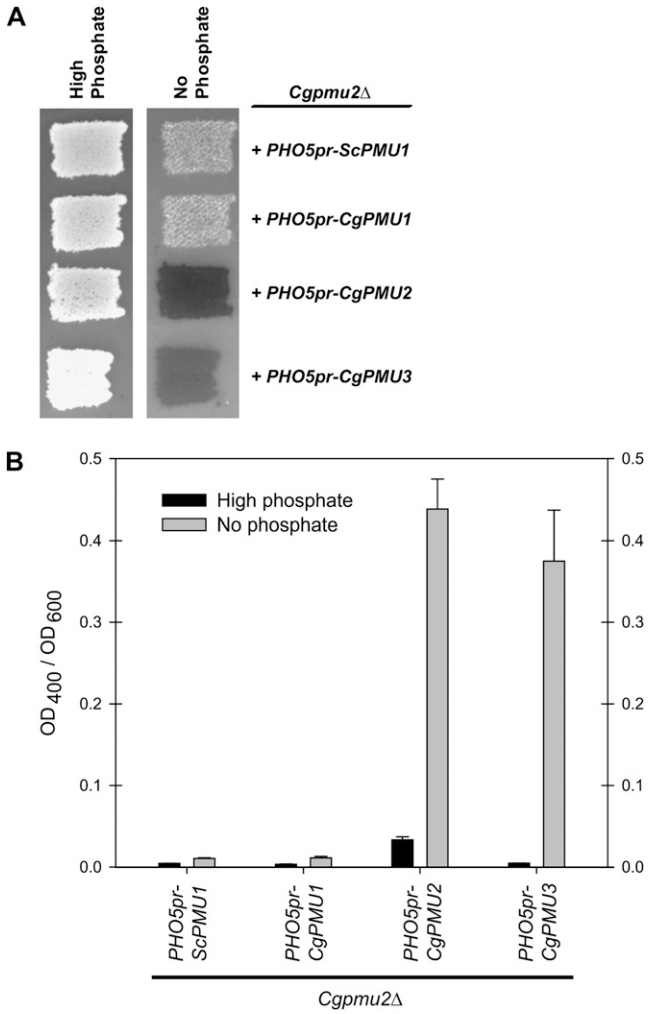

FIGURE 5.—The *CgPMU2* and *CgPMU3* ORFs are able to hydrolyze 1-NP or PNPP efficiently. All four ORFs were placed under the control of the phosphate starvation-regulated *ScPHO5* promoter and transformed into *Cgpmu2*Δ strain. Expression of each ORF was verified by RT–qPCR (Figure S4). Phosphatase activity is restored when *CgPMU2* and *CgPMU3* are expressed as judged by hydrolysis of 1-NP (A) or PNPP (B). Additionally, these ORFs were placed under the control of the *CgPMU2* promoter and exhibited similar results (Figure S5). Multiple isolates were subjected to the phosphatase plate assay and these are representative results.

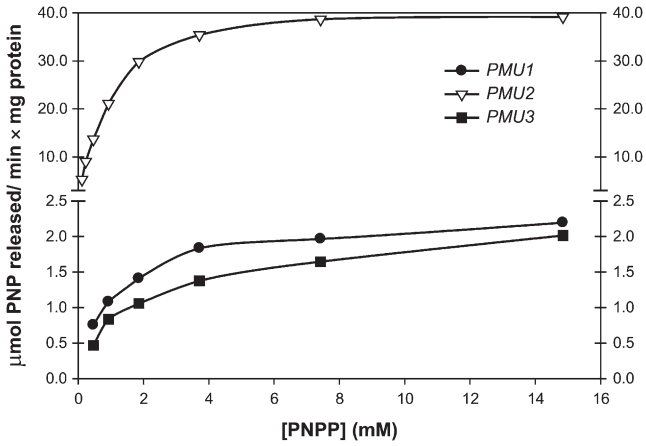

FIGURE 6.—Substrate vs. velocity curve with PNPP. Equal amounts of purified enzyme were incubated with various concentrations of PNPP and the amount of PNP formed was monitored at OD_{400}. All three exhibit Michaelis–Menten kinetics, and protein from a mock purification exhibited a velocity of ~0.2 mmol PNP released/min × mg protein regardless of substrate concentration (data not shown).

matter (LOTT *et al.* 2000). However, release of phosphate from this compound requires a specialized phosphatase, and *Sc*Pho5 has this phytase activity (OLSTORPE *et al.* 2009) and we wanted to determine whether the Pmu paralogs possessed phytase activity. Purified proteins did not exhibit detectable phytase activity in our assay conditions; therefore, we examined phytase activity from phosphate-starved *S. cerevisiae* and *C. glabrata* whole cells. Phytic acid is cleaved slowly, but detectably, by *S. cerevisiae* (28 fmol phosphate released/cell/hr ± 3.0, $n = 2$) and much slower by *C. glabrata* (0.43 fmol phosphate released/cell/hr ± 0.34, $n = 2$). We hypothesized that *C. glabrata* would be unable to grow in medium with phytic acid as the only phosphate source. Because *C. glabrata* grows on mammalian tissue, which is unlikely to be rich in phytic acid (GRASES *et al.* 2001), we expected there was no selective pressure to possess phytase activity. *C. glabrata* does not grow well in medium with phytic acid as the sole phosphate source; however, when *C. glabrata* contains *ScPHO5*, the cells are now able to grow (Figure 7, A and B).

Loss of *ScPHO5* is understandable in the context of not requiring phytase activity, but the gain of phosphatase activity through *CgPMU2* suggests that there must have been selective pressure for *C. glabrata* to acquire or maintain secreted phosphatase activity. To determine whether *C. glabrata* requires phosphatase activity, we grew *C. glabrata* in media containing 1-NP, guanosine monophosphate, or glycerol-2-phosphate as the sole source of phosphate (Figure 8). Because phosphatase activity is required to release inorganic phosphate from these substrates, we hypothesized that *C. glabrata* growth should depend on *CgPMU2* when these substrates are the only phosphate provided. *Cg*Pmu2 is required for growth on these organic phosphates as deletion of *CgPMU2* lowers the ability of cells to grow, utilizing these substrates (Figure 8). However, these strains grow well with similar amounts of inorganic phosphate. It seems reasonable that it is advantageous for *C. glabrata* to be able to access inorganic phosphate from an organic compound, although the precise identity of that compound is unknown.

DISCUSSION

We have isolated the gene encoding the major phosphate starvation-inducible secreted acid phosphatase in *C. glabrata*. This gene, *CgPMU2*, is a part of a three gene family of phosphomutase domain-containing genes.

TABLE 2

Activity of His_{10}-tagged purified proteins (μmol phosphate released/min × mg protein) against various phosphate-containing compounds measured in millimolar concentrations

	Pmu1		Pmu2		Pmu3	
Substrate	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
PNPP	1.7 ± 0.83	2.6 ± 0.39	0.81 ± 0.03	46 ± 6.1	1.7 ± 0.2	2.2 ± 0.16
Glycerol-1-phosphate	24 ± 0.03	29 ± 2.2	2.2 ± 0.64	11 ± 1.0	98 ± 30	42 ± 8.0
Glycerol-2-phosphate	23 ± 3.3	29 ± 5.7	25 ± 11	14 ± 3.3	ND	ND
IMP	ND	ND	6.4 ± 3.4	11 ± 0.87	ND	ND
Trehalose-6-phosphate	0.26 ± 0.001	0.29 ± 0.006	ND	ND	1.1 ± 0.38	0.24 ± 0.08

ND, no activity detected.

CgPMU2 is the only member of this gene family that is regulated by the phosphate starvation-regulated transcription factor *Cg*Pho4. Furthermore, it is the only gene that has a high V_{max} for hydrolysis of phosphate from *p*-nitrophenylphosphate and the only gene that substantially hydrolyzes inosine-5′-monophosphate. *Cg*Pmu2 is the functional analog of *Sc*Pho5; however, unlike *Sc*Pho5, it is unable to liberate phosphate from phytic acid.

Our data allow for a hypothetical reconstruction of events that dictated some niche specificity—the ability of *S. cerevisiae* to grow in phytic acid-rich environments and the inability of *C. glabrata* to grow in these environments. The ancestral Pmu gene product likely bound and possibly cleaved phosphate from intracellular phosphate-containing compounds, such as glycerol phosphate or trehalose phosphate. After speciation, *Cg*Pmu1 acquired a signal peptide, and a small-scale duplication event generated two copies of the *PMU* gene. This allowed for drift and sequence divergence of one copy of the gene, with *Cg*Pmu1 maintaining its ancestral function. The divergent copy then experienced a second duplication event generating *CgPMU2* and *CgPMU3*. Both acquired the ability to hydrolyze 1-NP and PNPP but only *CgPMU2* acquired regulation by phosphate starvation. *Cg*Pmu2 also acquired the ability to hydrolyze at least one additional organic phosphate molecule (inosine-5′-monophosphate).

There is no genomic sequence similar to *ScPHO5* in the *C. glabrata* genome, but most Ascomycetes contain *PHO5* homologs, suggesting that in most niches there is selective pressure to maintain a secreted acid phosphatase. *C. glabrata* also likely experienced selective pressure for secreted acid phosphatase but did not experience selective pressure to encode a phytase. Growth on mammalian tissue may have allowed for *CgPMU2* to replace *ScPHO5*. Interestingly, only *Aspergillus nidulans* appears to have both duplicated *PMU1* and lost *PHO5*, and future studies could explore whether a similar process has occurred in this species (Wapinski *et al.* 2007).

Evolution through natural selection suggests that unnecessary genes are usually lost, and there are excellent examples of this process, such as the loss of the genes encoding galactose catabolism when there is no galactose present over an evolutionary timescale (Butler *et al.* 2004; Hittinger *et al.* 2004). This "use it or lose it" process is likely what we are observing through the loss of *ScPHO5*. However, our data suggest a corollary to this process. In evolution, necessity can

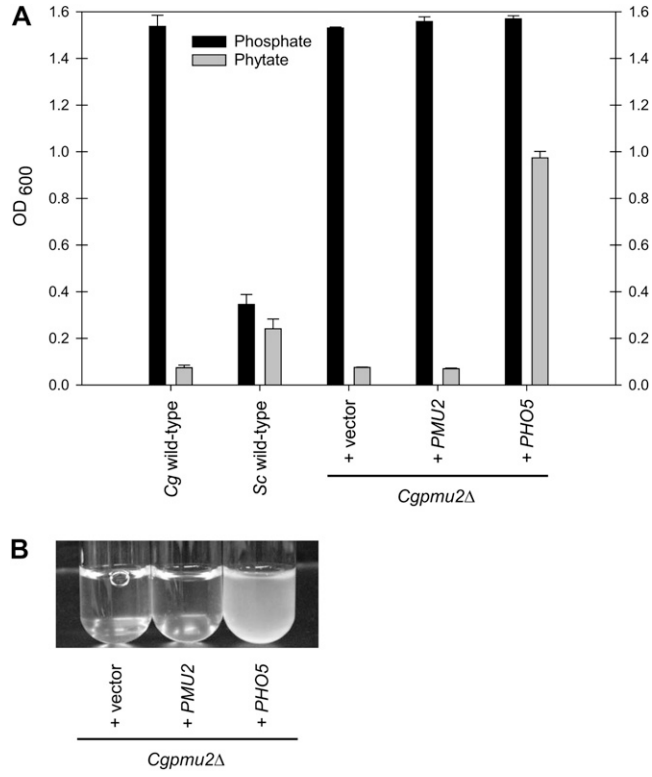

Figure 7.—*ScPHO5* encodes a phytase that allows *C. glabrata* to grow with phytic acid as a sole phosphate source. (A) Strains were inoculated at an OD_{600} of 0.001 and grown for 24 hr in SD + 1.2 mM phosphate or 60 hr in SD + 200 μM phytic acid and monitored by measuring OD_{600}. The strains at these times points were not dividing rapidly. *C. glabrata* consistently grows to a higher OD_{600} than *S. cerevisiae*. Deletion of *ScPHO5* has a modest effect on growth in phytic acid (data not shown) because there are other phytases in the *S. cerevisiae* genome (Olstorpe *et al.* 2009). (B) A photograph of strains from A, but inoculated at a density of 0.0001 in SD + 200 μM phytic acid and grown for 4 days at 30°.

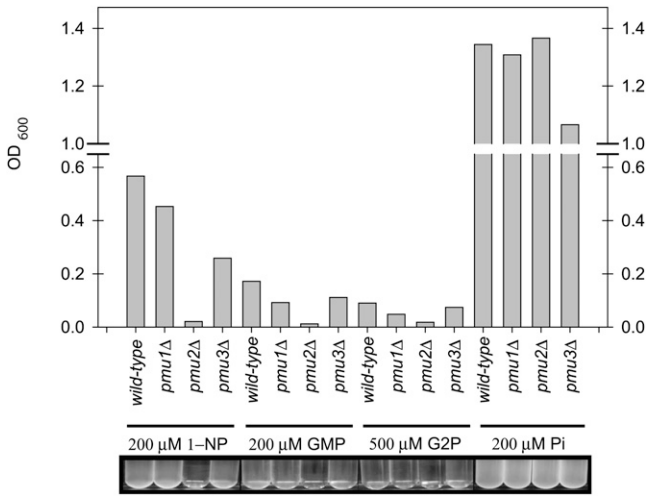

FIGURE 8.—*CgPMU2* is required for the growth of *C. glabrata* in media with organic phosphate as the sole phosphate source. Strains were inoculated at OD_{600} of 0.001 and grown for 20 hr in SD + 0.2 mM phosphate or 72 hr in SD + organic phosphate. GMP, guanosine monophosphate; G2P, glycerol-2-phosphate; P_i, inorganic phosphate. Strains grew much slower in organic phosphate sources and 500 μM G2P was required for measurable growth. This is one representative experiment, but was reproducible.

exert a strong selective pressure. If the *C. glabrata* genome encoded Pmu proteins with weak phosphatase activity and, at some point, no *ScPHO5* homolog was present, there would be a strong selective pressure on these Pmu proteins for the acquisition of broad-range specificity for organic phosphate compounds in the environment to provide essential inorganic phosphate for the cell. We cannot determine when *ScPHO5* was lost in speciation, but because it was replaced with the functional analog *CgPMU2* in *C. glabrata*, we can hypothesize that neofunctionalization coupled with gene loss has acted on the phosphate signal transduction pathway. The coupling of gene loss and neofunctionalization could also reinforce speciation by isolating closely related species—*i.e.*, allowing only one of the species to grow in phytic acid-rich environments. Similar small scale processes with other signal transduction pathways may be a key process in speciation.

We thank members of the Wykoff laboratory and Todd Jackman, Louise Russo, and Erin O'Shea for comments on this work. We thank Juliette Power for helping to generate plasmids used in this study. We also thank Brendan Cormack for *C. glabrata* strains. We also appreciate comments from anonymous reviewers that enhanced this work. This work was funded by the Department of Biology, the College of Arts and Sciences, and the Center for Undergraduate Research and Fellowships at Villanova University and by a grant from the National Science Foundation (RUI-MCB-0747799).

LITERATURE CITED

BARBARIC, S., M. MUNSTERKOTTER, C. GODING and W. HORZ, 1998 Cooperative Pho2-Pho4 interactions at the PHO5 promoter are critical for binding of Pho4 to UASp1 and for efficient transactivation by Pho4 at UASp2. Mol. Cell. Biol. **18:** 2629–2639.

BEISSWANGER, S., and W. STEPHAN, 2008 Evidence that strong positive selection drives neofunctionalization in the tandemly duplicated polyhomeotic genes in Drosophila. Proc. Natl. Acad. Sci. USA **105:** 5447–5452.

BENDTSEN, J. D., H. NIELSEN, G. VON HEIJNE and S. BRUNAK, 2004 Improved prediction of signal peptides: signalP 3.0. J. Mol. Biol. **340:** 783–795.

BUTLER, G., C. KENNY, A. FAGAN, C. KURISCHKO, C. GAILLARDIN *et al.*, 2004 Evolution of the MAT locus and its Ho endonuclease in yeast species. Proc. Natl. Acad. Sci. USA **101:** 1632–1637.

BYRNE, K. P., and K. H. WOLFE, 2006 Visualizing syntenic relationships among the hemiascomycetes with the Yeast Gene Order Browser. Nucleic Acids Res. **34:** D452–D455.

CHEN, P., T. TORIBARA and H. WARNER, 1956 Microdetermination of phosphorus. Anal. Chem. **28:** 1756–1758.

CONANT, G. C., and K. H. WOLFE, 2008 Turning a hobby into a job: how duplicated genes find new functions. Nat. Rev. Genet. **9:** 938–950.

CORMACK, B. P., and S. FALKOW, 1999 Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen *Candida glabrata*. Genetics **151:** 979–987.

DOMERGUE, R., I. CASTANO, A. DE LAS PENAS, M. ZUPANCIC, V. LOCKATELL *et al.*, 2005 Nicotinic acid limitation regulates silencing of Candida adhesins during UTI. Science **308:** 866–870.

ELLIOTT, B., R. S. HALTIWANGER and B. FUTCHER, 1996 Synergy between trehalose and Hsp104 for thermotolerance in *Saccharomyces cerevisiae*. Genetics **144:** 923–933.

FERMINAN, E., and A. DOMINGUEZ, 1997 The KIPHO5 gene encoding a repressible acid phosphatase in the yeast Kluyveromyces lactis: cloning, sequencing and transcriptional analysis of the gene, and purification and properties of the enzyme. Microbiology **143**(Pt 8): 2615–2625.

GRASES, F., B. M. SIMONET, R. M. PRIETO and J. G. MARCH, 2001 Phytate levels in diverse rat tissues: influence of dietary phytate. Br. J. Nutr. **86:** 225–231.

HE, X., and J. ZHANG, 2005 Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution. Genetics **169:** 1157–1164.

HENTGES, P., B. V. DRIESSCHE, L. TAFFOREAU, J. VANDENHAUTE and A. M. CARR, 2005 Three novel antibiotic marker cassettes for gene disruption and marker switching in Schizosaccharomyces pombe. Yeast **22:** 1013–1019.

HITTINGER, C. T., A. ROKAS and S. B. CARROLL, 2004 Parallel inactivation of multiple GAL pathway genes and ecological diversification in yeasts. Proc. Natl. Acad. Sci. USA **101:** 14144–14149.

HOLMES, A. R., S. TSAO, S. W. ONG, E. LAMPING, K. NIIMI *et al.*, 2006 Heterozygosity and functional allelic variation in the Candida albicans efflux pump genes CDR1 and CDR2. Mol. Microbiol. **62:** 170–186.

HUANG, S., and E. K. O'SHEA, 2005 A systematic high-throughput screen of a yeast deletion collection for mutants defective in PHO5 regulation. Genetics **169:** 1859–1871.

KERWIN, C. L., and D. D. WYKOFF, 2009 *Candida glabrata* PHO4 is necessary and sufficient for Pho2-independent transcription of phosphate starvation genes. Genetics **182:** 471–479.

LONGTINE, M. S., A. MCKENZIE, III, D. J. DEMARINI, N. G. SHAH, A. WACH *et al.*, 1998 Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14:** 953–961.

LOTT, J. N. A., I. OCKENDEN, V. RABOY and G. D. BATTEN, 2000 Phytic acid and phosphorus in crop seeds and fruits: a global estimate. Seed Sci. Res. **10:** 11–33.

O'NEILL, E. M., A. KAFFMAN, E. R. JOLLY and E. K. O'SHEA, 1996 Regulation of PHO4 nuclear localization by the PHO80–PHO85 cyclin-CDK complex. Science **271:** 209–212.

OLSTORPE, M., J. SCHNÜRER and V. PASSOTH, 2009 Screening of yeast strains for phytase activity. FEMS Yeast Res. **9:** 478–488.

REBORA, K., B. LALOO and B. DAIGNAN-FORNIER, 2005 Revisiting purine-histidine cross-pathway regulation in *Saccharomyces cerevisiae*: a central role for a small molecule. Genetics **170:** 61–70.

REDONDO-LOPEZ, V., M. LYNCH, C. SCHMITT, R. COOK and J. D. SOBEL, 1990 Torulopsis glabrata vaginitis: clinical aspects and susceptibility to antifungal agents. Obstet. Gynecol. **76:** 651–655.

Sanglard, D., F. Ischer, D. Calabrese, P. A. Majcherczyk and J. Bille, 1999 The ATP binding cassette transporter gene CgCDR1 from Candida glabrata is involved in the resistance of clinical isolates to azole antifungal agents. Antimicrob. Agents Chemother. **43:** 2753–2765.

Schneider, K. R., R. L. Smith and E. K. O'Shea, 1994 Phosphate-regulated inactivation of the kinase PHO80–PHO85 by the CDK inhibitor PHO81. Science **266:** 122–126.

Springer, M., D. D. Wykoff, N. Miller and E. K. O'Shea, 2003 Partially phosphorylated Pho4 activates transcription of a subset of phosphate-responsive genes. PLoS Biol. **1:** E28.

Ting, C. T., S. C. Tsaur, S. Sun, W. E. Browne, Y. C. Chen *et al.*, 2004 Gene duplication and speciation in Drosophila: evidence from the Odysseus locus. Proc. Natl. Acad. Sci. USA **101:** 12232–12235.

Wapinski, I., A. Pfeffer, N. Friedman and A. Regev, 2007 Natural history and evolutionary principles of gene duplication in fungi. Nature **449:** 54–61.

Wykoff, D. D., and E. K. O'Shea, 2001 Phosphate transport and sensing in *Saccharomyces cerevisiae*. Genetics **159:** 1491–1499.

Wykoff, D. D., A. H. Rizvi, J. M. Raser, B. Margolin and E. K. O'Shea, 2007 Positive feedback regulates switching of phosphate transporters in S. cerevisiae. Mol. Cell **27:** 1005–1013.

Zhang, J., 2003 Evolution by gene duplication: an update. Trends Ecol. Evol. **18:** 292–298.

Communicating editor: O. Cohen-Fix

GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.110.120824/DC1

Novel Acid Phosphatase in *Candida glabrata* Suggests Selective Pressure and Niche Specialization in the Phosphate Signal Transduction Pathway

Brianne R. Orkwis, Danielle L. Davies, Christine L. Kerwin, Dominique Sanglard and Dennis D. Wykoff

DOI: 10.1534/genetics.110.120824

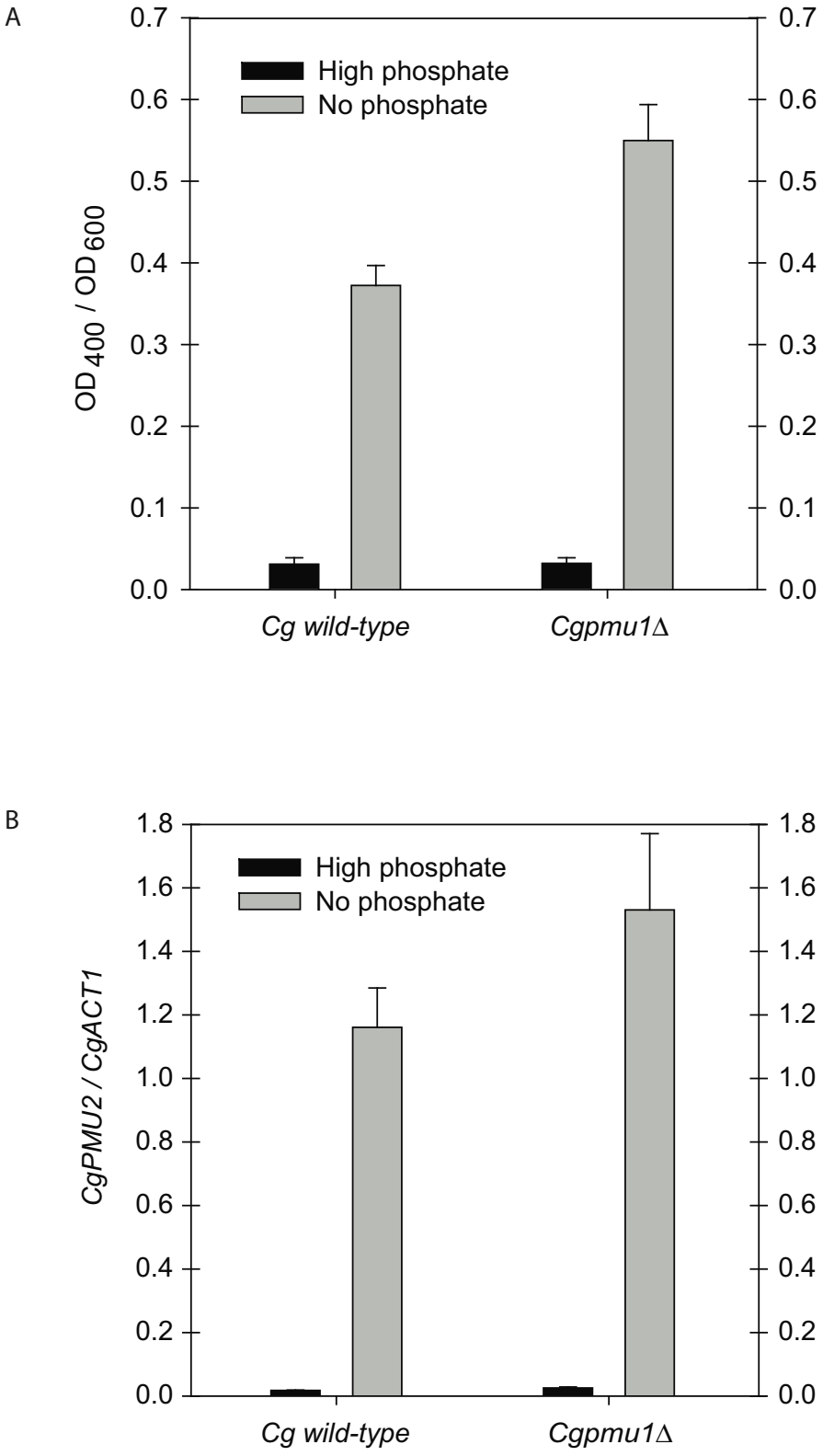

FIGURE S1.—Alternative *Cgpmu1*Δ strain induces PNPP phosphate activity better than original mutant. Strain DG87 was generated by deleting only the promoter of *CgPMU1* and the start codon. See Table S1 for exact primers. (A) PNPP hydrolysis was measured as described previously for three independently grown samples. (B) qPCR analysis of DG87 was performed in the same manner as Figure 3C.

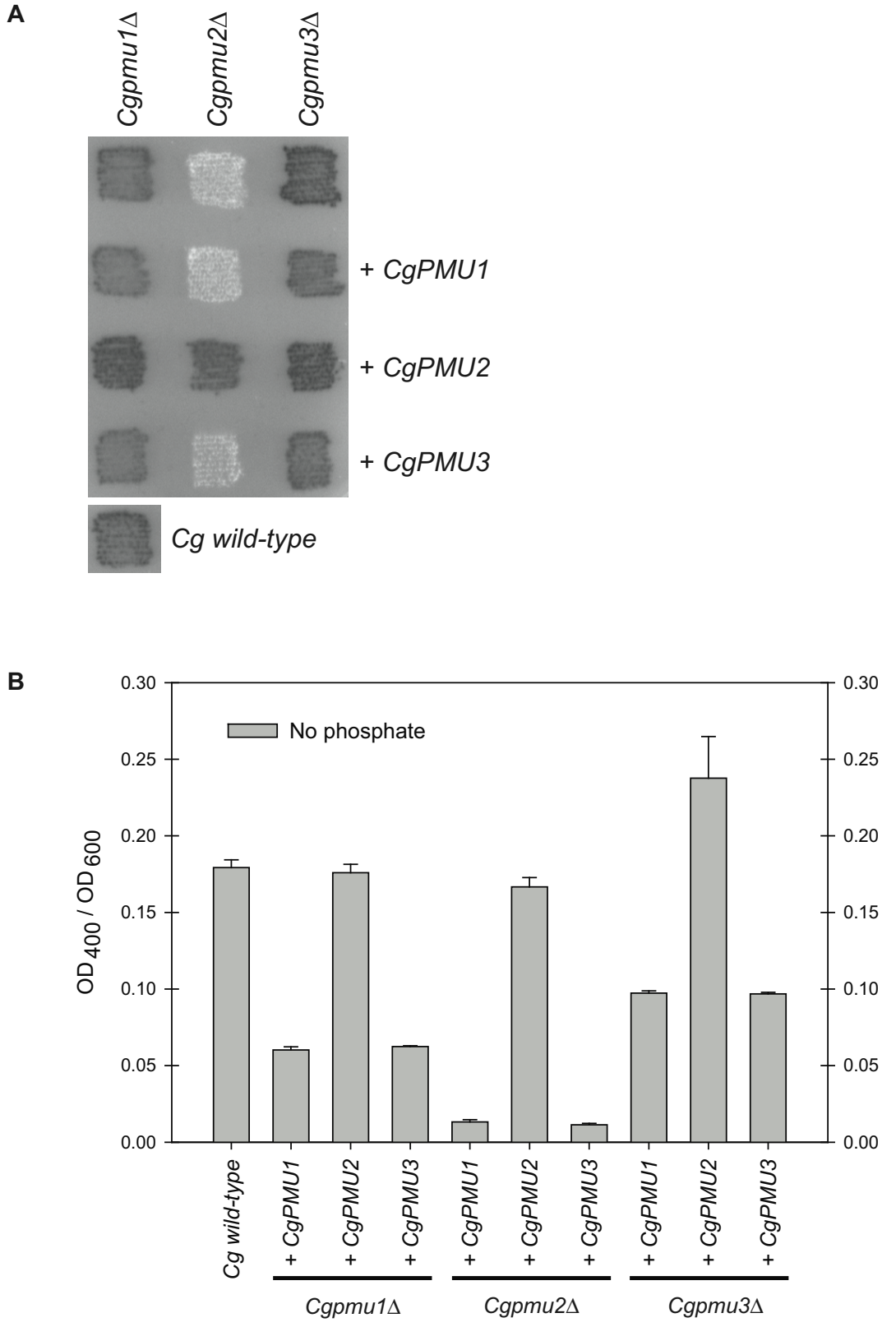

FIGURE S2.—Only genomic clones of *CgPMU2* complement the phosphatase defect of a *Cgpmu2*Δ strain. (A) Strains with each genomic clone were selected for on SD − histidine and replica plated onto medium lacking phosphate and subjected to a visual plate assay with 1-NP. (B) These same strains were grown in triplicate and assayed for PNPP hydrolysis after ~ 16 h of growth in medium lacking phosphate.

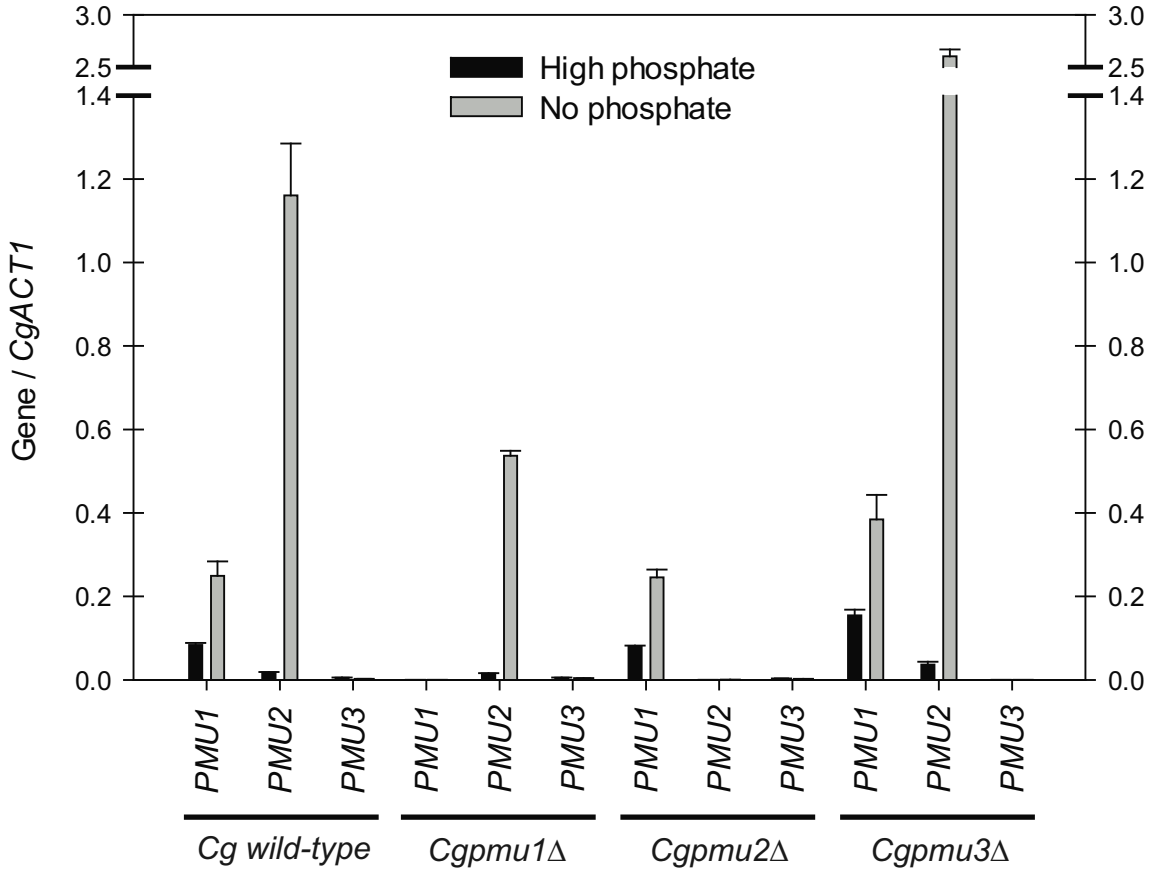

FIGURE S3.—qPCR on reverse-transcribed RNA from deletion strains. Transcription of each *PMU* gene was quantified and normalized to *CgACT1* expression in each deletion strain and wild-type in high and no phosphate conditions. The expression of *CgPMU2* is decreased in the original *Cgpmu1*Δ strain but is restored in the newer deletion strain (Figure S1). The level of *CgPMU2* in the *Cgpmu3*Δ strain is approximately twice the wild-type level, and could result from an unknown feedback mechanism. The increase in phosphatase activity and *CgPMU2* expression is consistently elevated in the *Cgpmu3*Δ strain, but because of error it is complicated to dissect at this time.

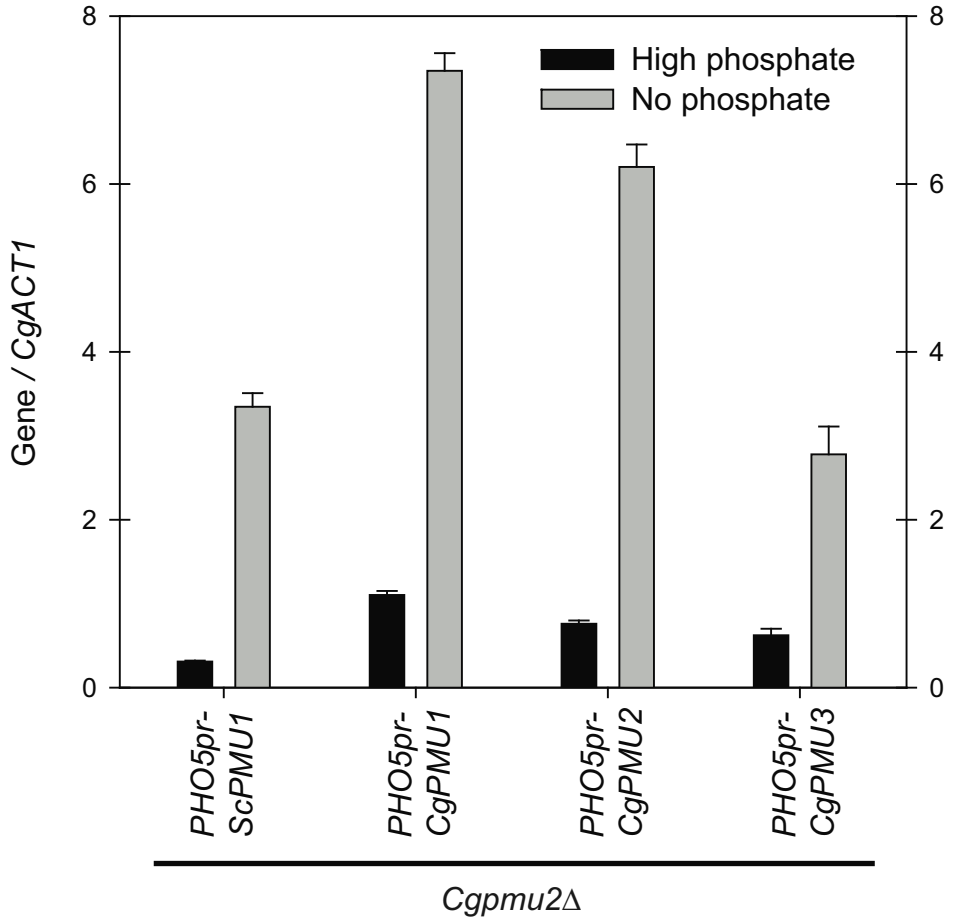

FIGURE S4.—Expression of *PMU* genes under the control of the *ScPHO5* promoter. Strains in Figure 5 were grown in high and no phosphate medium and subjected to rt-qPCR analysis. The *ScPHO5* promoter induces expression of each of the *PMU* constructs although to a varied extent. These results demonstrate that *ScPMU1* and *CgPMU1* are being expressed but do not result in dramatic phosphatase activity (see Figure 5) and suggest that *CgPMU3* is capable of significant hydrolysis of 1-NP and PNPP. We caution that conclusions on the absolute amount of phosphatase activity should be avoided based on Figure S5.

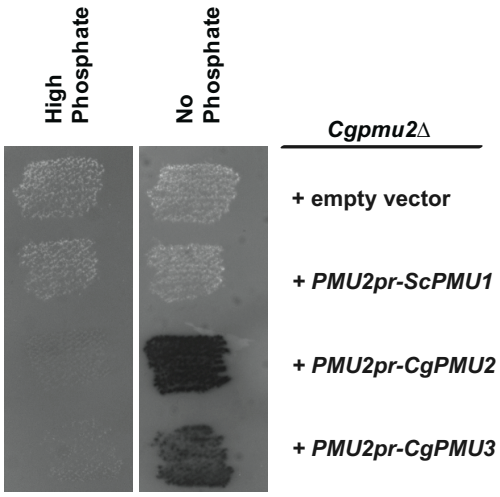

FIGURE S5.—The *CgPMU2* and *CgPMU3* ORFs are able to hydrolyze 1-NP or PNPP efficiently. The three indicated ORFs were placed under the control of the *CgPMU2* promoter and transformed into a *Cgpmu2*Δ strain and assayed for 1-NP phosphatase activity in high and no phosphate conditions. These results confirm Figure 5 eliminating any possible promoter effect from *ScPHO5*. The *CgPMU1* ORF was not able to be stably cloned into this vector.

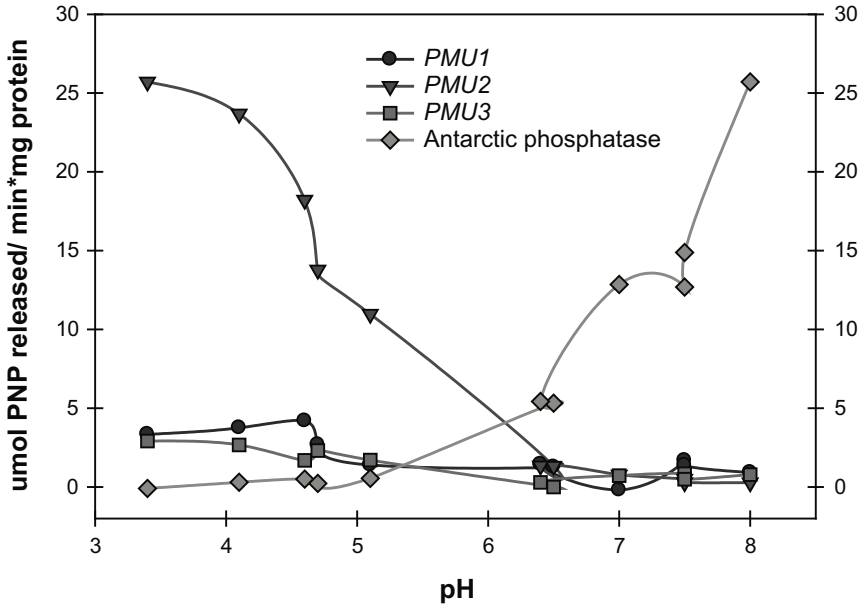

FIGURE S6.—Determination of pH optimum of each enzyme. A PNPP assay using different pH buffers was performed on the purified His_{10}-tagged proteins to determine the optimum pH at which *Cg*Pmu1, *Cg*Pmu2, and *Cg*Pmu3 function. pH levels ranging from 3.4 to 8.0 were tested. Although Pmu2 has the most phosphatase activity, all three enzymes function best at acidic pH levels. Pmu2 and Pmu3 function optimally at pH 3.4 where as Pmu1 functions best at pH 4.6. As a control, a known phosphatase (Antarctic phosphatase, Bio-Rad, USA) which is active at a neutral pH, was tested. The concentration of the Antarctic phosphatase enzyme is unknown. We arbitrarily set its' maximal activity to the same maximal activity as *Cg*Pmu2. The Antarctic phosphatase functions optimally at pH 8.

TABLE S1

Primers used in this study

Primer	Sequence	Purpose
	qPCR primers	
O75	gaccaaactacttacaactcc	CgACT1 5'
O76	ccactttcgtcgtattcttgcttg	CgACT1 3'
O155	TCCTACGAACTTCCAGATGGT	Sc ACT1 -RTPCR-F2
O156	GGCAGATTCCAAACCCAAAA	Sc ACT1 -RTPCR-R2
O193	cccagaagatgatgaattgtggc	qPCR on ScPMU1 gene 5'
O194	tgccgcgatcggccgtgttgac	qPCR on ScPMU1 gene 3'
O195	ctctagcagaataccaggatc	qPCR on CgPMU3 5'
O196	ctacgagaattgattgatcatc	qPCR on CgPMU3 3'
O236	ggccagacacggcgagggc	qPCR of CgPMU1 5'
O237	caccggaatggcatgtcaatg	qPCR of CgPMU1 3'
O664	gtgatgttactgttcattttgattatcctg	qPCR Cg PMU2
O641	gatacatcgatcacagtgtaaactag	qPCR Cg PMU2
	Primers to delete genes and check for deletion	
O182	ctctcgttacaatgatccttcataaattgctattgttgctCGGATCCCCGGGTTAATTAA	inactivation with KAN CgPMU2
O183	cgttatattaggatactacgatgaccgattggtcatcagcGAATTCGAGCTCGTTTAAAC	inactivation with KAN
O226	catgcttaacctctaaatgatcctacatagactgcttctgCGGATCCCCGGGTTAATTAA	k.o. of CgPMU1
O227	gaattccgggcattctttatgacacaacaagcattggttgGAATTCGAGCTCGTTTAAAC	
O286	gcttgttgtgtcataaagaatgc	Make a 3' extension on CgPMU1 delete
O287	ggaggaggcatacacggggc	to enhance transformation efficiency
O228	tcgttacaatgatccttcataaattgctattgttgctaaaCGGATCCCCGGGTTAATTAA	k.o of CgPMU3
O229	tgatatagatgatcggagttttaagatactacgagaattgGAATTCGAGCTCGTTTAAAC	
O780	TTAATTAACCCGGGGATCCG	PringleChk
O230	cccaccaacttcacaaaacac	CHECK PCR FOR CGPMU1
O231	gctgattgatgcgaactc	CHECK PCR FOR CG7568
O742	gggtattctttaaccaaggggacacacgcgtaacatccccCGGATCCCCGGGTTAATTAA	delete CgPMU1 ATG with KAN/NAT cassette
O743	ttccagtccaggttcagcaacagaagcagtctatgtaggaGAATTCGAGCTCGTTTAAAC	new deletion primer set
O744	cacgtggttactatttccatttgtgc	fus primers to use on 5' end for PMU1 ko
O745	gttacgcgtgtgtccccttggttaaag	rcomplelent of row 3
	Primers to clone PMUs into pET16b	
O218	GCTCGAGGATCCGatgatcctacatagactgcttc	CgPMU1 ORF for pET16b (BamHI sites)
O219	GCTCGAGGATCCGttatgacacaacaagcattgg	
O220	GCTACGCTCGAGatgatccttcataaattgc	CgPMU2 for pET (XhoI/BamHI site)

O221	GCTCGAGGATCCGttaggatactacgatgaccg	
O222	GCTACGCTCGAGatgatccttcataaattgc	CgPMU3 for pET (XhoI/BamHI site)
O223	GCTCGAGGATCCGttaagatactacgagaattg	

Primers to make pRS313 derived plasmids by gap repair		
O308	**ggtggcggccgctctagaactagtggatcc**aataacaaaatgtctaaagg	Inserts YFP into pRS313 with a BamHI site just upstream of start codon
O309	**gatatcgaattcctgcagcccggggctacc**cgaggcaagctaaacagatc	
O460	ggtggcggccgctctagaactagtggat**cgaagtcgagatgacagtgc**	Inserts 2 kb of PMU2 promoter into BamHI site from above plasmid
O461	cagtgaataattcttcacctttagacatGGATCC**tgtaacgagagtggatatt**	
O626	**ggagctccaccgcggtggcggccgctctagaactagt**taaatacaatgttccttggt	Inserts 1 kb of PHO5 promoter into BamHI site from above plasmid
O627a	cagtgaataattcttcacctttagacat**ttaattaa**tggtaatctcgaatttgcttg	

Primers to place PMU genes under control of PMU2p and PHO5p		
PMU2 promoted constructs		
O211	CAAATAAATATCCACTCTCGTTACAATGatcctacatagactgcttctg	Putting CgPMU1 under control of CgPase promoter
O213	CAAATAAATATCCACTCTCGTTACAATGatccttcataaattgctattgttg	Putting CgPMU3 or PMU2 under control of CgPase promoter
O215	CAAATAAATATCCACTCTCGTTACAATGtcactcagagcagtcccagg	Putting ScPMU1 under control of CgPase promoter
O482	accagtgaataattcttcacctttagacat**TTATGACACAACAAGCATTGG**	PMU1 stop codon 3' for gap repair with O211
O483	accagtgaataattcttcacctttagacatTTA**ggatactacgatgaccgat**	PMU2 stop codon 3' for gap repair with O213
O484	accagtgaataattcttcacctttagacat**ttaagatactacgagaattga**	PMU3 stop codon 3' for gap repair with O213
O492	CAAATAAATATCCACTCTCGTTACAATG**TCACTCAGAGCAGTCCCAGGATAT**	PCR ScPMU1 5' gap repair under control of CgPMU2 promoter
O493	accagtgaataattcttcacctttagacat**GAGCCTGCCGCGATCGGCCGTG**	PCR ScPMU1 3' gap repair under control of CgPMU2 promoter
PHO5 promoted constructs		
O628	caaatagagcaagcaaattcgagattaccaATGatcctacatagactgcttctg	PMU1 under control of PHO5 promoter in gap repair
O629	caaatagagcaagcaaattcgagattaccaATGatccttcataaattgctatt	PMU2 or PMU3 under control of PHO5 promoter in gap repair use above with PMU1 with O482, PMU2 with O483, PMU3 with O484
O630	caaatagagcaagcaaattcgagattaccaATGtcactcagagcagtcccagg	PCR ScPMU1 to put into PHO5p-YFP plasmid
O631	accagtgaataattcttcacctttagacatTCAgagcctgccgcgatcgg	with stop codon

Primers to place PMU genes into pRS313 (genomic clones)		
O746	**gaacaaaagctggagctccaccgcggt**caacttgcatcctttccgtac	PCR up PMU1 to put into pRS to check
O747	cgggggatccactagttctagagcggccgcTTAtgacacaacaagcattggttg	for complementation
O748	**gaacaaaagctggagctccaccgcggt**ggtacgattggaaatttaaaatgg	PCR up PMU3 to put into pRS to check
O749	cgggggatccactagttctagagcggccgcTTAagatactacgagaattgattg	for complementation

Primer for sequencing		
p24-seq1	cgactacgcgatcatggcgacc	for YEp24 sequencing