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CgCYN1, a Plasma Membrane Cystine-specific Transporter of *Candida glabrata* with Orthologues Prevalent among Pathogenic Yeast and Fungi*^[5]

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We describe a novel plasma membrane cystine transporter, CgCYN1, from *Candida glabrata*, the first such transporter to be described from yeast and fungi. *C. glabrata met15Δ* strains, organic sulfur auxotrophs, were observed to utilize cystine as a sulfur source, and this phenotype was exploited in the discovery of CgCYN1. Heterologous expression of CgCYN1 in *Saccharomyces cerevisiae met15Δ* strains conferred the ability of *S. cerevisiae* strains to grow on cystine. Deletion of the CgCYN1 ORF (CAGL0M00154g) in *C. glabrata met15Δ* strains caused abrogation of growth on cystine with growth being restored when CgCYN1 was reintroduced. The CgCYN1 protein belongs to the amino acid permease family of transporters, with no similarity to known plasma membrane cystine transporters of bacteria and humans, or lysosomal cystine transporters of humans/yeast. Kinetic studies revealed a K_m of $18 \pm 5 \mu\text{M}$ for cystine. Cystine uptake was inhibited by cystine, but not by other amino acids, including cysteine. The structurally similar cystathionine, lanthionine, and selenocystine alone inhibited transport, confirming that the transporter was specific for cystine. CgCYN1 localized to the plasma membrane and transport was energy-dependent. Functional orthologues could be demonstrated from other pathogenic yeast like *Candida albicans* and *Histoplasma capsulatum*, but were absent in *Schizosaccharomyces pombe* and *S. cerevisiae*.

Candida glabrata and *Candida albicans* are the two most prominent yeast causing bloodstream infections in humans (1). Of these, *C. glabrata* represents an intriguing evolutionary example of a pathogen where it is found to be more closely related to its nonpathogenic counterpart *Saccharomyces cerevisiae* than to *C. albicans* and in terms of genome size is found to have a significantly trimmer genome than *S. cerevisiae* (1, 2). Thus, despite a significantly smaller genome size it has evolved

the ability to survive within the human host, and any new proteins acquired by this yeast can be considered likely to play a very significant role in pathogenesis within the human host.

Like all living forms, yeast pathogens require essential nutrients for their survival. Sulfur is one of the essential requirements being a part of two important amino acids cysteine and methionine, as well as several other essential sulfur-containing compounds including glutathione and coenzyme A. Yeast pathogens must fulfill this requirement of sulfur by retrieving them from their human host. *C. glabrata*, like many yeast, can use both inorganic and organic forms of sulfur and have been demonstrated to have both forward and reverse transsulfuration pathways for the conversion of cysteine to methionine and also from methionine to cysteine (3). Although the sulfur assimilatory pathways were found to be very similar to that of *S. cerevisiae*, one important difference was found in the utilization of glutathione where *C. glabrata* was found to lack the ability to transport glutathione because of the absence of a high affinity glutathione transporter thus relying solely on the endogenous biosynthesis of glutathione for its survival (3). Interestingly, when one looks at the presence of sulfur compounds in the human blood plasma, glutathione is found in negligible amounts, whereas the most predominant organic sulfur form found in blood plasma is cystine (the oxidized form of cysteine) (4).

The preponderance of cystine in human plasma suggests that sulfur requirements in pathogenic yeast might involve their utilization of cystine. Interestingly, in a study done four decades ago, in another important yeast pathogen, *Histoplasma capsulatum*, cystine uptake was demonstrated in the yeast phase of this dimorphic yeast. However, the gene responsible for the phenotype was never discovered (5), despite plasma membrane cystine transporters having been described in both humans (6–8) and bacteria (9–12).

C. glabrata is a pathogen whose molecular genetics have now been reasonably well developed. Further, its haploid nature makes it much easier to manipulate than *C. albicans*. We therefore investigated the ability of *C. glabrata* to utilize cystine and exploited the *met15Δ* strains that are organic sulfur auxotrophs. We describe here the identification and characterization of a high affinity, plasma membrane cystine-specific transporter CgCYN1 that confers the ability of this yeast to utilize cystine. The transporter was demonstrated to have functional orthologues in the pathogenic yeast *C. albicans* and *H. capsula-*

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 1 and Figs. 1–3.

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TABLE 1
Yeast strains used in the study

Strain	Genotype	Parent	Source
ABC 733	<i>S. cerevisiae</i> MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0		Euroscarf
ABC 1904	<i>S. cerevisiae</i> MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δyct1::HIS4	ABC733	Laboratory strain
ABG 2370	<i>C. glabrata</i> met15Δ::hph Hyg ^R ura3Δ::Tn903 G418 ^R		R. Kaur
ABA 2240	<i>C. albicans</i> wt SCS134		K. Ganesan
ABA 2490	<i>C. albicans</i> met15Δ::FRT/ met15Δ::FRT	ABA2240	Laboratory strain
ABA 3062	<i>C. albicans</i> cyn1Δ::FRT/cyn1Δ::FRT	ABA2240	This study
ABA 3063	<i>C. albicans</i> met15Δ::FRT/ met15Δ::FRT, cyn1Δ::FRT/cyn1Δ::FRT	ABA2490	This study
ABG 2894	<i>C. glabrata</i> met15Δ::hph Hyg ^R ura3Δ::Tn903 G418 ^R cyn1Δ::hisG-URA3-hisG	ABG2370	This study
ABG 2903	<i>C. glabrata</i> met15Δ::hph Hyg ^R ura3Δ::Tn903 G418 ^R cyn1Δ::hisG	ABG2370	This study

tum, but was absent in the nonpathogenic yeast *S. cerevisiae* and *Schizosaccharomyces pombe*.

MATERIALS AND METHODS

All chemicals and reagents were of analytical reagent grade and were procured from different commercial sources. Medium components were purchased from BD (Difco). Oligonucleotide primers were synthesized from Sigma-Genosys (see supplemental Table 1). Restriction enzymes, DNA polymerases, and other DNA-modifying enzymes were obtained from New England Biolabs. Gel extraction kits and plasmid miniprep columns were obtained from Qiagen. [³⁵S]Cysteine was obtained from Bhabha Atomic Research Centre, and [³⁵S]cystine was obtained from American Radiolabeled Chemicals (ARC).³ Cys-Cys dipeptide was custom synthesized from an in-house peptide synthesizer.

Strains, Media, and Growth Conditions—The *Escherichia coli* strain DH5α was used as a cloning host. *S. cerevisiae*, *C. glabrata*, *C. albicans*, *S. pombe* strains used in the study are described in Table 1. *S. cerevisiae*, *C. albicans*, and *C. glabrata* strains were regularly maintained on yeast extract, peptone, and dextrose medium (YPD) whereas *S. pombe* was maintained on YES medium. Synthetic defined minimal medium contained yeast nitrogen base, ammonium sulfate, dextrose supplemented with methionine, cysteine, cystine, cystathionine, histidine, leucine, lysine (as per requirement), and uracil at 50 mg/liter. Yeast transformations were carried out using the modified lithium acetate method as described for *C. glabrata* (13) and for *C. albicans* using SAT1 flipper as disruption cassette (14).

Cloning and Tagging of CYN1 Gene from C. glabrata—CYN1 (*CAGL0M00154g*) gene was PCR-amplified from the genomic DNA of *C. glabrata* using primers CgCYN1F and CgCYN1R to obtain a 1.6-kb fragment, which was then cloned in *C. glabrata* (pGRB2.2)- and *S. cerevisiae* (p416TEF)-specific vectors in BamHI and XhoI sites to obtain plasmid pGRB2.2-CgCYN1 and p416TEF-ScCYN1, respectively.

For hemagglutinin (HA) tagging CYN1 gene pGRB2.2-CgCYN1 was taken as the template for PCR and HA tag was introduced just before the end codon of CgCYN1 using the PCR where the reverse primer had the HA tag (CgCYN1HAR) to yield plasmid pGRB2.2-CgCYN1HA.

Cloning of CYN1 Gene from C. albicans, H. capsulatum, and S. pombe—A putative orthologue of CgCYN1 in *C. albicans* (ORF Ca019.9873) was PCR-amplified from the genomic DNA

of *C. albicans* with primers CaCYN1F and CaCYN1R and cloned in the EcoRI and XhoI sites of p416TEF to obtain plasmid p416TEF-CaCYN1.

A putative orthologue of CgCYN1 in *H. capsulatum*, HCAG_06385, had two introns. Its cDNA was custom synthesized by Genescript which was then cloned in the BamHI and XhoI sites of p416TEF to obtain p416TEF-HcCYN1.

S. pombe ORF SPCPB1C11.02 was PCR-amplified from the genomic DNA of *S. pombe* using primers SpC11.02F and SpC11.02R and cloned in to the XbaI and SmaI sites of p416TEF to obtain plasmid p416TEF-Sp11.02.

Construction of C. glabrata Cgcyn1Δ—Plasmid p416TEF-CgCYN1 prepared from a *dam*[−]*dcm*[−] *E. coli* strain was digested with BclI and a HisG-Ura3-HisG cassette inserted in this site. This cassette was released from plasmid PHUKH2M (derived from pHUKH2 (15) and lacks the BamHI and XhoI sites), and the resulting ligation yielded p416TEF-CgCYN1M:HisG-Ura3-HisG.

The disruption cassette was released from plasmid p416TEF-CgCYN1M:HisG-Ura3-HisG by BamHI-XhoI digestion and transformed into *C. glabrata* met15Δura3Δ (ABG 2370). Transformants were selected on minimal medium lacking uracil with methionine as the sulfur source. The disruption was confirmed by diagnostic PCR. To remove the URA3 marker from the disruptant 5-fluoroorotic acid selection was performed using 0.1% 5-fluoroorotic acid and selecting the transformants on minimal medium with uracil (1.2 mg/100 ml) and methionine.

Construction of C. albicans Cacyn1Δ/Cacyn1Δ—To delete both the alleles of CaCYN1 (ORF Ca019.9873) in *C. albicans* we employed the nourseothricin disruption cassette and the SAT1 flipper strategy (using primers CaCYN1DF1, CaCYN1DF2, CaCYN1DR1, and CaCYN1DR2). The absence of a third allele for CaCYN1 was confirmed by the absence of a PCR product with primers CaCYN1 + 450F (anneals in the region which is removed in the two disrupted alleles) and CaCYN1R.

Growth Assays by Dilution Spotting—For dilution spotting assays, the different transformants were grown overnight in minimal medium with supplements but without uracil and reinoculated in fresh medium to an A₆₀₀ of 0.1 and grown for 6 h. The exponential phase cells were harvested washed with water and resuspend in water to an A₆₀₀ of 0.2. These were serially diluted to 1:10, 1:100, and 1:1000. Of these cell suspensions, 10 μl of the each dilution was spotted on the desired plates. Plates were incubated for 2 days, and photographs were taken.

Cellular Localization of HA-tagged CgCYN1—For the localization studies *Cgmet15ΔCYN1Δ* was transformed with the

³ The abbreviations used are: ARC, American Radiolabeled Chemicals; PGK, phosphoglycerate kinase; TEF, translation elongation factor.

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HA-tagged CgCYN1, and the transformants were used for the indirect immunofluorescence as described earlier (16).

Construction of Phylogenetic Tree—The protein sequence of *C. glabrata* CYN1 was used as a query sequence in BLAST to retrieve a current list of homologues of CgCYN1 in the fungal taxa. The protein sequences were then retrieved and aligned using ClustalW (17). The evolutionary history was inferred using the Neighbor-Joining method (18) using MEGA4 (19).

Radioactive Cystine Transport Assay—*S. cerevisiae* met15Δyct1Δ strain (ABC 1904) was transformed with *C. glabrata* CYN1 gene under the TEF promoter in plasmid p416TEF and with the vector control p416TEF. The transformants were grown in minimal medium containing methionine and other supplements overnight. Overnight cultures were reinoculated in the same fresh medium at an A_{600} of 0.1 and were allowed to grow until the cells reached the exponential phase, after which they were harvested, washed with ice-cold MES buffer, and then resuspended in the same MES buffer containing 2% glucose. After a 5-min preincubation of cells at 30 °C the transport experiment was initiated by adding radioactive cystine supplied by ARC, having a specific activity of 850 mCi/mmol) in MES buffer at a final concentration of 20 μM, and transport was monitored for 11 min. At selected time points (1, 3, 5, 7, 9, and 11 min) cells were harvested on a glass fiber prefilter (Advanced Microdevices, Ambala, India) and washed with excess cold water by vacuum filtration. The cells were then immersed in 3 ml of scintillation fluid (Sigma Flour Universal LSC mixture), and radioactivity was measured on a liquid scintillation counter (Wallac Microbeta, 1450 LSC and luminescence counter, PerkinElmer Life Sciences), and the results were expressed as nmol of cystine/mg of protein/min. Total protein was measured as previously described (16).

For saturation kinetics, the initial rate of cystine uptake was measured at different concentrations ranging from 2.5 to 160 μM (160, 80, 40, 20, 10, 5, and 2.5 μM), with specific activity being kept constant at each concentration. The initial rate of cystine uptake was determined by measuring the radioactive cystine accumulated in the cells up to 2 min.

For inhibition studies done with different sulfur compounds, amino acids, and structural analogues of cystine, the inhibitors were added at 20-fold excess along with the assay medium, and initial rates of cystine uptake were measured. For the studies done with the metabolic inhibitors cells were preincubated with the metabolic inhibitors for 15 min at 30 °C before measuring the initial rate of cystine uptake.

RESULTS

Isolation of CAGL0M00154g as an ORF That Allows Utilization of Cystine as a Sulfur Source in *C. glabrata*—*C. glabrata* met15Δ strains, like *S. cerevisiae* met15Δ strains, are organic sulfur auxotrophs and have been previously shown to utilize both cysteine and methionine as a sulfur source suggesting the existence of both forward and reverse transsulfuration pathways in *C. glabrata* (3). We used this *C. glabrata* met15Δ strain to test for the growth on cystine. Interestingly, we observed that *C. glabrata* met15Δ could utilize cystine as a sulfur source whereas *S. cerevisiae* met15Δ failed to do so (Fig. 1A).

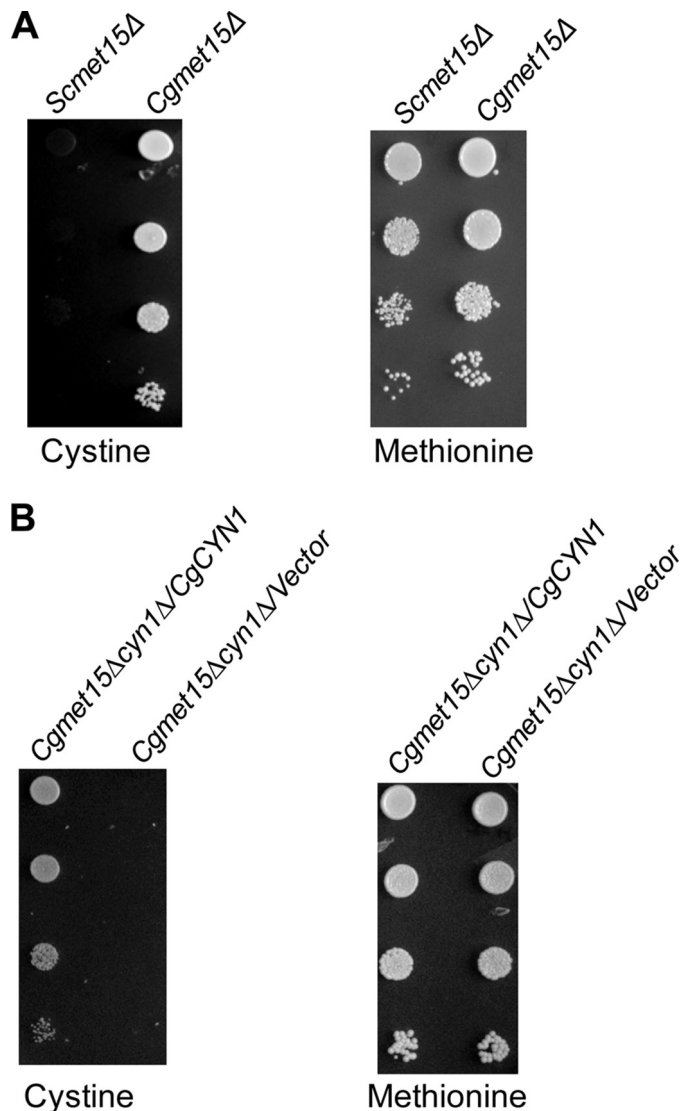


FIGURE 1. A, *C. glabrata* met15Δ, but not *S. cerevisiae* met15Δ, can utilize cystine as a sulfur source. Growth of *S. cerevisiae* and *C. glabrata* met15Δ strains on minimal medium supplemented with 200 μM cystine and 200 μM methionine is shown. B, ORF CAGL0M00154g (CgCYN1) of *C. glabrata* encodes for a functional cystine transporter. Growth of *C. glabrata* met15Δcyn1Δ strain transformed with CgCYN1 under the PGK promoter of *C. glabrata*-specific vector pGRB2.2 (Cgmet15Δcyn1Δ/CgCYN1) and with the corresponding vector control plasmid pGRB2.2 (Cgmet15Δcyn1Δ/Vector) on 200 μM cystine and 200 μM methionine is shown.

To identify the gene(s) in *C. glabrata* that were conferring the ability to grow on cystine, we complemented the *S. cerevisiae* met15Δ strain with a genomic library of *C. glabrata* made in a *S. cerevisiae*-specific vector YEp24. Transformants were selected on minimal medium with cystine as the sole organic sulfur source. A total of 20 transformants were obtained. These transformants were replica-patched on ammonium sulfate, and 16 transformants were found to grow on ammonium sulfate. These were not followed further because they were likely to contain the CgMET15 gene complementing in the Scmet15Δ strain. Plasmids were isolated from those transformants that could grow only on cystine and not on ammonium sulfate and reconfirmed for their phenotype following retransformation in *S. cerevisiae* met15Δ strain.

Sequencing of the inserts using YEp24-specific primers (YEp24F and YEp24R) in these four plasmids revealed a common ORF, *CAGL0M00154g*. The ORF *CAGL0M00154g* was amplified from the genome of *C. glabrata* and cloned into the yeast expression vector p416TEF. Heterologous expression of *CAGL0M00154g* conferred the capability of *S. cerevisiae met15Δ* strain to grow on cystine (supplemental Fig. 1).

To further ascertain that ORF *CAGL0M00154g* was indeed the ORF of *C. glabrata* that was conferring the ability of *C. glabrata* to grow on cystine, we deleted the ORF in a *C. glabrata met15Δ* strain. A complete growth defect was seen on cystine which could be restored by reintroducing ORF *CAGL0M00154g* expressed from the constitutive PGK promoter in *C. glabrata*-specific vector pGRB2.2 (Fig. 1B).

ORF *CAGL0M00154g* (*CgCYN1*) Is a High Affinity Cystine Transporter—The sequence analysis of ORF *CAGL0M00154g* revealed that it encoded a protein of 550 amino acids having 12 predicted transmembrane domains (see Fig. 8A) and belongs to the amino acid permease family of proteins. Based on the phenotypes, it appeared that it was likely to be a cystine transporter. Uptake experiments were performed using ^{35}S -labeled cystine to examine this possibility in *C. glabrata*. However, our initial experiments were complicated by the observation of the presence of radioactive cystine that was apparent in the radioactive cystine supplied by ARC. This was confirmed by a variety of transport experiments (data not shown) exploiting the *S. cerevisiae yct1Δmet15Δ* strain background. *YCT1* is a high affinity cystine-specific transporter that does not transport cystine as described previously (17). Briefly, TEF-*YCT1* transformed into *S. cerevisiae yct1Δmet15Δ* also revealed radioactive uptake using the ARC-supplied radioactive cystine (blocked by cold cystine, but not cystine). In contrast, TEF-*CgCYN1*-transformed strains also showed uptake (but blocked by cold cystine, not cystine). Furthermore, TEF-*YCT1* (but not TEF-*CgCYN1*) transformants could take up [^{35}S]cystine (from Bhabha Atomic Research Centre). The presence of specific cystine (and now cystine) transporters has allowed us to develop this as a simple bioassay for the presence of cystine and cystine in these radioactive samples.⁴ Because of the presence of contaminating radioactive cystine, the kinetic parameters for the *C. glabrata* ORF was determined in the *S. cerevisiae yct1Δ met15Δ* strains thus eliminating any interference from the contaminating cystine in the radioactive cystine supplied by ARC. We thus could get rid of the background given by the radioactive cystine. Thus, when *yct1Δmet15Δ* strain was transformed with TEF-*CgCYN1* a clear difference could be seen between the experimental transformant (*yct1Δmet15Δ/TEF-CgCYN1*) as opposed to the control transformant (*yct1Δmet15Δ/TEF*) (Fig. 2A).

To determine the K_m and the V_{\max} values for *CgCYN1*, *S. cerevisiae yct1Δmet15Δ* strain was again transformed with the cystine transporter *CgCYN1* under the TEF promoter in *S. cerevisiae yct1Δmet15Δ* strain along with the vector control p416TEF. Substrate concentrations ranging from 2.5 to 160 μM were used for determining the kinetic parameters. The K_m

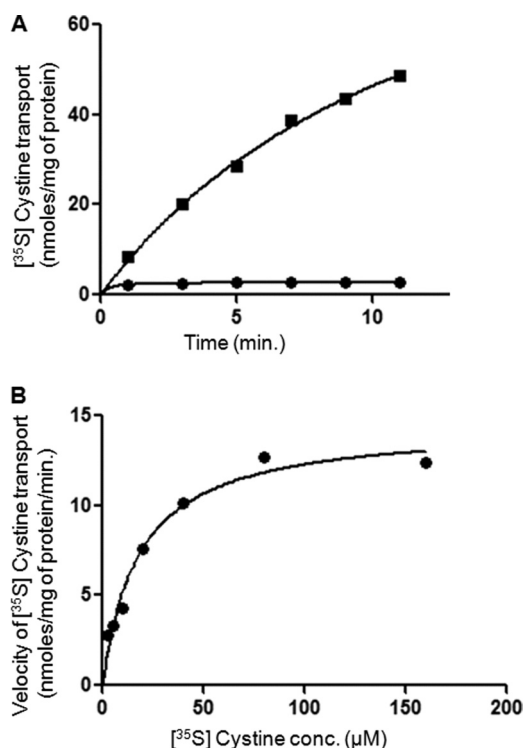


FIGURE 2. *CgCYN1* expressed in *S. cerevisiae met15Δyct1Δ* strain leads to uptake of [^{35}S]cystine. A, *Sc met15Δyct1Δ* strain was transformed with TEF-*CgCYN1* (■) and with the corresponding vector control p416TEF (●). Transformants were grown in simple defined medium (1.7 g/liter yeast nitrogen base, 20 g/liter glucose, 5 g/liter ammonium sulfate, and amino acid supplements at 70 mg/liter) containing 200 μM methionine, and log phase-grown cells were then harvested and used for the transport experiment as described under "Materials and Methods." Data shown are the mean of three experiments, with each experiment done in duplicate. B, Michaelis-Menten curve for the cystine uptake mediated by *CgCYN1* is shown. *Sc met15Δyct1Δ* strain was transformed with TEF-*CgCYN1* and with the corresponding vector control p416TEF. Transformants were then grown in simple defined medium containing 200 μM methionine followed by harvesting log phase cells. The initial rate of cystine uptake was then measured at concentrations ranging from 2.5 to 160 μM (2.5, 5, 10, 20, 40, 80, and 160 μM) by harvesting cells after their incubation at different concentrations of cystine at two time points (1 and 2 min). Data are representative of three experiments. Kinetic parameters and statistical analysis were done by nonlinear regression analysis of Michaelis-Menten plots using Prism version 5.02 (GraphPad Software, San Diego, CA).

value of $18 \pm 5 \mu\text{M}$ and a V_{\max} of 14 ± 4 nmol of cystine/mg of protein/min were found for the newly found cystine transporter. A K_m value of $18 \pm 5 \mu\text{M}$ suggests that the newly found cystine transporter is a high affinity cystine transporter (Fig. 2B). We have accordingly named ORF *CAGL0M00154g* as *CYN1*.

***CgCYN1* Is a Cystine-specific Transporter**—To examine the substrate specificity of *CgCYN1*, the rate of uptake of cystine was measured in *S. cerevisiae yct1Δmet15Δ* strain transformed with *CgCYN1* in the presence of different amino acids, cystine analogues, and various sulfur compounds. These inhibitors were added at >20-fold excess over the labeled substrate. The cells were harvested at 1- and 2-min intervals, and the results were normalized to the rate of uptake measured in the absence of any inhibitor.

Among the various amino acids like glycine, glutamic acid, cysteine, homocysteine, methionine, leucine, proline, valine, phenylalanine, serine, glutamine, and lysine, none could inhibit the transport for cystine thus showing the specificity of *CYN1*

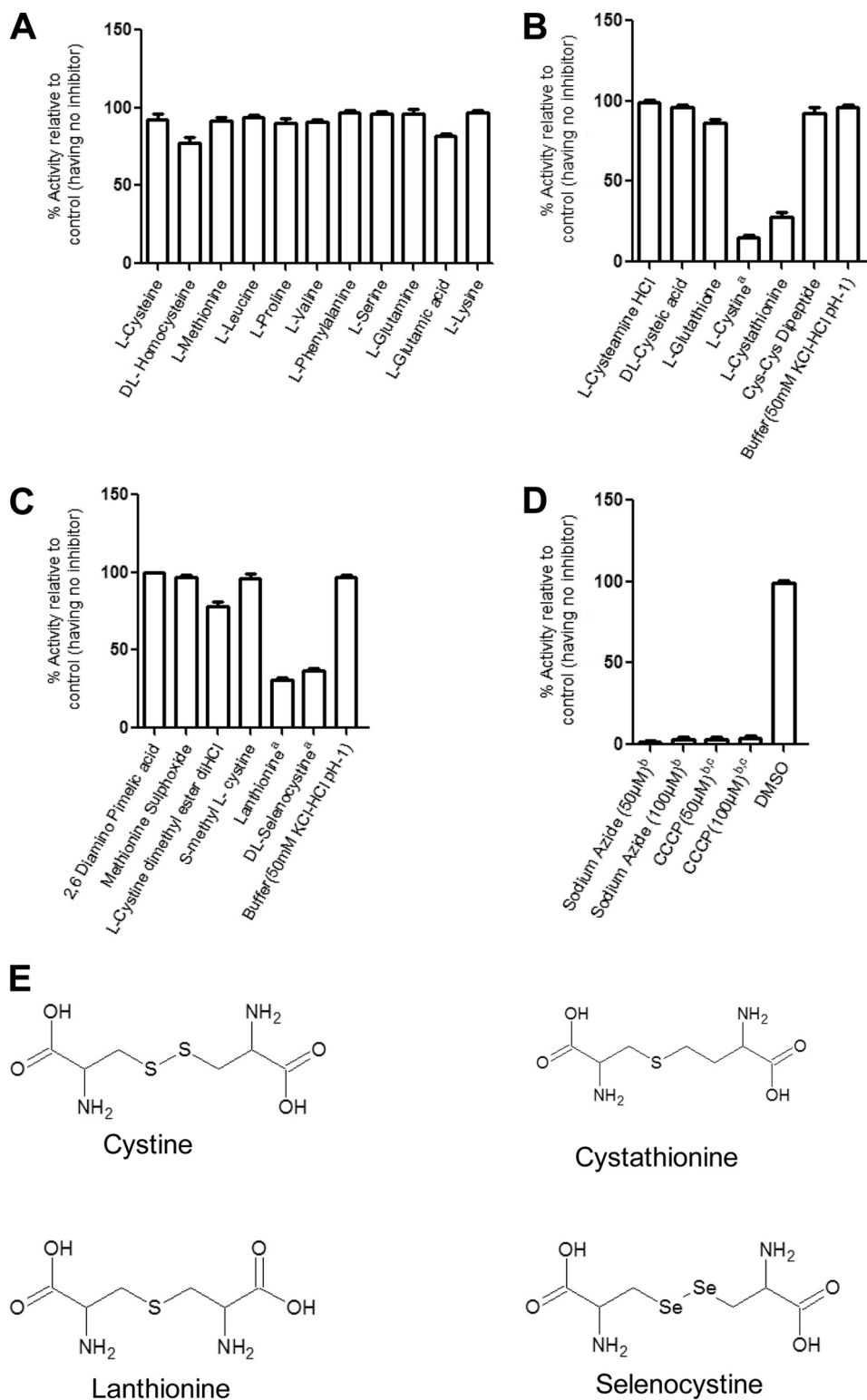
⁴ A. K. Yadav and A. K. Bachhawat, unpublished data.

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for cystine (Fig. 3A). Among the sulfur-containing compounds such as cysteamine hydrochloride, cystic acid, glutathione, cystathionine, and Cys-Cys dipeptide, only cystine and cystathionine could inhibit the transport (Fig. 3B). When one looks at the structure of these two compounds the structures are very similar and differ only in that the cystathionine has a single sulfur atom instead of two in cystine (Fig. 3E). Seeing the sig-

nificant transport inhibition by cystathionine, the growth of *Cgmet15Δcyn1Δ* was checked on cystathionine, and we observed that *Cgmet15Δ* strain could utilize cystathionine as a sole source of sulfur in a *CYN1*-dependent manner (Fig. 4).

Among the structural homologues to cystine, various compounds like 2,6-diamino pimelic acid, methionine sulfoxide, cystine dimethyl ester dihydrochloride, lanthionine, and sel-



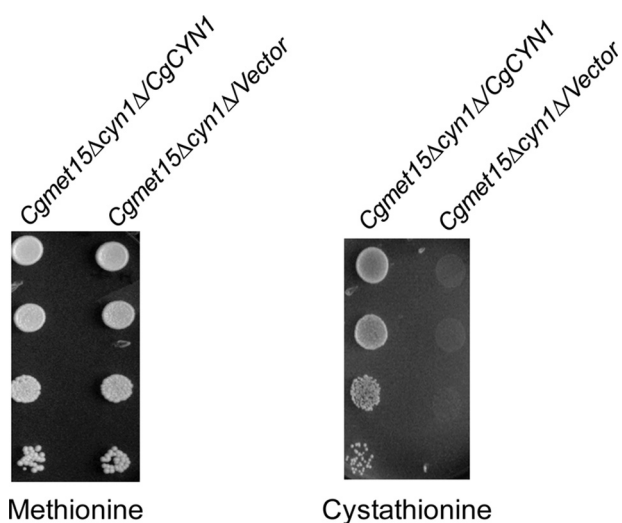


FIGURE 4. *C. glabrata* requires *CgCYN1* to utilize cystathionine as a sole source of sulfur. Growth of *C. glabrata met15Δcyn1Δ* strain transformed with *CgCYN1* under the PGK promoter of *C. glabrata*-specific vector pGRB2.2 (*Cgmet15Δcyn1Δ/CgCYN1*) and with the corresponding vector control plasmid pGRB2.2 (*Cgmet15Δcyn1Δ/Vector*) on 200 μ M cystathionine and 200 μ M methionine.

enocystine were taken for the competitive experiments at a 20-fold molar excess over the substrate, and it was found that only lanthionine and selenocystine could inhibit the transport by *CgCYN1* (Fig. 3C). Structurally, selenocystine is like cystine where the sulfur atoms of cystine have been replaced with selenium in selenocystine, and lanthionine is more similar to cystathionine and differs in that it lacks one carbon atom compared with cystathionine (Fig. 3E).

To determine whether the uptake of cystine by *CgCYN1* is energy-dependent, metabolic inhibitors like sodium azide and carbonyl cyanide *p*-chlorophenylhydrazone were used at 50 and 100 μ M concentrations. A 15-min preincubation of these inhibitors with the transformants expressing the cystine transporter (*Scyct1Δmet15Δ/TEF-CgCYN1*) resulted in a considerable loss in the cystine uptake thus suggesting that cystine transport by *CgCYN1* is energy mediated (Fig. 3D).

***CgCYN1* Localizes to the Plasma Membrane**—The ability of *CgCYN1* to take up cystine with high affinity from whole cells suggested that it was a plasma membrane transporter. To confirm this, *CgCYN1* was tagged with a nine-amino acid HA tag at the C terminus and expressed from the constitutive PGK promoter in the *C. glabrata* vector pGRB2.2. The tagged protein was functional as seen by complementation in the

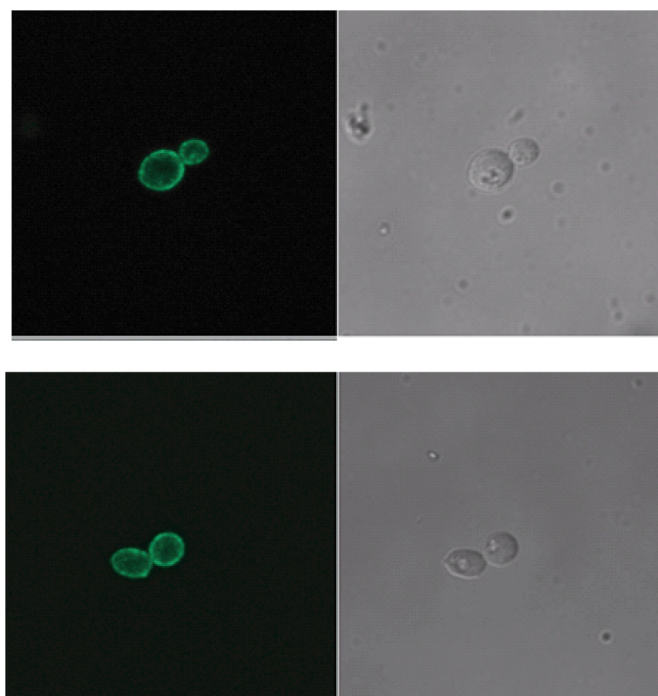


FIGURE 5. ***CgCYN1* is a plasma membrane-localized protein.** *Cgmet15Δcyn1Δ* strain complemented with HA-tagged *CgCYN1* under the constitutive PGK promoter was labeled by indirect immunofluorescence using mouse anti-HA primary antibody (1:250) and goat anti-mouse IgG HRP-conjugated secondary antibody followed by visualization using the confocal microscope as described under “Materials and Methods.”

Cgmet15Δcyn1Δ strain (supplemental Fig. 2). Indirect immunofluorescence was done using confocal microscopy as described under “Materials and Methods.” We observed a signal only on the plasma membrane (Fig. 5). No intracellular signal was seen in all cells. This confirmed that *CgCYN1* is exclusively localized to the plasma membrane.

***H. capsulatum* and *C. albicans* Orthologues of *CgCYN1* Also Function as Cystine Transporters**—We examined for the presence of *CgCYN1* orthologues in the pathogenic yeast, *H. capsulatum* and *C. albicans*. In *C. albicans* ORF *Ca019.9873* (similarity of 74% and identity of 57%) appeared as the putative orthologue. This ORF was cloned, and transformed in an *S. cerevisiae met15Δ* background. We observed that *Ca019.9873* (*CaCYN1*) could complement in *S. cerevisiae* very effectively for growth on cystine (Fig. 6A). To further validate the role of *C. albicans* ORF *Ca019.9873* in cystine transport we deleted this ORF in a *met15Δ* background of *C. albicans* and

FIGURE 3. *A*, inhibition of cystine uptake by different amino acids. The rate of cystine uptake was measured in *S. cerevisiae met15Δyct1Δ* strain transformed with the cystine transporter *CYN1* in the presence of different inhibitors added at 20-fold excess over the labeled substrate (15 μ M). The cells were harvested at 1- and 2-min intervals. The results were normalized to the rate of uptake measured in the absence of any inhibitor. Data are shown as mean \pm S.D. ($n = 2$). *B*, inhibition of cystine uptake by different sulfur compounds. The rate of cystine uptake was measured in *S. cerevisiae met15Δyct1Δ* strain transformed with the cystine transporter *CYN1* in the presence of different inhibitors added at 20-fold excess over the labeled substrate (15 μ M). The cells were harvested at 1- and 2-min intervals. The results were normalized to the rate of uptake measured in the absence of any inhibitor. Data are shown as mean \pm S.D. ($n = 2$). Inhibitors L-cystine, lanthionine, and DL-selenocystine were dissolved in a buffer of pH 1 (50 mM KCl-HCl, pH 1), hence the buffer was analyzed for its effect on cystine uptake. *C*, inhibition of cystine uptake by different cystine analog. The rate of cystine uptake was measured in *S. cerevisiae met15Δyct1Δ* strain transformed with the cystine transporter *CYN1* in the presence of different inhibitors added at 20-fold excess over the labeled substrate (15 μ M). The cells were harvested at 1- and 2-min intervals. The results were normalized to the rate of uptake measured in the absence of any inhibitor. Data are shown as mean \pm S.D. ($n = 2$). *D*, inhibition of cystine uptake by metabolic inhibitors. The rate of cystine uptake was measured in *S. cerevisiae met15Δyct1Δ* strain transformed with the cystine transporter *CYN1* which was preincubated with the metabolic inhibitors as described under “Materials and Methods.” Data are shown as mean \pm S.D. ($n = 2$). Cells were preincubated with the indicated concentrations of metabolic inhibitors for 15 min prior to the addition of the uptake medium. Carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) was dissolved in dimethyl sulfoxide (DMSO), and thus it was analyzed for its effect on cystine uptake. *E*, structure of different inhibitors inhibiting the cystine transport by *CgCYN1*.

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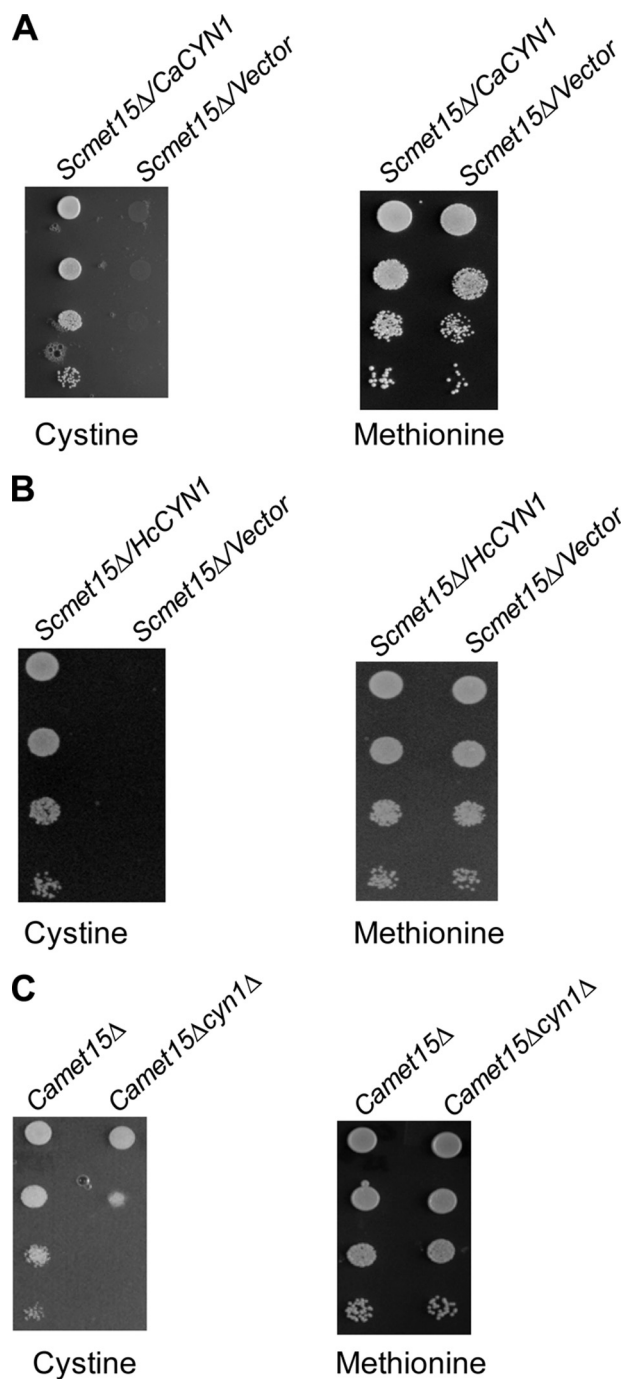


FIGURE 6. *C. albicans* orthologue of CgCYN1 (ORF Ca019.9873) and *H. capsulatum* orthologue of CgCYN1 (ORF HCAG_06385) encode for a functional cystine transporter. A, growth of *S. cerevisiae* met15Δ strain transformed with *C. albicans* ORF Ca019.9873 (CaCYN1) under the TEF promoter of *S. cerevisiae*-specific shuttle vector p416TEF (Scmet15Δ/CaCYN1) and with the corresponding vector control plasmid p416TEF (Scmet15Δ/Vector) on 200 μM cystine and 200 μM methionine. B, growth of *S. cerevisiae* met15Δ strain transformed with the *H. capsulatum* ORF HCAG_06385 (HcCYN1) under the TEF promoter in *S. cerevisiae*-specific shuttle vector p416TEF (Scmet15Δ/HcCYN1) and with the corresponding vector control plasmid p416TEF (Scmet15Δ/Vector) on 200 μM cystine and 200 μM methionine. C, growth of *C. albicans* met15Δ and met15Δcyn1Δ strain on 200 μM cystine and 200 μM methionine.

found that the deletion caused abrogation of growth on cystine. The residual growth seen on cystine plates is the result of only partial organic sulfur auxotrophy seen in *C. albicans* met15Δ strain (20) and not a consequence of growth on cystine. This

clearly indicated that ORF Ca019.9873 encodes for a functional cystine transporter in *C. albicans* (Fig. 6C).

H. capsulatum is a dimorphic fungus where cystine uptake has been seen in the yeast phase (4). In *H. capsulatum* ORF HCAG_06385 appeared to be the orthologue of the *C. glabrata* CYN1 protein (identity of 56% and similarity of 73%). ORF HCAG_06385 contained two introns, so we custom synthesized the cDNA and expressed it downstream of the TEF promoter in p416TEF followed by transforming it in *S. cerevisiae* met15Δ strain. We observed that the ORF complemented for growth on cystine in *S. cerevisiae*, indicating that ORF HCAG_06385 of *H. capsulatum* is also a cystine transporter (Fig. 6B). Thus, it can be concluded that CgCYN1 has functional homologues in *C. albicans* and *H. capsulatum* (multiple sequence alignment is shown in Fig. 8B).

The S. cerevisiae Homologue of CgCYN1 Is a Lysine Permease (LYP1), and the S. pombe Orthologue for CgCYN1 (SPCPB1C11.02) Has No Role in Cystine Transport—S. cerevisiae lacks the ability to grow on cystine as described earlier and was thus likely to lack an orthologue of CgCYN1. The closest homologue of CgCYN1 in *S. cerevisiae* is LYP1 which is a lysine transporter (identity 37%). LYP1 is also a member of the AAP family whose orthologue exists in *C. glabrata* (CAGL0J08162g).

When seeking the orthologue of CYN1 in the *S. pombe* genome, ORF SPCPB1C11.02 was picked up (having a identity of 41% and similarity of 62%). This also appeared to be the orthologue from reverse BLAST analysis. *S. pombe* also has a distinct lysine permease distinct from this ORF. To examine whether ORF SPCPB1C11.02 functioned as a cystine transporter we cloned and expressed this in *S. cerevisiae* met15Δ strain to see whether it could enable *S. cerevisiae* to utilize cystine. However, even when SPCPB1C11.02 was overexpressed under the TEF promoter and transformed in *S. cerevisiae* met15Δ strain, it could not confer growth on cystine (supplemental Fig. 3A). To eliminate the possibility that it might be a problem of heterologous expression, we examined the *cys1a*Δ strain of *S. pombe* which is a cysteine auxotroph (21) for growth on cystine. We found that it could not grow on cystine even at high concentrations of cystine (600 μM), further confirming that *S. pombe* lacked a cystine transporter (supplemental Fig. 3B).

*CgCYN1 Represents a Novel Family of Cystine Transporters—*The presence of cystine transporters in three pathogenic yeast species, and its absence among *S. cerevisiae* and *S. pombe*, prompted us to do a phylogenetic analysis of these transporters in different yeast and fungi. The lysine permease, LYP1 of *S. cerevisiae*, was the closest homologue of CgCYN1 in *S. cerevisiae* (37% identity, 57% similarity), and *C. glabrata* also contained a separate LYP1 orthologue. We therefore extracted the close homologues of CgCYN1 in different yeast and fungi, while also extracting the lysine permease homologues of these different species. When we look at the phylogenetic tree there seems to be clear distinction between the lysine transporters and the cystine transporters clusters (Fig. 7). Furthermore, the CgCYN1 orthologues appeared to be present among many *Aspergillus* species. These organism, like *C. glabrata*, also contained orthologues of the *S. cerevisiae* lysine permease, LYP1. *S. pombe* contained a LYP1 orthologue, but also contained an ORF

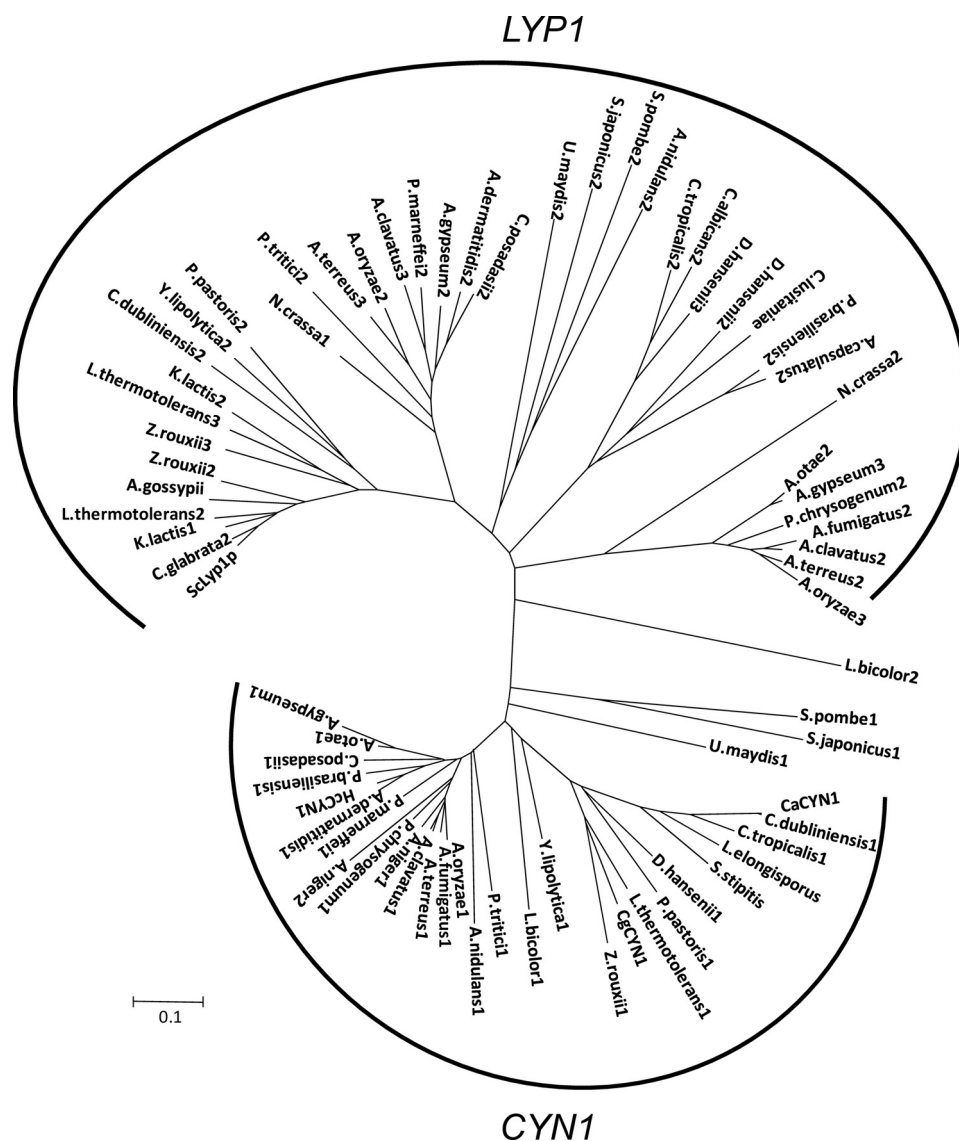


FIGURE 7. **Phylogenetic tree of the CYN1 and LYP1 families.** Homologues of *C. glabrata* CYN1 and *S. cerevisiae* LYP1 were retrieved and aligned using ClustalW, and phylogenetic analysis was carried out by MEGA4 software. The scale bar corresponds to 0.1 estimated amino acid substitutions per site.

SPCPB11.02 lying somewhere between these two clusters. However, this ORF did not appear to function as a cystine transporter as described above (Fig. 8).

DISCUSSION

The work presented here describes a novel plasma membrane cystine transporter of yeast. The transporter belongs to a distinctly different family from that previously reported for cystine transporters of humans and bacteria. Humans have the heterodimeric Na^+ -independent cystine/glutamate transporter (system x_c^-) that mediates the uptake the cystine against the exchange of glutamate (22), and a second b^{0+} transporter that takes up cystine (or dibasic amino acids) against the efflux of neutral amino acids (23). The lysosomal cystine transporter of mammals, CTNS, which has functional homologues in the yeast vacuoles (ERS1) (24) and is involved in H^+ -driven efflux of cystine from these organelles, also belongs to a separate family of transporters (LCT). Bacteria have both high and low affinity

transporters of cystine, and most of them belong to the ABC uptake system. However, in *Bacillus subtilis*, out of three transporters transporting cystine (TcyP, TcyJKLMN, and TcyABC) TcyP belongs to the dicarboxylate amino acid cation symporter family.

The existence of a pathway for cystine utilization in pathogenic yeast was not unexpected, considering that cystine is the most abundant sulfur compound in blood plasma. Cystine transport has been described in *H. capsulatum* 4 decades ago (4), but the gene was never identified. Cystine utilization has also been documented in dermatophytes like *Microsporum gypseum* (25) which secrete sulfite that reduces the cystine in keratin (present in abundance in their ecological niche) to cysteine and *S*-sulfocysteine, which are then subsequently taken up following proteolysis (26, 27). Which of these or other pathways might operate in *C. glabrata* was not clear. The findings described here have revealed that a novel transporter was mediating the utilization in not only *C. glabrata* but also in *C. albi-*

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FIGURE 8. A, pictorial representation of the putative topology of *CgCYN1* as predicted by HMMTOP software. B, multiple sequence alignment of *CgCYN1* and its homologues (*CaCYN1* and *HcCYN1*). The shaded regions correspond to the 12 transmembrane domains as predicted by HMMTOP software.

cans and *H. capsulatum*. The discovery thus also takes to a satisfying conclusion the observations made with *H. capsulatum* 40 years ago.

There are two possible reasons that might have delayed the discovery of the identity of this transporter. The first is that much of the work on sulfur and amino acid transporters has

focused on *S. cerevisiae* and *S. pombe*, and the transporter is lacking in these yeast species. A second reason might be due to the observation made in this work that the radioactive cystine had substantial levels of radioactive cysteine. It is possible that samples of radioactive cystine may sometimes carry this contamination with cysteine. However, as yeast species also carry

transporters of cysteine, the identification of a cystine transporter can be masked or complicated in such a situation. In the present study we were greatly assisted in our studies by our use of the *S. cerevisiae* strain defective in high affinity cysteine uptake (*ycf1Δ*). Using this strain has eliminated background cysteine uptake and has been a powerful aid to uncovering this cystine transporter from *C. glabrata*.

Cystathionine, an intermediate in cysteine biosynthesis in yeast, was found to be a potent inhibitor of transport along with cystine, and when one looks at the structure of these two compounds the structures are very similar except that the cystathionine has a single sulfur atom instead of two in cystine (Fig. 3E). Cystathionine is also present at low levels in blood plasma (3). Not surprisingly, we observed that cystathionine could also be used efficiently as a sulfur source in this yeast. The only other two inhibitors were known cystine analogues, lanthionine and selenocystine, which indicates the high specificity of the transporter for cystine. No other amino acid, including cysteine or even lysine, could inhibit cysteine transport through this transporter.

The K_m of CgCYN1 for cystine was found to be 18 μM , a value very close to the value of 30 μM described for *H. capsulatum* many years ago (5) and close to the reported concentrations of cystine (50 μM) in the blood plasma (4). Although bacterial cystine transporters have been reported of still higher affinity, the affinity of CgCYN1 is also reasonably high considering the fact that it is in the micromolar range and thus it would be adequate for this transporter to be effective *in vivo*.

Because *C. glabrata* lacks a glutathione transporter and relies solely on the endogenous production of glutathione for its survival (3) the presence of a cystine transporter would be important for the cell not only for meeting the cysteine requirements, but also to meet the glutathione requirements, because cysteine is rate-limiting for glutathione synthesis *in vivo*. Increased glutathione requirements become very crucial under the conditions of stress as survival in the host is in itself a stress for the pathogen.

CYN1 orthologues were absent in *S. cerevisiae*, and despite an apparent orthologue in *S. pombe*, there was no cystine transporter in the fission yeast as well. In contrast, cystine transporters seem to have been acquired by a large number of pathogenic yeast, with orthologues also appearing to be present in many aspergilli. Whether the presence of this gene actually leads to a defect in virulence in these organisms, however, remains to be established. Nevertheless, the acquisition of a new gene (CYN1) in an otherwise slimmer *C. glabrata* genome (*C. glabrata* has 398 membrane proteins whereas *S. cerevisiae* has 502 membrane proteins) (28) and the prevalence of this transporter among pathogenic yeast and fungi seem to suggest that it may play an important role in their *in vivo* survival.

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REFERENCES

- Kaur, R., Domergue, R., Zupancic, M. L., and Cormack, B. P. (2005) *Curr. Opin. Microbiol.* **8**, 378–384
- Dujon, B., Sherman, D., Fischer, G., Durrens, P., Casaregola, S., Lafontaine, I., De Montigny, J., Marck, C., Neuvéglise, C., Talla, E., Goffard, N., Franke, L., Aigle, M., Anthouard, V., Babour, A., Barbe, V., Barnay, S., Blanchin, S., Beckerich, J. M., Beyne, E., Bleykasten, C., Boisramé, A., Boyer, J., Cattolico, L., Confaniolero, F., De Daruvar, A., Despons, L., Fabre, E., Fairhead, C., Ferry-Dumazet, H., Groppi, A., Hantraye, F., Hennequin, C., Jauniaux, N., Joyet, P., Kachouri, R., Kerrest, A., Koszul, R., Lemaire, M., Lesur, I., Ma, L., Muller, H., Nicaud, J. M., Nikolski, M., Oztas, S., Ozier-Kalogeropoulos, O., Pellenz, S., Potier, S., Richard, G. F., Straub, M. L., Suleau, A., Swennen, D., Tekai, F., Wésolowski-Louvel, M., Westhof, E., Wirth, B., Zeniou-Meyer, M., Zivanovic, I., Bolotin-Fukuhara, M., Thierry, A., Bouchier, C., Caudron, B., Scarpelli, C., Gaillardin, C., Weissenbach, J., Wincker, P., and Souciet, J. L. (2004) *Nature* **430**, 35–44
- Yadav, A. K., Desai, P. R., Rai, M. N., Kaur, R., Ganesan, K., and Bachhawat, A. K. (2011) *Microbiology* **157**, 484–495
- Jones, D. P., Carlson, J. L., Mody, V. C., Cai, J., Lynn, M. J., and Sternberg, P. (2000) *Free Radic. Biol. Med.* **28**, 625–635
- Gilbert, B. E., and Howard, D. H. (1970) *Infect. Immun.* **2**, 139–144
- Rajan, D. P., Kekuda, R., Huang, W., Wang, H., Devoe, L. D., Leibach, F. H., Prasad, P. D., and Ganapathy, V. (1999) *J. Biol. Chem.* **274**, 29005–29010
- Rajan, D. P., Huang, W., Kekuda, R., George, R. L., Wang, J., Conway, S. J., Devoe, L. D., Leibach, F. H., Prasad, P. D., and Ganapathy, V. (2000) *J. Biol. Chem.* **275**, 14331–14335
- Sato, H., Tamba, M., Ishii, T., and Bannai, S. (1999) *J. Biol. Chem.* **274**, 11455–11458
- Burguière, P., Auger, S., Hullo, M. F., Danchin, A., and Martin-Verstraete, I. (2004) *J. Bacteriol.* **186**, 4875–4884
- Turner, M. S., Woodberry, T., Hafner, L. M., and Giffard, P. M. (1999) *J. Bacteriol.* **181**, 2192–2198
- Baptist, E. W., and Kredich, N. M. (1977) *J. Bacteriol.* **131**, 111–118
- Butler, J. D., Levin, S. W., Facchiano, A., Miele, L., and Mukherjee, A. B. (1993) *Life Sci.* **52**, 1209–1215
- Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20**, 1425
- Reuss, O., Vik, A., Kolter, R., and Morschhäuser, J. (2004) *Gene* **341**, 119–127
- Earley, M. C., and Crouse, G. F. (1996) *Gene* **169**, 111–113
- Kaur, J., and Bachhawat, A. K. (2009) *J. Biol. Chem.* **284**, 23872–23884
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673–4680
- Saitou, N., and Nei, M. (1987) *Mol. Biol. Evol.* **4**, 406–425
- Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) *Mol. Biol. Evol.* **24**, 1596–1599
- Viaene, J., Tiels, P., Logghe, M., Dewaele, S., Martinet, W., and Contreras, R. (2000) *Yeast* **16**, 1205–1215
- Fujita, Y., and Takegawa, K. (2004) *Biosci. Biotechnol. Biochem.* **68**, 306–311
- Bannai, S. (1986) *J. Biol. Chem.* **261**, 2256–2263
- Feliubadaló, L., Font, M., Purroy, J., Rousaud, F., Estivill, X., Nunes, V., Golomb, E., Centola, M., Aksentijevich, I., Kreiss, Y., Goldman, B., Pras, M., Kastner, D. L., Pras, E., Gasparini, P., Bisceglia, L., Beccia, E., Gallucci, M., de Sanctis, L., Ponzzone, A., Rizzoni, G. F., Zelante, L., Bassi, M. T., George, A. L., Jr., Manzoni, M., De Grandi, A., Riboni, M., Endsley, J. K., Ballabio, A., Borsani, G., Reig, N., Fernández, E., Estévez, R., Pineda, M., Torrents, D., Camps, M., Lloberas, J., Zorzano, A., and Palacín, M. (1999) *Nat. Genet.* **23**, 52–57
- Gao, X. D., Wang, J., Keppler-Ross, S., and Dean, N. (2005) *FEBS J.* **272**, 2497–2511
- Stahl, W. H., and McQue, B. (1949) *Arch. Biochem.* **20**, 422–432
- Kunert, J. (1972) *Experientia* **28**, 1025–1026
- Kunert, J. (1976) *Z. Allg. Mikrobiol.* **16**, 97–105
- De Hertogh, B., Hancy, F., Goffeau, A., and Baret, P. V. (2006) *Genetics* **172**, 771–781