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Yct1p, a Novel, High-Affinity, Cysteine-Specific Transporter From the Yeast *Saccharomyces cerevisiae*

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ABSTRACT

Cysteine transport in the yeast *Saccharomyces cerevisiae* is mediated by at least eight different permeases, none of which are specific for cysteine. We describe a novel, high-affinity, ($K_m = 55$ μM), cysteine-specific transporter encoded by the ORF *YLL055w* that was initially identified by a combined strategy of data mining, bioinformatics, and genetic analysis. Null mutants of *YLL055w*, but not of the other genes encoding for transporters that mediate cysteine uptake such as *GAP1*, *GNP1*, *MUP1*, or *AGP1* in a *met15*Δ background, resulted in a growth defect when cysteine, at low concentrations, was provided as the sole sulfur source. Transport experiments further revealed that Yll055wp was the major contributor to cysteine transport under these conditions. The contributions of the other transporters became relevant only at higher concentrations of cysteine or when *YLL055w* was either deleted or repressed. *YLL055w* expression was repressed by organic sulfur sources and was mediated by the Met4p-dependent sulfur regulatory network. The results reveal that *YLL055w* encodes the principal cysteine transporter in *S. cerevisiae*, which we have named *YCT1* (*y*east *c*ysteine *t*ransporter). Interestingly, Yct1p belongs to the Dal5p family of transporters rather than the amino acid permease family to which all the known amino acid transporters belong.

CYSTEINE, with its free sulphydryl group, is an important amino acid residue for the structural and functional properties of proteins. The free thiol group of cysteine is involved in the formation of disulphide bonds, crucial for the stability of certain proteins, and is also an important catalytic and redox center in various enzymes, cofactors, and regulatory proteins. Cysteine is also the rate-limiting nutrient in glutathione biosynthesis (Alfafara *et al.* 1992; Wen *et al.* 2004), the major redox buffer and detoxification molecule in the cell. Studies have revealed that the entire sulfur assimilation pathway leading to cysteine biosynthesis is upregulated during increased cellular demands of glutathione upon exposure to heavy metals or other toxic compounds in yeasts and plants (Vido *et al.* 2001; Fauchon *et al.* 2002; Aranda and del Olmo 2004; Mendoza-Cozatl *et al.* 2005). Despite the importance of cysteine in cellular metabolism, detoxification, and stress response, an increased cysteine level has been shown to be toxic to cells (Ono *et al.* 1991; Kumar *et al.* 2006). The intracellular cysteine levels are thus tightly regulated. In addition to the *de novo* synthesis of cysteine from inorganic sulfur in *Saccharomyces cerevisiae*, the transport of cysteine from the extracellular medium also contributes to the cellular cysteine homeostasis.

Several studies have been carried out to biochemically characterize cysteine transport and to identify the transporter proteins responsible for uptake of cysteine in *S. cerevisiae*. However, these studies have been complicated by the different strain backgrounds and growth conditions employed and have led to quite contradictory conclusions. In one of the earliest reports, cysteine uptake by a brewing strain of *S. cerevisiae* was found to be slow and nonsaturable over a large concentration range of cysteine (Maw 1963). Similarly, During-Olsen *et al.* (1999) showed that cysteine uptake was nonsaturable in the presence of ammonia as the nitrogen source and that two or more transport systems for cysteine uptake existed. In contrast, Ono and Naito (1991) reported the presence of a specific and a saturable cysteine transport system in a wild-type S288C strain background, which is derepressed only in the absence of sulfur in the growth medium.

Several broad-specificity transporter proteins that can mediate the transport of cysteine in *S. cerevisiae* have been identified (During-Olsen *et al.* 1999; Regenberg *et al.* 1999; Kosugi *et al.* 2001). During-Olsen *et al.* (1999) employed an overexpression strategy that identified several members of the amino acid permease (AAP) family that could mediate cysteine uptake under different growth conditions. However, these transporters mediated the uptake of cysteine in a nonspecific manner. They included the general amino acid permease, Gap1p, and the asparagine and glutamine amino acid permease, Agp1p, as the main transporters in

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TABLE 1

Strains used in this study

Strain	Genotype	Source
ABC 734 (BY4742)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	J. Boeke
ABC 733 (BY4741)	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	J. Boeke
ABC 1080 (CC718-1A)	*MAT***a** *his 3 leu2 ura3 ade2 trp1 cbf1::TRP1*	Y. Surdin-Kerjan
ABC 1081 (CC950-2A)	*MAT***a** *his 3 leu2 ura3 ade2 trp1 met 4::TRP1*	Y. Surdin-Kerjan
ABC 1082 (CD130-7D)	*MAT***a** *his 3 leu2 ura3 ade2 trp1 met 28::LEU2*	Y. Surdin-Kerjan
ABC 1097	*MAT***a** *his3Δ1 leu 2Δ0 lys2Δ 0 ura3Δ 0 met31Δ::KanMX4*	EUROSCARF
ABC 1098	*MAT***a** *his3Δ1 leu 2Δ 0 lys2Δ 0 ura3Δ 0 met32Δ::KanMX4*	EUROSCARF
ABC 1381 (W303)	*MAT***a** *his 3 leu2 ura3 ade2 trp1*	Y. Surdin-Kerjan
ABC 1580	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yll055wΔ::KanMX4*	EUROSCARF
ABC 1814	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLL055w::GFP-HIS3MX*	Invitrogen (San Diego)
ABC 1827	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gnp1Δ::KanMX4*	EUROSCARF
ABC 1839	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mup1Δ::KanMX4*	EUROSCARF
ABC 1842	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gap1Δ::KanMX4*	EUROSCARF
ABC 1846	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 agp1Δ::KanMX4*	EUROSCARF
ABC 1902	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gnp1Δ::KanMX4 yll055w::HIS4*	This study
ABC 1903	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mup1Δ::KanMX4 yll055w::HIS4*	This study
ABC 1904	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yll055w::HIS4*	This study
ABC 1905	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 agp1Δ::KanMX4 yll055w::HIS4*	This study
ABC 2090	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gnp1Δ::KanMX4 mup1Δ::LEU2*	This study
ABC 2091	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gnp1Δ::KanMX4 mup1Δ::LEU2 yll055w::HIS4*	This study
ABC 2067	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yal067cΔ::KanMX4*	EUROSCARF
ABC 2072	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yil166cΔ::KanMX4*	EUROSCARF

proline medium (non-nitrogen repressing); the glutamine high-affinity permease, Gnp1p; the branched-chain amino acid transporters, Bap1p and Bap2p; and the amino acid transporters, Tat1p and Tat2p, which contributed to cysteine transport under ammonia-rich conditions (During-Olsen *et al.* 1999). In another study, Kosugi *et al.* (2001) employed a genetic strategy to show the involvement of Mup1p, a high-affinity methionine permease, also belonging to the AAP family, in cysteine uptake. On the basis of these studies, it appears that cysteine is not taken up by a specific permease, but rather by multiple permeases with broad specificity, each active under different sets of growth conditions. However, no specific high-affinity transporter for cysteine uptake has been proposed or characterized in the yeast *S. cerevisiae* so far.

The absence of a specific trasnporter for this important amino acid has appeared puzzling to us, and we have therefore considered it worthwhile to reexamine the yeast genome for the existence of a cysteine-specific transporter. Starting with an analysis that mined existing genomewide data, we sought out membrane transporters that were being derepressed under conditions of increased cysteine requirements in the cell. This analysis identified a candidate transporter, Yll055wp of unassigned function, belonging to the Dal5p transporter family. Although all the amino acid transporters described so far in *S. cerevisiae* fall into the amino acid permease family, we nevertheless investigated the possibility that *YLL055w* might encode a cysteine trasnporter. Detailed genetic, molecular, and biochemical analyses of this protein along with studies on its regulation, which are described in this report, reveal that the *YLL055w* ORF encodes a high-affinity, cysteine-specific transporter.

MATERIALS AND METHODS

Chemicals and reagents: All the chemicals used in this study were obtained from commercial sources and were of analytical grade. Media components were purchased from Difco (Detroit). Oligonucleotides were purchased from Biobasic (Markham, ON, Canada). Restriction enzymes, Vent DNA polymerase, and *Taq* DNA polymerase and other modifying enzymes were obtained from New England Biolabs (Beverly, MA). A DNA sequencing kit (ABI PRISM 310 with dye termination cycle-sequencing ready-reaction kit) was obtained from Perkin-Elmer (Norwalk, CT). Gel-extraction kits and plasmid miniprep columns were obtained from QIAGEN (Valencia, CA) or Sigma (St. Louis). [^{35}S]Cysteine (specific activity 37MBq mmol^{-1}) was obtained from Bhabha Atomic Research Centre, Mumbai, India.

Strains, media, and growth conditions: The *Escherichia coli* strain *DH5α* was used as a cloning host. Yeast strains used in the study are described in Table 1. The yeast was regularly maintained on nonselective yeast extract, peptone and dextrose medium. For yeast transformation and induction experiments, synthetically defined minimal medium containing yeast nitrogen base and dextrose supplemented with adenine, histidine, leucine, lysine, tryptophan, and uracil (when not used as auxotrophic marker) at 50 mg/liter was used with either 5 g/liter ammonium sulfate as the nitrogen source (minimal ammonium medium) or 2 g/liter proline as nitrogen source (minimal proline medium; During-Olsen *et al.* 1999). Growth, handling of bacteria and yeast, and all the molecular techniques

TABLE 2

Oligonucleotides and their sequences in this study

Oligomer name	Sequence (5′–3′)
YLL055W-F	GCT AGC TCT AGA AAT GTC AAA AGT TGA CGT AAA AATTG
YLL055W-R	CGA GTC TCG AGT GTC AGA TAG AAC ATT TAC ACA AC
MUP1dLEU2-F	TCA ACA AGG AGA ACT ATC AAT TTT CTT CTT CTA CTACAA ATC GAC TAC GTC GTA AGG CCG
MUP1dLEU2-R	GGC AAT TTT GAC TCT CCA GAA CCC ATC TTC ACC AAG CAC AAA ATG GAA TCC CAA CAA TTA C
Xho1YLL055wP600F	AAC ACT CGA GTT CAG CAT CCG AGC
XhoYLLwP-387F	ATT GCT CGA GCA AAA ATG TGT GGC TTC TG
XhoYLLwP-372F	TGT GCT CGA GTC TGA AAA AAA AAA TAG GCA CCC C
BamH1YLL055wP-R	AAT CGG ATC CCA TTT CTT TTT TGT TAT ATT TTC

used in the study were according to the standard protocols (Sambrook *et al.* 1989; Guthrie and Fink 1991).

Cloning of *YLL055w* and construction of the yll055w::HIS3 disruption plasmid: The ORF *YLL055w* was PCR amplified from yeast genomic DNA using the primer pair YLL055W-F and YLL055W-R (Table 2). The 1.6-kb PCR product obtained was digested with *Xba*I and *Xho*I and cloned downstream of the TEF promoter in the single copy, *URA3*-based expression vector (p416TEF; Mumberg *et al.* 1995).

The *yll055w::HIS3* disruption plasmid was prepared by cloning the *HIS3* disruption cassette at the *Bam*HI site in the ORF *YLL055w*, leading to insertional disruption of *YLL055w*. The *HIS3* disruption cassette was released by *Bam*HI digestion of the plasmid p*CEN3-HIS3* (Futcher and Carbon 1986).

Construction of strains: The *YLL055w* ORF was disrupted in the *met15*Δ, *met15*Δ *agp1*Δ, *met15*Δ *gap1*Δ, *met15*Δ *gnp1*Δ, and *met15*Δ *mup1*Δ strain backgrounds using the yll055w::HIS3 disruption plasmid. The pyll055w::HIS3 disruption plasmid was digested with *Xba*I and *Eco*RI and the resulting 2.9-kb fragment was transformed into the strains. The transformants were selected on minimal ammonia medium without histidine and confirmed for the disruption by diagnostic PCR using the primer pair YLL055W-F and YLL055W-R.

The *MUP1* gene was disrupted in *met15*Δ *gnp1*Δ and *met15*Δ *gnp1*Δ *yll055w*Δ strains using one-step PCR-mediated gene disruption (Baudin *et al.* 1993). The *mup1*Δ*::*LEU2 disruption cassette was generated using the plasmid pair MUP1dLEU2-F and MUP1dLEU2-R and the plasmid pSP1 as a template (Cottarel *et al.* 1993). The 2.2-kb PCR product obtained was transformed into the strains and the resulting transformants were selected on minimal ammonia medium without leucine. The transformants were confirmed by their growth defect on a low concentration of methionine (2 μM) as a sole source of sulfur.

Construction of the *YLL055w* promoter–LacZ fusion constructs for the β-galactosidase reporter assay: The *YLL055w* upstream activating sequences were PCR amplified using different primer pairs, using oligonucleotides listed in Table 2. Briefly, for pYLL055w-600, pYLL055w-387, and pYLL055w-372 reporter constructs, the 600, 387, and 372 bp upstream of *YLL055w* were amplified from yeast genomic DNA, using the forward primers XhoIYLL055wP-600F, XhoYLLwP-387F, and XhoYLLwP-372F, respectively, and the reverse primer BamH1YLL055wP-R. The PCR products were purified, digested with *Xho*I and *Bam*HI, and cloned into pLG669z (Guarente and Ptashne 1981) (Figure 8).

For construction of an integrative *YLL055w* promoter–LacZ fusion construct, the 600-bp *Bam*HI–*Xho*I *YLL055w* promoter fragment cloned above was excised out and cloned into *Bam*HI–*Xho*I sites of the integrative vector pLacZi (CLONTECH, Palo Alto, CA). This integrative plasmid pYLL055w-600i was linearized by digestion with *Sph*I, which cuts in the *YLL055w* promoter. The linearized plasmid was transformed into the *met15*Δ (ABC 733) strain and the transformants were selected on minimal media plates without uracil. The β-galactosidase reporter assay was done with eight independent transformants.

Growth assay by dilution spotting: For growth assay, the different strains were grown overnight in minimal ammonia medium without uracil and reinoculated in fresh medium to an OD_{600} of 0.1 and grown for 6 hr. The exponential-phase cells were harvested, washed with water, and resuspended in water to an OD_{600} of 0.2. These were serially diluted to 1:10, 1:100, and 1:1000. Of these cell resuspensions, 10 μl were spotted on minimal medium containing cysteine, DL-homocysteine, or methionine as sole sulfur source. The plates were incubated at 30° for 3 days and photographs were taken.

Induction conditions and β-galactosidase assay: Fresh yeast transformants were used in all β-galactosidase experiments. The transformants were picked, grown overnight in minimal ammonia medium without uracil, and reinoculated in fresh minimal ammonia medium (without any organic sulfur source or with 0.25 mM cysteine, 0.25 mM methionine, or 0.25 mM glutathione) to an initial OD_{600} of 0.1. They were grown for an additional 6–7 hr to induce β-galactosidase. Since the *met15*Δ, *met31*Δ, *met32*Δ, *met28*Δ, cbf1Δ, and *met4*Δ strains used in this study were methionine/organic sulfur auxotrophs, the transformants were initially grown in medium containing methionine and were washed during induction and resuspended in medium containing a very low concentration of methionine (0.02 mM, a concentration that did not affect induction). The cells were then harvested and washed twice in Lac-Z buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, 0.27% 2-mercaptoethanol, pH 7) and $\sim 5 \times 10^7$ cells were taken for the reporter gene assay. β-Galactosidase activity was assayed in permeabilized yeast cells essentially as described previously (Guarente and Ptashne 1981). Briefly, the cells were resuspended in Lac-Z buffer, permeabilized by the addition of 50 μl chloroform and 20 μl SDS (0.1%). They were then vigorously vortexed for 20 sec. These samples were equilibrated for 10 min at 30° and then *o*-nitrophenyl β-galactopyranoside was added sequentially to the reaction samples. β-Galactosidase units are given as $OD_{420} \times 1000\ min^{-1}\ ml^{-1}/OD_{600}$ at 30°. The experiments were repeated with a minimum of three independent colonies.

Cysteine uptake measurement: Yeast transformants were grown overnight in minimal ammonia medium without uracil and reinoculated to an OD_{600} of 0.1 in fresh medium containing 2 μM methionine (nonrepressive sulfur conditions) or 200 μM methionine (repressive sulfur conditions). Cultures were incubated at 30° for 6 hr and exponentially growing cells

TABLE 3

***DAL5* gene family members: sulphur amino acid composition and regulation pattern**

ORF	Gene name	Reported substrate	Cys + Met residues (% Cys + Met residues)	Regulation	Reference
YJR152w	*DAL5*	Allantoate ureidosuccinate	8 + 18 (4.9)	Nitrogen catabolite repression	Rai *et al.* (1987, 1988)
YLR004c	*THI73*	Unknown	14 + 15 (5.6)	Thiamine	Llorente and Dujon (2000)
YLL055w	—	Unknown	4 + 12 (3.0)	Sulphur source	Boer *et al.* (2003)
YGR260w	*TNA1*	Nicotinic acid	13 + 18 (5.8)	Nicotinic acid	Llorente and Dujon (2000)
YIL166c	—	Unknown	13 + 18 (3.7)	Sulphur source	Boer *et al.* (2003)
YCR028c	*FEN2*	Pantothenate	9 + 20 (5.7)	Pantothenate	Stolz and Sauer (1999)
YAL067c	*SEO1*	Unknown	8 + 11 (3.2)	—	Isnard *et al.* (1996)
YGR065c	*VHT1*	Biotin	13 + 11 (4.0)	Biotin	Stolz *et al.* (1999)

were harvested and washed with a large volume of ice-cold MES buffer (20 mm MES/KOH, 0.5 mm $CaCl_2$, 0.25 mm $MgCl_2$, pH 5.5). Cells were finally resuspended in the MES buffer containing 2% glucose (resuspension buffer) at 2 OD_{600}/ml, aliquoted in 100-μl samples, and kept on ice. After a 5-min preincubation of cells at 30°, cysteine uptake was initiated by addition of 100 μl of assay medium. The assay medium contained radiolabeled cysteine ([^{35}S]cysteine, specific activity 37MBq $mmol^{-1}$) in MES resuspension buffer at a concentration of 100 μm, such that the final concentration of cysteine was 50 μm in the reaction vial. At selected times, the uptake was stopped by diluting the medium with a 20-fold volume of ice-cold water and cells were collected on the glass fiber prefilter (Advanced Microdevices, Ambala, India) by vacuum filtration. The harvested cells were washed twice with the same volume of ice-cold water. The filters were immersed in 3 ml of scintillation fluid (Sigma-Fluor Universal LSC cocktail, Sigma) and radioactivity was measured using a liquid scintillation counter (Wallac Microbeta, 1450 LSC and luminescence counter, Perkin-Elmer Life Sciences, Groningen, The Netherlands). The results were expressed as nanomoles of cysteine · mg · protein^{-1} min^{-1}.

For saturation kinetics, the initial rate of cysteine uptake was measured at a range of cysteine concentrations from 10 to 100 μm, with specific activity being kept constant at each concentration. The initial rate of cysteine uptake was determined by measuring the radioactive cysteine accumulated in the cells at 30- and 90-sec time points.

For inhibition studies with different sulfur compounds and amino acids, the competing ligand was added at 20-fold excess along with assay medium and initial rate of cysteine uptake was measured. The metabolic inhibitors were preincubated with the cells for 15 min at 30°, before measuring the initial rate of cysteine uptake.

As we observed that the cysteine transport activity rapidly fell when the cells were incubated in resuspension buffer on ice, the uptake experiments were performed within half an hour of the incubation of cells on ice.

Phylogenetic footprinting: The identification of conserved sequences in the multiple sequence alignment of *YLL055w* ortholog promoters of the Saccharomyces species was carried out using the webserver (http://203.90.127.21/anand/sacch_prom_pat.html; Kohli *et al.* 2004).

RESULTS

Identification of Yll055wp, a member of the Dal5p transporter family, as a possible cysteine transporter: To identify putative transporters in the yeast genome that were induced under conditions leading to insufficiency for cysteine in the cell, we examined the published literature for different genomewide expression profiling studies carried out in *S. cerevisiae*. This analysis revealed an uncharacterized ORF, *YLL055w*, encoding a putative transporter that was specifically upregulated under sulfur-limiting conditions under both aerobiosis and anaerobiosis, similarly to other sulfur assimilatory enzymes (Boer *et al.* 2003; Tai *et al.* 2005). In addition, this ORF was also found to be induced in a *gsh1*Δ strain background (Wheeler *et al.* 2003) (which has reduced intracellular levels of glutathione) and upon exposure to cadmium (Vido *et al.* 2001; Fauchon *et al.* 2002) or acetaldehyde (Aranda and del Olmo 2004), conditions under which most of the genes in the sulfur assimilation pathway were upregulated.

The induction profiles of the *YLL055w* ORF in these different studies were consistent with a role in sulfur assimilation, and therefore indicated a candidate cysteine transporter. However, this ORF did not belong to the amino acid permease family, to which all the known amino acid transporters have been found to belong. Yll055wp, which is predicted to encode a polypeptide of 531 amino acids, shows homology to Dal5p, an allantoate permease, in *S. cerevisiae* and is a member of the *DAL5* gene family. The *DAL5* gene family is a subfamily of the anion:cation symporter family, which in turn belongs to the major facilitator superfamily (Nelissen *et al.* 1997; Saier *et al.* 1999). The members encode putative weak acid permeases, which share between 20 and 30% amino acid identity. Some of the eight Dal5p family members have been functionally characterized and shown to transport substrates such as allantoate, biotin, pantothenate, and nicotinic acid in *S. cerevisiae* (Table 3). Although none have been shown to transport amino acids, two—*YLL055w* and *YIL166c* with unknown function—have been shown to be under sulfur regulation (Boer *et al.* 2003) while a third, *YAL067c*, has been picked in a mutagenic screen based on a toxic analog of methionine, the same screen in which Mup1p was identified as the high-affinity methionine permease (Isnard

et al. 1996). These observations raised the possibility that one or more of these uncharacterized ORFs could probably encode for the transporters involved in uptake of sulfate or sulfur-containing molecules, such as cysteine.

A characteristic feature of the sulfur assimilatory enzymes and proteins of yeast is that they have a lower sulfur amino acid content (Baudouin-Cornu *et al.* 2001). We therefore analyzed the sulfur amino acid content of all the members of the Dal5p family to see if further possibilities for these ORFs being a part of the sulfur assimilatory pathway might be suggested. Among the members of the Dal5p family, Yll055wp showed the lowest sulfur content, further suggesting that the protein indeed might play a role in sulfur assimilation (Table 3).

On the basis of these analyses, a preliminary genetic investigation into the role of these uncharacterized ORFs of the Dal5p family was carried out to determine their role in cysteine or homocysteine uptake. A *met15*Δ strain is unable to assimilate inorganic sulfur and is an organic sulfur auxotroph (Thomas and Surdin-Kerjan 1997). It can grow on organic sulfur sources such as methionine, glutathione, cysteine, and homocysteine. The deletion strains *yll055w*Δ, *yil166c*Δ, and *yal067c*Δ, each in a *met15*Δ background, were procured from the EUROSCARF (European *S. cerevisiae* Archive for Functional Analysis) deletion collection and checked for their ability to utilize cysteine or homocysteine as a sole sulfur source (Figure 1). The disruptant strains along with the wild type (*met15*Δ) were serially diluted and spotted on minimal ammonia medium with cysteine or homocysteine as the sole source of organic sulfur over a range of concentrations. The wild type and the different disruptants were able to use methionine as an organic sulfur source efficiently. However, a clear growth defect was observed for the *yll055w*Δ mutant at low concentrations of cysteine (100 and 200 μM), as compared to the wild type (Figure 1). At higher cysteine concentrations, both strains grew equally well. As multiple permeases have been shown to be capable of transporting cysteine (During-Olsen *et al.* 1999; Regenberg *et al.* 1999; Kosugi *et al.* 2001), it is likely that the phenotype of *yll055w* disruption was being masked by the activity of these permeases at the higher cysteine concentrations. The disruptions of the other two ORFs, *YIL166c* and *YAL067c*, did not result in any growth defect at any of the cysteine concentrations examined. Furthermore, no difference was observed in the utilization of homocysteine between the wild type and the three disruptants. Taken together, these results strongly suggested the possibility that Yll055wp might in fact be a cysteine transporter.

***YLL055w* encodes the major cysteine transporter of *S. cerevisiae*:** To evaluate the role of *YLL055w* in cysteine uptake, the *YLL055w* ORF was cloned downstream of the TEF promoter in a centromeric expression vector (p416TEF). Upon introduction into a *yll055w*Δ mutant, the gene complemented the growth defect at low cysteine concentrations (Figure 2), confirming the role of

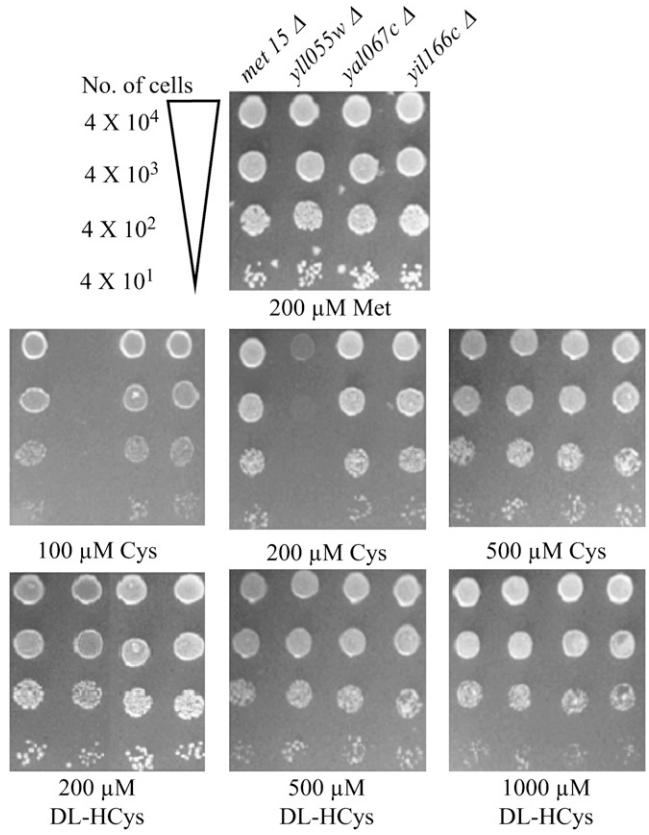

Figure 1.—*YLL055w* disruption in the *met15*Δ background results in a growth defect at low cysteine concentrations. The *met15*Δ, *met15*Δ *yll055w*Δ, *met15*Δ *yil166c*Δ, and *met15*Δ *yal067c*Δ strains were grown to exponential phase in minimal ammonia medium containing methionine, harvested, washed, and resuspended in water and serially diluted to give 0.2, 0.02, 0.002, and 0.0002 OD_{600} of cells. A total of 10 μl of these dilutions was spotted on minimal ammonia medium containing different concentrations of cysteine (Cys) and dl-homocysteine (dl-Hcys). The photographs were taken after 3 days of incubation at 30°.

the *YLL055w* ORF in the utilization of cysteine in this strain. At high concentrations of cysteine, transformation with the *YLL055w* gene expressed from the strong TEF promoter also showed cessation of growth of both the *met15*Δ *yll055w*Δ and the *met15*Δ strain. This is likely to be a consequence of the increased cysteine uptake and cysteine toxicity, a phenomenon similar, although less acute, than what was observed with the yeast glutathione transporter (Srikanth *et al.* 2005). Complementation of the growth defect of the *met15*Δ *yll055w*Δ strain and the cysteine-mediated toxicity upon overexpression of *YLL055w* gave further support to the notion that Yll055wp might indeed be a cysteine transporter.

To gain more direct information on the role of *YLL055w*-encoded protein in cysteine uptake, we next measured the accumulation of radiolabeled cysteine ([^{35}S]cysteine) in the *met15*Δ cells and *met15*Δ *yll055w*Δ cells. Cells were grown in a limiting organic sulfur source (20 μM methionine) and cysteine uptake was measured at different time intervals as described in materials

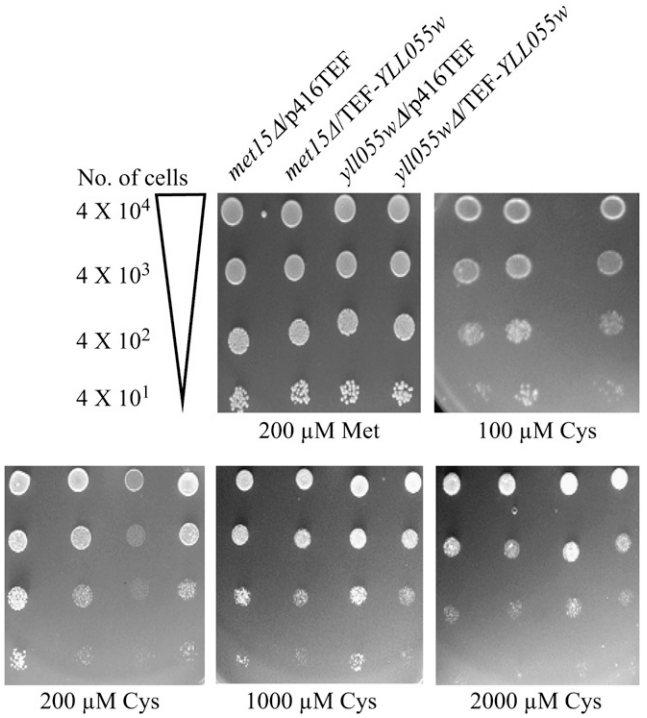

FIGURE 2.—The *YLL055w* ORF expressed from TEF promoter complements the growth defect observed in *met15*Δ *yll055w*Δ at low cysteine concentrations. Plasmid-bearing *YLL055w* under the TEF and the corresponding vector were transformed into *met15*Δ and *met15*Δ *yll055w*Δ (*yll055w*Δ). The dilution spotting was done as described in MATERIALS AND METHODS and in the legend to Figure 1.

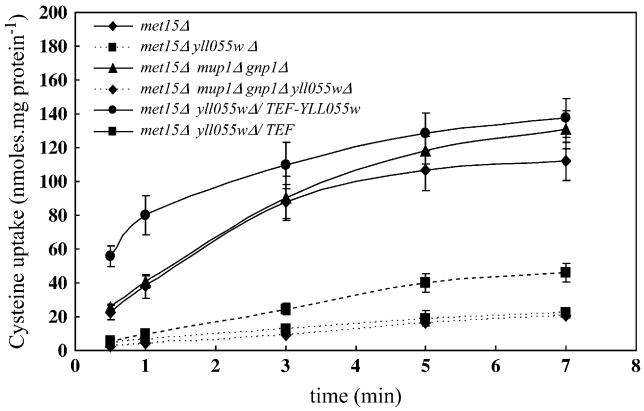

FIGURE 3.—*YLL055w* disruption in *met15*Δ and *met15*Δ *gnp1*Δ *mup1*Δ results in decreased uptake of [^{35}S]cysteine, which is restored by the *YLL055w* ORF expressed from TEF promoter. Strains—*met15*Δ, *met15*Δ *yll055w*Δ, *met15*Δ *gnp1*Δ *mup1*Δ, *met15*Δ *gnp1*Δ *mup1*Δ *yll055w*Δ, and *met15*Δ *yll055w*Δ complemented with *YLL055w* under the TEF promoter and the corresponding vector—were grown in minimal ammonia medium containing 2 μM methionine and used for the transport assay as described in MATERIALS AND METHODS. Log-phase cells were incubated with 50 μM [^{35}S]cysteine for different time intervals and counts were taken to determine the intracellular cysteine accumulation with time. Data are shown as mean ± SD ($n = 3$).

AND METHODS. In agreement with the growth assays done on the plates, the disruption of *YLL055w* in the *met15*Δ background led to a significant decrease in the uptake of radiolabeled cysteine (50 μM cysteine) as compared to the wild-type strain (Figure 3). The initial rate of cysteine uptake in *met15*Δ cells was 31.4 nmol · mg protein^{-1} min^{-1} compared to 5.3 nmol · mg protein^{-1} min^{-1} in the *met15*Δ *yll055w*Δ cells. We attributed the residual uptake observed in the deletant strain to the presence of the other nonspecific permeases and nonspecific binding of the radiolabeled ligand to the cell. However, the amount of cysteine taken up by the other permeases appeared to be insufficient in meeting the sulfur demands of the cells needed for growth when cysteine is present at low concentrations. Complementation of the *yll055w*Δ strain with *YLL055w* expressed from the TEF promoter led to a two- to threefold higher accumulation of [^{35}S]cysteine in cells as compared to the wild-type cells (Figure 3). These observations confirm that *YLL055w* encodes a major *y*east *c*ysteine *t*ransporter, and we refer to the gene as *YCT1* in the remaining sections of this article.

The previously reported cysteine permeases such as Gap1p, Gnp1p, Mup1p, and Agp1p play a negligible role in cysteine uptake, as compared to Yct1p, in different nitrogen sources: Earlier studies have revealed Gap1p, the general amino acid permease, as the major cysteine transporter (GREASHAM and MOAT 1973; DURING-OLSEN *et al.* 1999). However, Gap1p is inactive in yeast growing on a preferred nitrogen source, *e.g.*, ammonia (COURCHESNE and MAGASANIK 1983; JAUNIAUX and GRENSON 1990). Gap1p, along with Agp1p, mediates cysteine uptake in media containing nonrepressive nitrogen sources (*e.g.*, proline medium), with Agp1p being induced in the presence of leucine in the medium (DURING-OLSEN *et al.* 1999; REGENBERG *et al.* 1999). In ammonia-based medium devoid of amino acids, Agp1p, Gnp1, and Mup1p have been shown to be the major participants in cysteine uptake (DURING-OLSEN *et al.* 1999; REGENBERG *et al.* 1999; KOSUGI *et al.* 2001). Hence, to evaluate the contribution of Yct1p in cysteine uptake in comparison to the other permeases mediating cysteine transport, we decided to disrupt the major cysteine transporters reported in the literature and check the growth on cysteine as the sulfur source. Accordingly, the deletions of *AGP1*, *GAP1*, *GNP1*, and *MUP1* in the *met15*Δ background were procured from the EUROSCARF deletion collection, and *YLL055w* (*YCT1*) deletions were also created in these backgrounds as described in MATERIALS AND METHODS. As the *HIS3* marker was used to disrupt the *YLL055w* gene in these strains, we used the same disruption cassette to make a *met15*Δ *yll055w::HIS3* strain. The *met15*Δ, *met15*Δ *agp1*Δ, *met15*Δ *gap1*Δ, *met15*Δ *gnp1*Δ, and *met15*Δ *mup1*Δ, as well as the *met15*Δ *yll055w*Δ, *met15*Δ *agp1*Δ *yll055w*Δ, *met15*Δ *gap1*Δ *yll055w*Δ, *met15*Δ *gnp1*Δ *yll055w*Δ, and *met15*Δ *mup1*Δ *yll055w*Δ disruptants, were analyzed for their ability to utilize cysteine as an organic sulfur source in the presence of different nitrogen sources.

strain (31.4 nmol · mg protein^{-1} min^{-1}). The disruption of *YCT1* in *met15*Δ *gnp1*Δ *mup1*Δ decreased the rate of cysteine uptake to 3.8 nmol · mg protein^{-1} min^{-1}, underlining the predominant role played by Yct1p in cysteine uptake at low concentrations.

As only Gap1p and Agp1p have been implicated in cysteine uptake in media with a nonpreferred nitrogen source (During-Olsen *et al.* 1999), independent *gap1* and *agp1* disruptants in the met15Δ *yll055w*Δ background were checked for their ability to grow on cysteine as a sulfur source in the presence of proline as the sole nitrogen source. Despite the presence of Gap1p in *met15*Δ *agp1*Δ *yll055w*Δ and vice versa, *yct1* disruption prevents growth at low concentrations and results in a marginal growth defect even at a higher cysteine concentration (500 μM cysteine) (Figure 4B). *met15*Δ *agp1*Δ and *met15*Δ *gap1*Δ by themselves did not show any obvious growth defect on cysteine and grew as well as *met15*Δ alone. From these results, it can be concluded that Yct1p is the major contributor to cysteine uptake in both nitrogen-rich and nitrogen-poor medium, especially at low-cysteine concentrations, at which the different cysteine permeases reported so far have a very limited role to play.

Biochemical characterization of Yct1p reveals that it is a high-affinity, cysteine-specific transporter: From our genetic data it is evident that at high-cysteine concentrations (>500 μM), multiple permeases are involved in cysteine uptake; this probably explains the observed nonsaturation in cysteine uptake by During-Olsen *et al.* (1999). However, at low-cysteine concentrations (<200 μM), only Yct1p mediates cysteine uptake. Hence, for kinetic studies of Yct1p, we chose a narrow range of substrate concentrations, 10–100 μM, and measured the initial rate of cysteine uptake in the *met15*Δ and the *met15*Δ *yll055w*Δ background. The Lineweaver–Burke plot, obtained after subtracting the values of the initial rate of cysteine uptake in the *met15*Δ *yll055w*Δ double-delete strain from the values for the *met15*Δ strain, was linear, implying that a single uptake system is involved in uptake in this narrow range of concentrations (Figure 5). A K_m of 55.4 ± 6.1 μM and a V_{max} of 22.4 ± 6.3 nmol of cysteine · mg · protein^{-1} min^{-1} were calculated from this curve.

To define the substrate specificity of the transporter, competitive transport studies were undertaken in which the initial rate of cysteine uptake was measured in the presence of 20-fold excess of unlabeled competing ligand. Taking into account the participation of other amino acid permeases in cysteine uptake, we undertook these studies in the *met15*Δ *gnp1*Δ *mup1*Δ triple-delete strain to decrease the interference from these permeases in the competition assay. Moreover, these studies were done at low substrate concentration (40 μM) so that we could preferentially measure the cysteine uptake activity of the Yct1p.

The effect of the different sulfur amino acids homocysteine and methionine and the nonsulfur amino acids

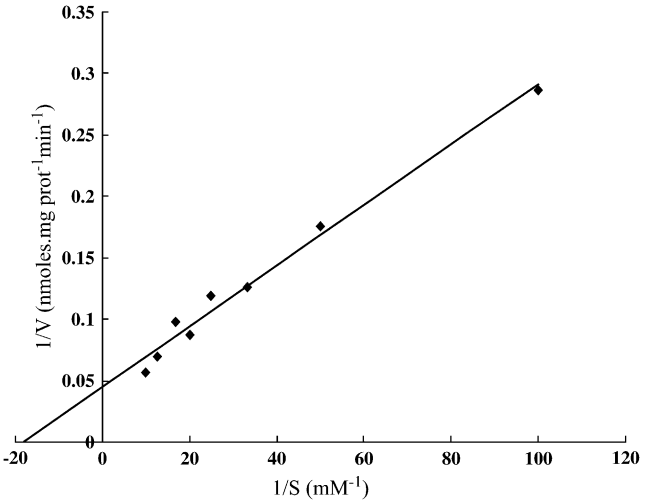

Figure 5.—Lineweaver–Burke plot for cysteine uptake in the *met15*Δ strain to determine kinetic parameters of Yct1p. The initial rate of cysteine uptake was measured at cysteine concentrations ranging from 0.01 to 0.1 mM by harvesting cells incubated with radiolabeled cysteine at 30- and 90-sec time intervals. Data are representative of three experiments (done in duplicates).

such as glycine, proline, valine, leucine, phenylalanine, serine, glutamate, glutamine, and lysine was evaluated on the cysteine uptake by Yct1p. Among the various amino acids used, only homocysteine (and, to a lesser extent, methionine) inhibited the cysteine uptake (Table 4). However, the other amino acids checked had little or no effect on the uptake of cysteine. Moreover, sulfur compounds with modified carboxy group (cysteamine), amino group (cysteic acid), or higher molecular weight (glutathione, cystathionine, cystine) did not have any effect on cysteine uptake in the *met15*Δ *gnp1*Δ *mup1*Δ triple-delete strain. Hence, unlike the other known amino acid permeases that transport cysteine, Yct1p is a highly cysteine-specific transporter.

The metabolic inhibitors, such as sodium azide and CCCP, which deplete the cellular ATP level and collapse the transmembrane proton gradient, were also examined for any inhibitory effect on cysteine uptake by Yct1p. A 15-min preincubation of the inhibitor with the cells resulted in a considerable loss in the cysteine uptake. This implies that Yct1p-mediated cysteine uptake is an energy-dependent process (Table 4).

The competitive transport studies done in the *met15*Δ *gnp1*Δ *mup1*Δ triple-delete strain retained ~40% activity with both cysteine (the strongest inhibitor) and metabolic inhibitors (even at sodium azide concentrations of 1 mM; data not shown) and is a consequence of background activity in the strain due to contribution from the other cysteine transporters as well as nonspecific binding of the radiolabeled ligand. (The cysteine uptake activity in the *met15*Δ *gnp1*Δ *mup1*Δ *yll055w*Δ tetra-delete strain was found to be nearly 15–25% of the cysteine uptake observed in the *met15*Δ *gnp1*Δ *mup1*Δ

TABLE 4

Effect of various compounds on cysteine uptake in the *met15*Δ *gnp1*Δ *mup1*Δ triple-delete strain

Compound	% activity
No inhibitor	100
Amino acids[a]	
L-cysteine	45 ± 2
DL-homocysteine	58 ± 2
L-methionine	76 ± 2
Glycine	90 ± 3
L-proline	100 ± 1
L-valine	85 ± 2
L-leucine	97 ± 1
L-phenylalanine	89 ± 1
L-serine	100 ± 1
L-glutamine	99 ± 4
L-gluatamic acid	98 ± 3
L-lysine	89 ± 1
Sulphur compounds[a]	
L-cysteamine HCl	92 ± 10
DL-cysteic acid	111 ± 15
L-glutathione	98 ± 2
L-cystine[b]	102 ± 3
L-cystathionine[b]	104 ± 4
Buffer (50 mM KCl–HCl pH 1)[b]	109 ± 2
Metabolic inhibitors[c]	
Sodium azide (40 μM)	64 ± 5
Sodium azide (100 μM)	42 ± 4
CCCP (40 μM)[d]	62 ± 5
CCCP (100 μM)[d]	48 ± 1
DMSO[d]	101 ± 5

The rate of uptake of cysteine (40 μM) was measured in *met15*Δ *gnp1*Δ *mup1*Δ in the presence of the different compounds listed in the table. The cells were harvested at 30- and 90-sec intervals. The results were normalized to the rate of uptake measured in the absence of any other compound (no inhibitor). Data are shown as mean ± SD ($n = 4$).

[a] All competitors were added to a final concentration of 800 μM in a 10-μl volume, *i.e.*, 20-fold excess over the labeled substrate, and were added simultaneously with uptake medium.

[b] L-cystine and L-cystathionine were dissolved in buffer (50 mM KCl–HCl, pH 1); hence the buffer was analyzed for its effect on cysteine uptake by the cells.

[c] Cells were preincubated with the indicated concentrations of metabolic inhibitors for 15 min prior to the addition of the uptake medium.

[d] CCCP was dissolved in DMSO and hence DMSO was analyzed for its effect on cysteine uptake by the cells.

triple-delete strain, hence contributing significantly to background cysteine observed in the triple-delete strain.)

***YCT1* is under sulfur regulation and is the major cysteine transporter under sulfur-derepressed conditions:** As mentioned earlier, genomewide expression profiling studies have revealed that *YCT1* is expressed under conditions of sulfur limitation, cadmium toxicity, low-glutathione levels, and acetaldehyde exposure, conditions under which the entire sulfur assimilation pathway has been shown to be upregulated (Vido *et al.* 2001; Fauchon *et al.* 2002; Boer *et al.* 2003; Wheeler *et al.* 2003; Aranda and del Olmo 2004; Tai *et al.* 2005). To gain further insights into the physiological role of Yct1p, we decided to undertake a more detailed study on the expression pattern of *YCT1* in response to different sulfur sources.

A *YCT1* promoter–β-galactosidase reporter fusion was constructed in which the 600-bp upstream sequences of *YCT1* were fused upstream of the β-galactosidase gene in the multicopy plasmid pLG699Z. The transformants were grown in different sulfur sources and examined for β-galactosidase activity in both a *met15*Δ and a *MET15* background. As shown in Figure 6A, expression of *YCT1* was maximum in nonrepressing sulfur conditions in both the *met15*Δ and the *MET15* background and considerably repressed in the presence of organic sulfur in the medium. Among the organic sulfur sources, methionine strongly repressed the expression of *YCT1*, whereas cysteine had a milder repressing effect in the *met15*Δ background. However, this differential repression pattern was not observed in the wild-type (*MET15*) background, in which methionine, cysteine, and glutathione resulted in mild repression of *YCT1*. Moreover, the fold induction in the *YCT1* expression between the poor methionine and the rich methionine medium was ~60-fold in the *met15*Δ background, whereas it was just 2-fold in the *MET15* background. This could be the result of sulfur starvation that is being experienced by cells in the *met15*Δ background due to the impairment of the sulfur assimilation pathway in this strain, implying that *YCT1* is under tight sulfur regulation.

To confirm if the sulfur repression seen with 2μ plasmids was also observed in a single-copy integrated promoter–LacZ fusion at the *YLL055w* site, we also constructed a 600-bp *YLL055w* promoter–β-galactosidase reporter fusion in an integrating plasmid and integrated this construct in the *YLL055w* promoter in a *met15*Δ strain (materials and methods). We observed >13-fold repression in the *YCT1* expression when the integrative transformants were grown in methionine-rich medium (data not shown). However, as the sensitivity obtained with the integrated reporter construct was very low, we worked with 2μ-based constructs for the subsequent studies on *YCT1* expression.

The repression of *YCT1* by organic sulfur sources was also observed in actual transport measurements. Cysteine uptake was measured in the *met15*Δ strain after growing it in rich methionine medium (Figure 6B). No difference was observed in cysteine uptake between the *met15*Δ strain and the *met15*Δ *yll055w*Δ when these strains were grown in 200 μM methionine containing minimal ammonia medium. Interestingly, although Mup1p, the methionine transporter that has also been shown to transport cysteine (Kosugi *et al.* 2001), is also derepressed in sulfur-limiting conditions (Boer *et al.* 2003), it did not appear to contribute to cysteine transport to any significant extent under these conditions. The tight sulfur

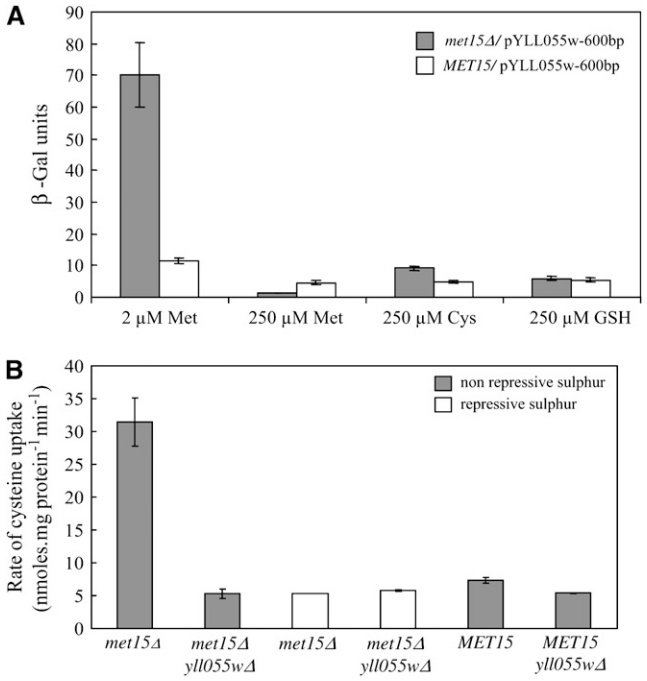

FIGURE 6.—Expression of *YLL055w* is under strong sulfur regulation in both *met15*Δ (ABC 733) and *MET15* (ABC 734) strains. (A) β-Galactosidase reporter assay–plasmid pYLL055w-600, bearing 600-bp upstream sequences of *YLL055w* fused upstream of the LacZ ORF, was transformed into *met15*Δ and *MET15* strains and the transformants were grown in the presence of different sulfur sources and assayed for β-galactosidase enzyme activity as described in MATERIALS AND METHODS. Data are shown as mean ± SD ($n = 5$). (B) Cysteine uptake. Strains *met15*Δ and *met15*Δ *yll055w*Δ were grown in minimal ammonia medium containing 2 μM methionine (nonrepressive sulfur) and 200 μM methionine (repressive sulfur) and the initial rate of [^{35}S]cysteine uptake was calculated by measuring the radiolabeled cysteine accumulated in the cells at 30- and 90-sec time intervals. Wild-type (*MET15*) and *yll055w*Δ strains were also grown in minimal ammonia medium and the initial rate of cysteine uptake was determined. Data are shown as mean ± SD ($n = 3$).

regulation of the *YCT1* gene was also reflected by the low rate of cysteine uptake in the *MET15* strain background, which was found to be just marginally more than the rate of cysteine uptake in the *yll055w*Δ background (Figure 6B). These results imply that the major role of Yct1p in cysteine transport is under conditions of sulfur limitations in the cell.

The *YCT1* gene is under regulation by the Met4p-based sulfur regulatory network and involves *cis*-elements corresponding to the known *cis*-regulatory elements: Studies on the regulation of the different structural genes in the sulfur assimilation pathway have led to identification of three *cis*-acting elements: TCACGTG, AAANTGTGG, and TGACTC, which are present in different combinations in the upstream regions of the sulfur assimilatory pathway genes (THOMAS and SURDIN-KERJAN 1997). Of these, the "TCACGTG" sequence is the binding site for the heteromeric transcriptional activation complex, Cbf1p–Met4p–Met28p (KURAS *et al.* 1996). Met4p is a leucine zipper transcriptional activator and is absolutely essential for activation of the sulfur assimilation genes in nonrepressing conditions (THOMAS *et al.* 1992). The second motif, "AAANTGTGG," is recognized by the Met4p–Met28p–Met31p/Met32p complex, although the role of this complex in gene regulation has been shown to be gene dependent (BLAISEAU *et al.* 1997). The role of the third motif and the *trans*-factors bound to it are yet unknown (THOMAS and SURDIN-KERJAN 1997). In addition, a recent study in our lab on regulation of *HGT1*, a high-affinity glutathione transporter, has led to the identification of a novel *cis*-element, CGCCACA, present in two copies, and it was shown that the *trans*-factors Met4p, Met28p, and Met31p control the regulation of this gene (SRIKANTH *et al.* 2005). Considering the strong derepression of *YCT1* in sulfur-limiting conditions, we decided to examine the *cis*-elements and *trans*-factors involved in the transcriptional activation of *YCT1* to check whether *YCT1* expression is regulated by the same mechanism as the other genes in the sulfur assimilation pathway.

A general sequence inspection of the 1-kb upstream sequence region of *YCT1* did not reveal the presence of any of the above *cis*-motifs reported in the sulfur regulation. Hence we resorted to the phylogenetic footprinting approach to identify any of the conserved motifs in upstream sequences of *YCT1*. Phylogenetic footprinting, a comparative genomics approach, has been successfully employed to identify regulatory motifs and other functional elements in the noncoding regions of the genomes on the basis of their high degree of conservation across the closely related species during the course of evolution due to selection pressure (CLIFTEN *et al.* 2003; KELLIS *et al.* 2003; LENHARD *et al.* 2003).

A web-based tool (KOHLI *et al.* 2004) was employed to perform the multiple sequence alignment of the 600-bp upstream sequences of *YCT1* with the orthologs of other closely related sensu stricto Saccharomyces species (*S. paradoxus*, *S. mikatae*, *S. bayanus*) and the more distant sensu stricto species *S. kudriavzevii* to identify any conserved sequence stretches (Figure 7). The analysis identified a conserved sequence stretch, AAATNTGTGGCT, at −385 bp upstream from the translation start site, which closely matched the sulfur regulatory motif II (AAANTGTGG). The similarity of this conserved sequence stretch in the *YCT1* orthologous promoters from the *Saccharomyces* spp. and the repression seen with both methionine and cysteine suggested that these conserved sequence stretches might act as the *cis* regulatory elements for the expression of *YCT1*. To test this hypothesis, constructs of *YCT1* promoter–β-galactosidase fusions were created only to include and exclude the conserved sequence "AAATNTGTGGCT," positioned at −385, thereby generating pYLL055w-387 and pYLL055w-372 reporter constructs (Figure 8). These constructs were transformed into the *met15*Δ strain and the β-galactosidase

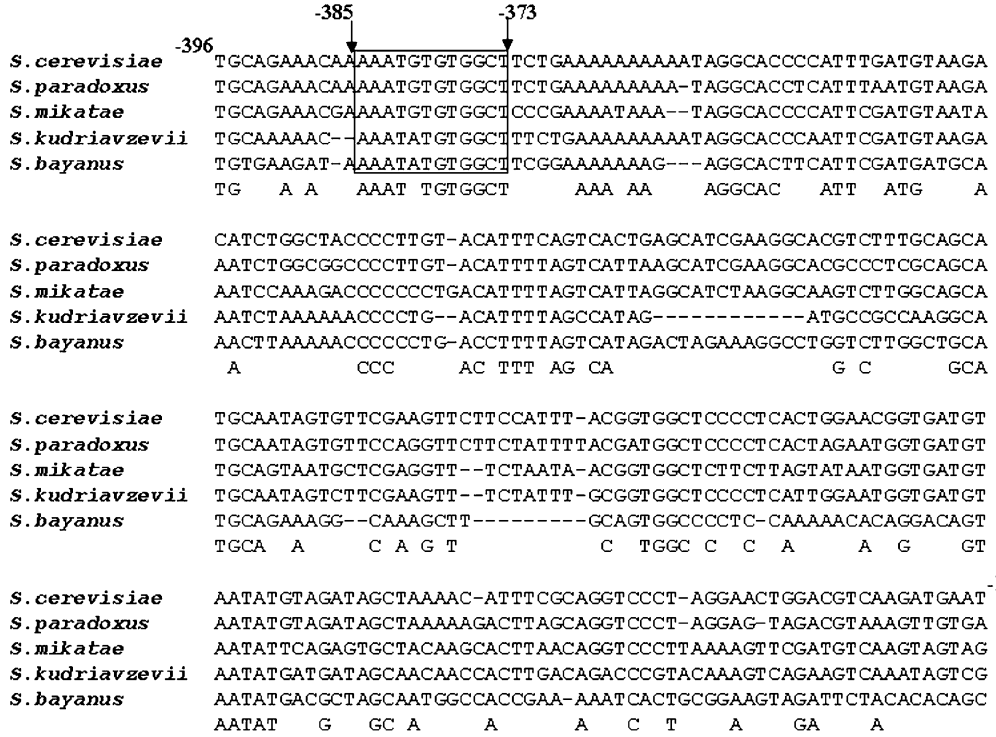

FIGURE 7.—Multiple sequence alignment of the *YLL055w* upstream sequences of different Saccharomyces species to identify conserved sequence stretches. The *YLL055w* upstream activating sequences of *S. cerevisiae*, *S. bayanus*, *S. mikatae*, *S. paradoxus*, and *S. kudriavzevii* were extracted and multiple sequence alignment was carried out using the webserver (http://203.90.127.21/anand/sacch_prom_pat.html; KOHLI *et al.* 2004). A sequence alignment stretch corresponding to the −396 to −157 upstream region of *YLL055w* from *S. cerevisiae* is shown. The conserved sequence stretch at −385, resembling the sulfur regulatory motif II-AAANTGTGG, is boxed.

activity was measured after growing cells in the sulfur-limiting and sulfur-rich medium (Figure 8). We observed that the deletion of the conserved sequence AAATNTGTGGCT in the pYLL055w-372 construct resulted in the complete loss of activation in the sulfur-limiting medium and also decreased the expression in cysteine medium compared to the control, the pYLL055w-600 construct. In contrast, the other deletion construct showed β-galactosidase activity comparable to the control in the sulfur-limiting conditions. This indicates that the motif AAATGTGTGG in the *YCT1* upstream region is absolutely essential for the activation of the gene in nonrepressing sulfur conditions, although the full expression of the gene in the sulfur-limiting and sulfur-rich medium requires the presence of some additional upstream elements in the *YCT1* promoter.

Since the *cis*-regulatory sequence that we identified was slightly different from the canonical *cis*-regulatory sequences involved in regulation of the sulfur assimilatory enzymes, we sought to examine if the *trans*-factors involved in the sulfur assimilatory pathway play a role in the regulation of *YCT1*. To examine this, the pYLL055w-600 construct was transformed into different genetic backgrounds bearing mutations in the different sulfur regulatory *trans*-factors (*met4*Δ, *met28*Δ, *cbf1*Δ, *met31*Δ, *met32*Δ) and *YCT1* expression was examined (Table 5). Consistent with the absolute requirement of the Met4p for the activation of sulfur assimilation genes in the sulfur-limiting conditions (THOMAS and SURDIN-KERJAN 1997), significantly reduced enzyme activity was detected in the *met4*Δ strain. A decreased activation of *YCT1* was also observed in the *met32*Δ strain, whereas expression was

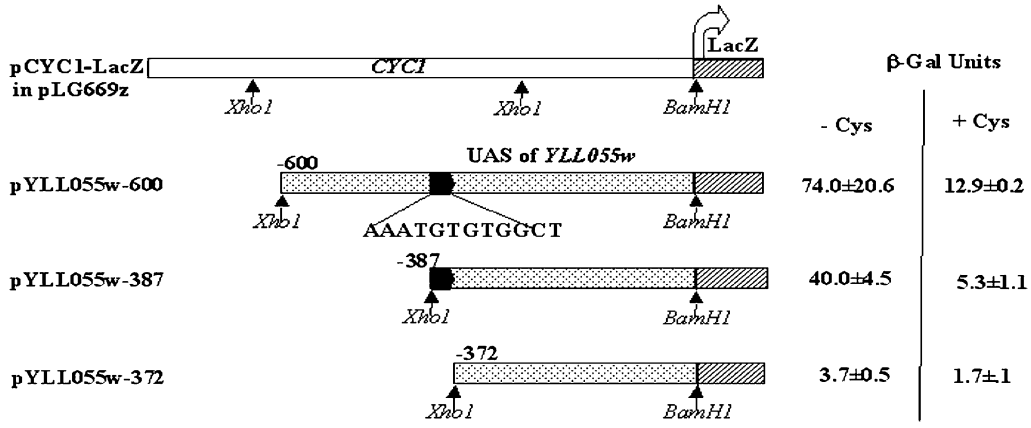

FIGURE 8.—β-Galactosidase reporter assay of different deletion constructs of the *YLL055w* promoter to identify the functionally important *cis* motifs in it. Multicopy plasmids bearing different upstream sequences fused to the LacZ ORF were transformed into the *met15*Δ strain and β-galactosidase enzyme activity was determined after growing cells in a poor sulfur source (2 μM methionine) and in a rich sulfur source (250 μM cysteine).

TABLE 5

Regulation of *YLL055w* by different sulphur regulatory *trans*-factors

Strain background	− Methionine	+ Methionine
Wild type (ABC 734)	8.4 ± 0.4	3.1 ± 0.4
Wild type (ABC 1381)	17.8 ± 1.1	5.6 ± 1.6
*met4*Δ (ABC1081)	3.7 ± 1.1	5.6 ± 1.5
*met28*Δ (ABC 1080)	212.5 ± 7.5	6.2 ± .08
*cbf1*Δ (ABC 1082)	15.0 ± 0.6	4.6 ± 2.6
*met31*Δ (ABC 1031)	15.0 ± 0.7	4.3 ± 0.25
*met32*Δ (ABC 1032)	2.9 ± 0.2	1.6 ± 0.2

The pYLL055w-600 multicopy plasmid bearing a 600-bp upstream region of *YLL055w* fused upstream of the β-galactosidase gene was transformed into strains deleted for the different sulphur regulatory *trans*-factors and their respective wild-type strains. The transformants were grown in the presence and absence of methionine and the β-galactosidase enzyme activity was measured. Data are shown as mean ± SD ($n = 3$).

significantly increased in the *met28*Δ strain under the same growth conditions. However, this high induction in enzyme activity in the *met28*Δ background was not observed in a sulfur-rich medium. Thus it appears that the sulfur regulatory factors Met4p and Met32p are absolutely essential for the expression of *YCT1* under nonrepressive sulfur conditions whereas Met28p seems to "restrict" the activity in these inducing conditions.

DISCUSSION

This study has revealed the existence of a high-affinity cysteine-specific transporter in *S. cerevisiae* that is also the major transporter of cysteine in the yeast. This transporter (Yct1p, Yll055wp) was initially picked up from data mining and bioinformatics analysis and has been demonstrated to function as a high-affinity, cysteine-specific transporter through both genetic and biochemical studies.

The plate-based loss-of-growth phenotype and the decreased cysteine uptake in the *yct1*Δ strain, which were complemented by a plasmid bearing *YCT1*, provided direct evidence for the role of the *YCT1*-encoded transporter in cysteine uptake. Although cysteine uptake was mediated by the other members of the AAP family at high concentration, the complete loss in growth of the *yct1*Δ strain at low concentrations of cysteine, coupled with the low K_m of the Yct1p for cysteine (55.4 μM), clearly makes it a high-affinity, major cysteine transporter in the yeast. The fact that the deduced K_m of Agp1p, the only cysteine transporter in yeast that shows saturation with respect to cysteine uptake, is 200 μM, further signifies the high affinity of Yct1p for cysteine uptake (During-Olsen *et al.* 1999). Apart from cysteine, significant inhibition in cysteine uptake by Yct1p was exhibited only by homocysteine, a close structural homolog of cysteine (and, to a lesser extent, by methionine). The growth experiments with the *yll055w*Δ strain, however, suggest that homocysteine is not transported principally by Yct1p (Figure 1).

The transport experiments, which were carried out with whole cells, suggest plasma membrane localization for Yct1p. The possibility that Yct1p might also be localized to other organelles, however, has not been examined. A genomewide analysis of protein localization in *S. cerevisiae*, using green fluorescent protein (GFP)-tagged proteins expressed from their native promoter in the chromosome itself, has predicted that Yct1p is restricted to the endoplasmic reticulum (Huh *et al.* 2003). However, functional analyses of the yeast strain bearing the chromosomal copy of the GFP-tagged *YLL055w* in the *met15*Δ background gave a *yll055w* null mutant phenotype for growth on cysteine (data not shown). Hence, it appears that the GFP-tagged Yll055wp is nonfunctional, and hence no conclusion can be drawn regarding the localization of the protein from such a construct.

The fact that this major cysteine transporter in *S. cerevisiae* has eluded identification so far is quite surprising since several attempts have been made to identify cysteine transporter genes, and several genetic studies have targeted the sulfur assimilation pathway, of which this forms a part (Cherest and Surdin-Kerjan *et al.* 1992; During-Olsen *et al.* 1999; Kosugi *et al.* 2001). One possibility could be that the *YLL055w* gene under its own promoter is toxic in *E. coli* (J. Kaur and A. K. Bachhawat, unpublished observations). As a result, *E. coli* colonies harboring plasmids bearing this gene with its native promoter form pin-point-sized colonies on plates, and hence the gene is likely to be absent from genomic libraries used to isolate this gene. A second aspect is that the phenotypic defect on cysteine plates is seen only at low concentrations of cysteine over a narrow concentration range. Finally, the fact that this gene is under tight regulation by the sulfur regulatory network makes significant contribution in cysteine uptake discernible only in sulfur-limiting conditions such as in the *met15*Δ background.

The observation that the major cysteine transporter of *S. cerevisiae* does not fall into the AAP family, to which all the other known amino acid transporters belong, is intriguing and shows that not all the transporters of the 20 common L-α-amino acids in *S. cerevisiae* need to belong to the AAP family (Regenberg *et al.* 1999). The Dal5p transporter family is unique to fungi. Its members, in addition to transporting cysteine as described in this report, have been reported to transport different metabolites like nicotinic acid, allantoin, pantothenic acid, and biotin (Table 3). The possibility of Yct1p homologs being cysteine transporters in other yeast and fungi was therefore evaluated by examining possible orthologs in other organisms. BLAST analysis and reverse Best Hit analysis suggests that orthologs of the cysteine transporter probably do exist in other yeasts

and fungi (data not shown), although this needs to be experimentally established.

The discovery of this transporter has helped resolve much of the confusion that abounds in the literature about the kinetic aspects of cysteine transport. Kinetic studies of transporters are always complicated when multiple transporters contribute to the transport of the substrate. Yct1p plays the predominant role at lower concentrations of cysteine, as seen from growth experiments and as reflected in the K_m of the transporter for cysteine. At higher concentrations, other transporters contribute in a much more significant manner, masking the contributions of Yct1p to a significant extent. The use of higher ranges of substrate concentrations has thus masked the kinetic behavior of cysteine transport (During-Olsen *et al.* 1999). Hence we have restricted ourselves to a much lower range of concentrations in determining K_m, a concentration range that was also employed by one earlier study that was able to observe saturating kinetics with respect to cysteine uptake and reported a K_m of 83.3 μM, which is reasonably close to what we observed in this study (Ono and Naito 1991).

A recent study has presented a rational basis for the presence of multiple permeases for methionine in the cell (Menant *et al.* 2006). Methionine transport is mediated by seven different transporters that include Mup1p, Mup3p, Agp3p, Agp1p, Bap2p, Bap3p, and Gnp1p. Three of them, Mup1p, Mup3p and Agp3p, have been shown to be under methionine repression, while the remaining Agp1p, Bap2p, Bap3p, and Gnp1p, which are nonspecific transporters, are induced severalfold in the presence of methionine (Menant *et al.* 2006). Hence, by manipulating the activity and regulation of these permeases in response to different extracellular and intracellular conditions, the cell is able to adjust itself to the prevailing environmental conditions and yet maintain the homeostasis of the amino acid inside. The presence of nine transporters mediating cysteine uptake in *S. cerevisiae*, which include Yct1p that is under tight regulation by sulfur, might suggest the need for a similar mechanism for maintaining cysteine homeostasis (Regenberg *et al.* 1999).

Studies on regulation of the sulfur assimilation pathway have revealed that between the two common organic sulfur sources, methionine and cysteine, methionine represses the gene transcription more efficiently, although the effector molecule for the transcriptional regulation of these genes is proposed to be cysteine or a derivative thereof (Thomas and Surdin-Kerjan 1997; Hansen and Johannesen 2000). This has been attributed to the slow rate of uptake of cysteine from the extracellular medium, due to the low affinity of the cysteine transporters in the plasma membrane (Ono *et al.* 1991; During-Olsen *et al.* 1999). However, the identification of Yct1p as a high-affinity, cysteine-specific transporter questions this assumption. It is also observed that a *met15*Δ strain grows better on glutathione as an organic sulfur source compared to cysteine, when both are present at the same concentration (Hansen and Johannesen 2000; Ganguli *et al.* 2006). As glutathione utilization proceeds through cysteine (Ganguli *et al.* 2006), this difference becomes difficult to explain considering that Hgt1p, the high-affinity glutathione transporter in yeast (Bourbouloux *et al.* 2000), and Yct1p, the high-affinity cysteine transporter in yeast, have similar K_m values. Whether this decreased ability to utilize cysteine as an efficient organic sulfur source arises due to some post-transcriptional regulation of Yct1p, and hence to decreased cysteine uptake or some other step in the cysteine utilization, could be an interesting subject for future studies.

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LITERATURE CITED

Alfafara, C. G., A. Kanda, T. Shio, H. Shimizu, S. Shioya *et al.*, 1992 Effect of amino acids on glutathione production by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **36:** 538–540.

Aranda, A., and M. L. del Olmo, 2004 Exposure of *Saccharomyces cerevisiae* to acetaldehyde induces sulfur amino acid metabolism and polyamine transporter genes, which depend on Met4p and Haa1p transcription factors, respectively. Appl. Environ. Microbiol. **70:** 1913–1922.

Baudin, A., O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute and C. Cullin, 1993 A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. Nucleic Acids Res. **21:** 3329–3330.

Baudouin-Cornu, P., Y. Surdin-Kerjan, P. Marliere and D. Thomas, 2001 Molecular evolution of protein atomic composition. Science **293:** 297–300.

Blaiseau, P., A. Isnard, Y. Surdin-Kerjan and D. Thomas, 1997 Met31p and Met32p, two related zinc finger proteins, are involved in transcriptional regulation of yeast sulfur amino acid metabolism. Mol. Cell. Biol. **17:** 3640–3648.

Boer, V. M., J. H. De Winde, J. T. Pronk and M. D. Piper, 2003 The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. J. Biol. Chem. **278:** 3265–3274.

Bourbouloux, A., P. Shahi, A. Chakladar, S. Delrot and A.K. Bachhawat, 2000 Hgt1p, a high affinity glutathione transporter from the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **275:** 13259–13265.

Cherest, H., and Y. Surdin-Kerjan, 1992 Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae*: updating of the sulfur metabolism pathway. Genetics **130:** 51–58.

Cliften, P. F., P. Sudarsanam, A. Desikan, L. Fulton, B. Fulton *et al.*, 2003 Finding functional features in Saccharomyces genomes by phylogenetic footprinting. Science **301:** 71–76.

Cottarel, G., D. Beach and U. Deuschle, 1993 Two new multipurpose multicopy *Schizosaccharomyces pombe* shuttle vectors, pSP1 and pSP2. Curr. Genet. **23:** 547–548.

Courchesne, W. E., and B. Magasanik, 1983 Ammonia regulation of amino acid permeases in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **3:** 672–683.

During-Olsen, L., B. Regenberg, C. Gjermansen, M. C. Kielland-Brandt and J. Hansen, 1999 Cysteine uptake by *Saccharomyces cerevisiae* is accomplished by multiple permeases. Curr. Genet. **35:** 609–617.

Fauchon, M., G. Lagniel, J. C. Aude, L. Lombardia, P. Soularue *et al.*, 2002 Sulfur sparing in the yeast proteome in response to sulfur demand. Mol. Cell **9:** 713–723.

Futcher, B., and J. Carbon, 1986 Toxic effects of excess cloned centromeres. Mol. Cell. Biol. **6:** 2213–2222.

Ganguli, D., C. Kumar and A. K. Bachhawat, 2006 The alternative pathway of glutathione degradation is mediated by a novel protein complex involving three new genes in *Saccharomyces cerevisiae*. Genetics **175:** 1137–1151.

Greasham, R. L., and A. G. Moat, 1973 Amino acid transport in a polyaromatic amino acid auxotroph of *Saccharomyces cerevisiae*. J. Bacteriol. **115:** 975–981.

Guarente, L., and M. Ptashne, 1981 Fusion of *Escherichia coli* lacZ to the cytochrome c gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **78:** 2199–2203.

Guthrie, C., and G. R. Fink, 1991 Guide to yeast genetics and molecular biology. Methods Enzymol. **194:** 3–37.

Hansen, J., and P. F. Johannesen, 2000 Cysteine is essential for transcriptional regulation of the sulfur assimilation genes in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **263:** 535–542.

Huh, W. K., J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson *et al.*, 2003 Global analysis of protein localization in budding yeast. Nature **425:** 686–691.

Isnard, A.-D., D. Thomas and Y. Surdin-Kerjan, 1996 The study of methionine uptake in *Saccharomyces cerevisiae* reveals a new family of amino acid permeases. J. Mol. Biol. **262:** 473–484.

Jauniaux, J-C., and M. Grenson, 1990 GAP1, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. Eur. J. Biochem. **190:** 39–44.

Kellis, M., N. Patterson, M. Endrizzi, B. Birren and E. S. Lander, 2003 Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature **423:** 241–254.

Kohli, D. K., C. V. Srikanth and A. K. Bachhawat, 2004 A search tool for identification and analysis of conserved sequence patterns in Saccharomyces spp. orthologous promoter. In Silico Biol. **4:** 411–415.

Kosugi, A., Y. Koizumi, F. Yanagida and S. Udaka, 2001 MUP1, high affinity methionine permease, is involved in cysteine uptake by *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem. **65:** 728–731.

Kumar, A., L. John, M. M. Alam, A. Gupta, G. Sharma *et al.*, 2006 Homocysteine- and cysteine-mediated growth defect is not associated with induction of oxidative stress response genes in yeast. Biochem. J. **396:** 61–69.

Kuras, L., H. Cherest, Y. Surdin Kerjan and D. Thomas, 1996 A heteromeric complex containing the centromere binding factor 1 and two basic leucine zipper factors, Met4 and Met28, mediates the transcription activation of yeast sulfur metabolism. EMBO J. **15:** 2519–2529.

Lenhard, B., A. Sandelin, L. Mendoza, P. Engstrom, N. Jareborg *et al.*, 2003 Identification of conserved regulatory elements by comparative genome analysis. J. Biol. **2:** 13–24.

Llorente, B., and B. Dujon, 2000 Transcriptional regulation of the *Saccharomyces cerevisiae* DAL5 gene family and identification of the high affinity nicotinic acid permease TNA1 (YGR260w). FEBS Lett. **23:** 237–241.

Maw, G. A., 1963 The uptake of some sulphur-containing amino acids by a brewer's yeast. J. Gen. Microbiol. **31:** 247–259.

Menant, A., R. Barbey and D. Thomas, 2006 Substrate-mediated remodeling of methionine transport by multiple ubiquitin-dependent mechanisms in yeast cells. EMBO J. **25:** 4436–4447.

Mendoza-Cozatl, D., H. Loza-Tavera, A. Hernandez-Navarro and R. Moreno-Sanchez, 2005 Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, protists and plants. FEMS Microbiol Rev. **29:** 653–671.

Mumberg, D., R. Muller and M. Funk, 1995 Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene **156:** 119–122.

Nelissen, B., R. De Wachter and A. Goffeau, 1997 Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. **21:** 113–134.

Ono, B., and K. Naito, 1991 The cysteine transport system of *Saccharomyces cerevisiae*. Yeast **7:** 849–855.

Ono, B., K. Naito, Y. Shirahige and M. Yamamoto, 1991 Regulation of cystathionine gamma-lyase in *Saccharomyces cerevisiae*. Yeast **7:** 843–848.

Rai, R., F. S. Genbauffe, H. Z. Lea and T. G. Cooper, 1987 Transcriptional regulation of the DAL5 gene in *Saccharomyces cerevisiae*. J. Bacteriol. **169:** 3521–3524.

Rai, R., F. S. Genbauffe and T. G. Cooper, 1988 Structure and transcription of the allantoate permease gene (DAL5) from *Saccharomyces cerevisiae*. J. Bacteriol. **170:** 266–271.

Regenberg, B., L. During-Olsen, M. C. Kielland-Brandt and S. Holmberg, 1999 Substrate specificity and gene expression of the amino-acid permeases in *Saccharomyces cerevisiae*. Curr. Genet. **36:** 317–328.

Saier, M. H., Jr., J. T. Beatty, A. Goffeau, K. T. Harley, W. H. Heijne *et al.* 1999 The major facilitator superfamily. J. Mol. Microbiol. Biotechnol. **1:** 257–279.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Srikanth, C. V., P. Vats, A. Bourbouloux, S. Delrot and A. K. Bachhawat, 2005 Multiple cis-regulatory elements and the yeast sulphur regulatory network are required for the regulation of the yeast glutathione transporter, Hgt1p. Curr. Genet. **47:** 345–358.

Stolz, J., and N. Sauer, 1999 The fenpropimorph resistance gene FEN2 from *Saccharomyces cerevisiae* encodes a plasma membrane H+-pantothenate symporter. J. Biol. Chem. **274:** 18747–18752.

Stolz, J., U. Hoja, S. Meier, N. Sauer and E. Schweizer, 1999 Identification of the plasma membrane H+-biotin symporter of *Saccharomyces cerevisiae* by rescue of a fatty acid-auxotrophic mutant. J. Biol. Chem. **274:** 18741–18746.

Tai, S. L., V. M. Boer, P. Daran-Lapujade, M. C. Walsh, J. H. de Winde *et al.*, 2005 Two-dimensional transcriptome analysis in chemostat cultures. Combinatorial effects of oxygen availability and macronutrient limitation in *Saccharomyces cerevisiae*. J. Biol. Chem. **280:** 437–447.

Thomas, D., and Y. Surdin-Kerjan, 1997 Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **61:** 503–532.

Thomas, D., I. Jacquemin and Y. Surdin Kerjan, 1992 MET4, a leucine zipper protein, and centromere-binding factor 1 are both required for transcriptional activation of sulfur metabolism in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:** 1719–1727.

Vido, K., D. Spector, G. Lagniel, S. Lopez, M. B. Toledano *et al.*, 2001 A proteome analysis of the cadmium response in *Saccharomyces cerevisiae*. J. Biol. Chem. **276:** 8469–8474.

Wen, S., T. Zhang and T. Tan, 2004 Utilization of amino acids to enhance glutathione production in *Saccharomyces cerevisiae*. Enzyme Microb. Technol. **35:** 501–507.

Wheeler, G. L., E. W. Trotter, I. W. Dawes and C. M. Grant, 2003 Coupling of the transcriptional regulation of glutathione biosynthesis to the availability of glutathione and methionine via the Met4 and Yap1 transcription factors. J. Biol. Chem. **278:** 49920–49928.

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