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Genomewide Screen Reveals a Wide Regulatory Network for Di/Tripeptide Utilization in *Saccharomyces cerevisiae*

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ABSTRACT

Small peptides of two to six residues serve as important sources of amino acids and nitrogen required for growth by a variety of organisms. In the yeast *Saccharomyces cerevisiae*, the membrane transport protein Ptr2p, encoded by *PTR2*, mediates the uptake of di/tripeptides. To identify genes involved in regulation of dipeptide utilization, we performed a systematic, functional examination of this process in a haploid, nonessential, single-gene deletion mutant library. We have identified 103 candidate genes: 57 genes whose deletion decreased dipeptide utilization and 46 genes whose deletion enhanced dipeptide utilization. On the basis of Ptr2p-GFP expression studies, together with *PTR2* expression analysis and dipeptide uptake assays, 42 genes were ascribed to the regulation of *PTR2* expression, 37 genes were involved in Ptr2p localization, and 24 genes did not apparently affect Ptr2p-GFP expression or localization. The 103 genes regulating dipeptide utilization were distributed among most of the Gene Ontology functional categories, indicating a very wide regulatory network involved in transport and utilization of dipeptides in yeast. It is anticipated that further characterization of how these genes affect peptide utilization should add new insights into the global mechanisms of regulation of transport systems in general and peptide utilization in particular.

PEPTIDES can serve as a major nutritional source of amino acids and nitrogen for microbial cell growth and for human nutrition as well (Pritchard and Coolbear 1993; Daniel 2004). Peptides are generated by the action of proteases and peptidases in environments where microbes reside (Gonzales and Robert-Baudouy 1996; Daniel 2004). All cellular organisms from bacteria to fungi, plants, and mammals are capable of taking up small peptides (two to six amino acids) through a system mediated by cytoplasmic membrane transporters specific for peptides (Hauser *et al.* 2001). In mammalian systems, two major peptide transporters, PepT1 and PepT2, have been found in the intestine and kidney, respectively (Fei *et al.* 1994; Liu *et al.* 1995). The PepT1 transporter provides the means for absorption of nutritional peptides and peptidomimetic drugs, such as β-lactam antibiotics, angiotensin-converting enzyme inhibitors, renin inhibitors, and antivirals. The substrate specificity of these transporters has provided an impetus to many pharmaceutical companies to develop peptide transporters as drug delivery systems (Lee 2000; Herrera-Ruiz and Knipp 2003).

Two peptide transport systems have been characterized in the model eukaryote *Saccharomyces cerevisiae*: the peptide transport (PTR) system transports di/tripeptides (Steiner *et al.* 1995) and the oligopeptide transport (OPT) system highly favors transport of peptides of four to five amino acid residues and glutathione (Lubkowitz *et al.* 1997; Miyake *et al.* 2002). While PTR family members are present in all cells studied to date and are in the same family as the mammalian transporters PepT1 and PepT2, OPT family members have been found only in plants and fungi (Hauser *et al.* 2001). Both the PTR and OPT transport systems are dependent on the proton-motive force, are predicted to have 12 transmembrane domains, and have specific signature sequences distinguishing them from one another and from all other proteins in the database (Hauser *et al.* 2001). Yet the amino acid sequence of the PTR and OPT members places them in different, unrelated families of transport proteins with presumably totally separate evolutionary origins. The only *S. cerevisiae* member of the PTR system is Ptr2p, encoded by the *PTR2* gene. Di/Tripeptide utilization is regulated by a number of means including *PTR2* transcriptional regulation.

In early studies predating the cloning of *PTR2*, peptide utilization was shown to be upregulated by growing cells on organic nitrogen sources such as allantoin, isoleucine, or proline, defined as poor nitrogen sources in comparison to the rich nitrogen source ammonium sulfate (Island *et al.* 1991). Peptide utilization was also

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stimulated markedly by addition of micromolar amounts of certain amino acids, most notably leucine and tryptophan, to the growth medium (Island *et al.* 1987; Baetz *et al.* 2004). These environmental conditions were later shown to upregulate the expression of *PTR2* (Perry *et al.* 1994). Although nitrogen source apparently regulates *PTR2* expression via nitrogen catabolite repression (Marzluf 1997), amino acids regulate *PTR2* expression via the Ssy1p-Ptr3p-Ssy5p (SPS) signal transduction pathway (Barnes *et al.* 1998; Forsberg and Ljungdahl 2001b; Forsberg *et al.* 2001a,b).

In the SPS complex, Ssy1p is a transmembrane receptor that senses extracellular amino acids. Ptr3p and Ssy5p interact with the cytoplasmically located N terminus of Ssy1p (Forsberg and Ljungdahl 2001a; Poulsen *et al.* 2005). Two related transcription factors, Stp1p and Stp2p, are downstream of the SPS complex and regulate the expression of *PTR2* and branched-chain amino acid permeases (de Boer *et al.* 2000). Stp1p and Stp2p are synthesized in an inactive form, and the activation of Stp1p and Stp2p depends on the endoproteolytic processing of their N-terminal domain mediated by Ptr3p and Ssy5p. The truncated forms of Stp1p and Stp2p are translocated into the nucleus to upregulate expression of downstream genes including *PTR2* (Andreasson and Ljungdahl 2002, 2004).

In addition to the SPS complex regulation, *PTR2* expression is positively regulated by the import of di/tripeptides with basic (Arg, His, or Lys) and bulky (Ile, Leu, Phe, Trp, or Tyr) N-terminal residues that bind to Ubr1p to allosterically activate Ubr1p-mediated degradation of the *PTR2* repressor Cup9p (Byrd *et al.* 1998; Turner *et al.* 2000; Du *et al.* 2002). Relief of Cup9p repression of *PTR2* results in enhanced *PTR2* expression. In a *cup9* null mutant strain, *PTR2* is overexpressed, resulting in a marked increase in dipeptide uptake (Byrd *et al.* 1998). Cup9p is degraded by Ptr1p through the ubiquitinylation pathway. In this pathway, Ptr1p acts as a scaffolding protein or ubiquitin ligase (E3) for two other proteins, Ubc2p and Ubc4p, which serve as ubiquitin-conjugating (E2) enzymes in the Cup9p degradation process (Xie and Varshavsky 1999). Deletion of *PTR1* results in the stabilization of Cup9p, lack of expression of *PTR2*, and a null peptide-uptake phenotype (Island *et al.* 1991; Turner *et al.* 2000; Du *et al.* 2002). Other transcription factors, such as Dal81p/Uga35p, have also been reported to be involved in *PTR2* expression, in ways apparently different from their involvement in the SPS or Cup9p pathways (Iraqui *et al.* 1999). Obviously, the systems regulating *PTR2* expression are numerous and complex, and there has not been a systematic study to uncover other proteins involved in the regulation of peptide utilization.

Systematic screening of yeast deletion mutant collections has been successfully applied in identifying genes related to drug resistance (Page *et al.* 2003; Aouida *et al.* 2004; Baetz *et al.* 2004), human disease (Steinmetz *et al.* 2002), telomere function (Askree *et al.* 2004), centromeric cohesion (Marston *et al.* 2004), vacuolar protein sorting (Bonangelino *et al.* 2002), and other biological processes (Scherens and Goffeau 2004). To identify other gene products involved in the regulation of peptide utilization in yeast, we conducted a genome-wide screen using the haploid, nonessential, single-gene deletion strain library. We have identified a total of 103 open reading frames (ORFs), accounting for ~2% of the nonessential deletion mutants as being involved in causing increased or decreased utilization of peptides for growth. We monitored Ptr2p-GFP expression in the identified 103 deletion mutant strains, and genes involved in multiple cellular functions including transcriptional regulation and membrane trafficking were revealed as being involved in dipeptide utilization. The data generated provide a global view of molecular components regulating dipeptide utilization by *S. cerevisiae*.

MATERIALS AND METHODS

Strains, media, and mutant library growth assay screen: A yeast haploid, nonessential, single-gene deletion mutant strain library was purchased from Open Biosystems (Huntsville, AL), containing 4827 deletion strains, 4750 in the BY4742 (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) background and 77 strains in the BY4739 (*MAT*α *leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) strain background. The list of strains contained in the library is available at the company's website (http://openbiosystems.com/yeast_knock_outs.php). The deletion mutants were arrayed in 96-well microtiter plates kept at −80° in YPD broth containing G418 and glycerol (1% yeast extract, 2% peptone, 2% dextrose, 200 μg/ml G418, and 15% glycerol). After thawing, the deletion mutants were inoculated into minimal medium supplemented with amino acids to satisfy the auxotrophic requirements, using a 96-well plate replicator. The minimal medium used in this study is referred to as MM + HLKU and contained (per liter) 20 g dextrose, 1.7 g yeast nitrogen base (Difco, Detroit) without $(NH_4)_2SO_4$ and amino acids, 1 g allantoin as nitrogen source, supplemented with 20 μg/ml His (H), 30 μg/ml Leu (L), 30 μg/ml Lys (K), and 20 μg/ml Ura (U). The plates were incubated at 30° for 2 days, and then the liquid medium growth assay was performed as follows. Five microliters from each well were inoculated into 200 μl of three different liquid media: (1) MM + HLKU medium as described above, (2) MM + His–Leu (H–L)HKU medium, or (3) MM + (H–L)HKU + Trp medium. The MM + (H–L)HKU and MM + (H–L)HKU + Trp media contained 80 μM His–Leu dipeptide in place of leucine with all other components identical to the MM + HLKU medium, except that tryptophan (30 μg/ml) was added to the MM + (H–L)HKU + Trp medium. The purpose of the tryptophan addition was to induce *PTR2* expression as shown in previous studies (Island *et al.* 1987). The efficiency of the utilization of His–Leu was reflected in cell growth. The minicultures in the microtiter plates were grown at 30° with shaking at 200 rpm. The OD_{620} was measured at incubation times of 0, 12, 24, 48, and 72 hr with a 96-well plate reader (Multiskan MCC/340; Labsystems, Helsinki). In some circumstances, growth at incubation times of 36, 60, 84, and 96 hr was also recorded. The *ptr2* deletion mutant was not able to grow in the His–Leu-supplemented medium, although its growth in the medium MM + HKLU was excellent. Strains either defective in peptide utilization (*ptr1* deletion) or overexpressing

PTR2 (*cup9* deletion) were inoculated into wells of each plate as controls showing defective or enhanced dipeptide utilization, respectively. In this manner, deletion strains were identified that grew more, or less, efficiently than wild type in MM + (H–L)HKU or MM + (H–L)HKU + Trp medium.

Solid medium growth assay: Using the same principle as that employed in the liquid medium growth assay, a solid medium growth assay was used to determine whether the candidate gene deletion strains obtained from the liquid medium growth screen could utilize dipeptide on solid medium as their substrate to satisfy auxotrophic requirements. The deletion mutant candidates from the screen, wild-type BY4742, *cup9*, and *ptr1* deletion mutants were grown overnight in MM + HLKU liquid medium at 30° while shaking at 200 rpm. Cells were harvested by centrifugation and washed three times with sterile, distilled water. Cells were then counted by hemacytometer and adjusted to the cell number of 5×10^6/ml. Five microliters of a suspension containing 2.5×10^4 and 2.5×10^3 yeast cells were spotted onto agar plates containing one of three different media: (1) MM + HLKU, (2) MM + (H–L)HKU, or (3) MM + (H–L)HKU + Trp. The plates were incubated at 30°, and growth was recorded after 2 days. A score was given to the growth of each strain compared to the growth of wild-type, *cup9*, and *ptr1* deletion mutant strains.

Toxic halo assays and osmotic sensitivity assay: For the toxic dipeptide halo assay, the sensitivity of deletion mutants to the toxic dipeptide Ala–Ethionine (Eth) was measured as previously described (Island *et al.* 1987). Eth is an analog of methionine, and utilization of Eth causes cell death. This assay is more sensitive than the previously described dipeptide growth assays. Yeast cells having a functional dipeptide transport system will take up the toxic dipeptide and die (indicated by a clear halo of growth inhibition on plates spotted with ethioninyl-containing peptides). Cells with a defective dipeptide uptake system will not take up the dipeptide efficiently and will survive (as indicated by a small halo or no halo). Cells were grown overnight in MM + HLKU medium and then harvested and washed three times with sterile, distilled water. Yeast cells were counted and adjusted to the cell number of 5×10^6/ml. One milliliter of the cell suspension was added to 0.8% noble agar (3 ml) and plated onto solid MM + HLKU medium. Two 6-mm sterile paper disks containing either 0.2 or 0.1 μmol of Ala–Eth were placed on the lawn of cells. The halo size was measured after 2 days of incubation at 30°. The halo size formed by each deletion mutant was compared to the halo size of the wild-type strain by dividing the halo size of the mutant by the halo size of the wild-type strain and multiplied by 100 to get a percentage. A strain that gave a >100% value was considered to be deleted for a gene that was involved in downregulation of peptide utilization, whereas a strain deleted for a gene involved in upregulation would give a <100% value in this test.

For the canavanine toxicity assay, the procedure was the same as that for the toxic dipeptide halo assay. Two 6-mm sterile paper disks containing 1 μg of canavanine were placed on the lawn of cells. The size of halo was measured and the image was taken after 2 days incubation at 30°.

For the osmotic sensitivity assay, the wild-type and deletion mutant strains were grown overnight in MM + HLKU medium and then harvested and washed three times with sterile, distilled water. Yeast cells were counted and adjusted to the cell number of 5×10^6/ml, and then cells were further diluted to 5×10^5/ml, 5×10^4/ml, and 5×10^3/ml. Ten microliters of each cell suspension were spotted into MM + HLKU media containing 0 m, 0.4 m, and 1.0 m NaCl.

Uptake assays: The strains were grown overnight in MM + HLKU and then subcultured into a fresh medium. Cells were harvested in log phase, washed with 2% glucose, and adjusted to a final concentration to 2×10^8 cells/ml. The uptake assay was initiated by combining equal volumes of prewarmed (30°) cells and uptake assay mixture [2% glucose, 20 mm sodium citrate/potassium phosphate, pH 5.5, 320 μm Leu–Leu (Sigma, St. Louis), and 2 μCi/ml [^{3}H]Leu–Leu (Island *et al.* 1987)]. After 10 min, portions (100 μl) were removed onto a membrane filter and washed four times by vacuum filtration with 1 ml ice-cold water. The radioactivity retained on the filter was determined by liquid scintillation spectrometry, and results were reported as nmol Leu–Leu uptake/1×10^9 cells/10 min. The accumulation of [^{3}H]Leu–Leu in the *ptr2* strain was subtracted from that of the tested strains, and the percentage of the accumulation of [^{3}H]Leu–Leu in each deletion mutant *vs.* that in the wild type was calculated.

Reporter gene assay: The centromeric plasmid pRD1, which contained a selectable *URA3* marker and the *lacZ* gene under the control of the *PTR2* promoter, was transformed into the tested deletion mutant strains. The reporter gene assay was performed using a protocol adapted from Hoffman (Hoffman *et al.* 2002). The strains were grown overnight in MM + HLK and subcultured into a fresh medium. Cells were harvested in log phase, washed with sterile water, and adjusted to a final concentration to 1×10^8 cells/ml. Cell suspension (100 μl) was added to a well of a 96-well plate, and then 20 μl fluorescein di-β-d-galactopyranoside (FDG) solution was added. The FDG solution was prepared by mixing solution 1 (0.1 mm FDG diluted in 25 mm PIPES, pH 7.2) and solution 2 (5% Triton X-100 diluted in 250 mm PIPES, pH 7.2) in equal amounts just prior to use. The plate was incubated at 37° for 1.5 hr and then read with a fluorescence multiwell plate reader (Wallac Victor2, 1420 multilabel counter; Perkin-Elmer Life and Analytical Sciences, Wellesley, MA), using 485 and 530 nm as excitation and emission wavelengths, respectively.

Classification of the obtained genes: The classification of the 103 genes identified through this screen was based on the Gene Ontology (GO) annotation found in the Saccharomyces Genome Database (SGD) (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper) (Christie *et al.* 2004), where 34 functional categories of biological process were given. Among the 103 genes, some genes fell into several categories due to their multiple known functions. The detailed gene function could also be referenced from the Yeast Proteome Database (YPD) (https://proteome.incyte.com/control/tools/proteome) (Hodges *et al.* 1999).

Northern analysis of *PTR2* mRNA in deletion mutant strains: Approximately 3×10^8 cells of select candidate deletion mutant strains were harvested after overnight growth in MM + HLKU medium. Yeast total RNA was isolated using an extraction kit (RiboPure-Yeast; Ambion, Austin, TX). Total RNA was quantified by monitoring absorbance at 260 nm, and 20 μg was loaded per lane into a formaldehyde-reducing gel. Gels were run in buffer containing MOPS (pH 7.0) for 5 hr at 75 V and then transferred by capillary action onto a nylon hybridization membrane in 20× SSC. Total RNA was UV crosslinked to the membrane with a Stratalinker UV source. The blot was prehybridized at 65° in Church buffer (7% SDS, 1% BSA, 1 mm EDTA, and 250 mm Na_2HPO4, pH 7.2) for 2 hr and hybridized overnight in the same buffer containing radioactive probes at 65°. Radioactive probes were prepared as follows. A 1.7-kb fragment of *PTR2* was obtained by PCR amplification using the primers PTR2-F (Northern) and PTR2-R (Northern), and a 0.92-kb fragment of *ACT1* was obtained by PCR amplification using the primers ACT1-F (Northern) and ACT1-R (Northern) (primer sequences are represented in supplemental Table S1 at http://www.genetics.org/supplemental/). DNA fragments were labeled with [α-^{32}P] dATP by random-primed probe synthesis. Blots were rinsed with posthybridization buffer (0.1× SSC, 0.1% SDS) at 65°,

and the RNA images were developed with a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA). The expression of *PTR2* in each mutant was analyzed at least twice in separate experiments, with similar expression found in each experiment. The quantification of *PTR2* and *ACT1* mRNA was performed using the software ImageQuant 5.0 (GE Healthcare Technologies, Waukesha, WI).

Real-time RT–PCR: Approximately 3×10^8 cells of the tested deletion mutant strains were harvested after overnight growth in MM + HLKU medium. Yeast total RNA was isolated using the RiboPure-Yeast extraction kit (Ambion) and then treated with the TURBO DNA-free kit (Ambion) to eliminate the genomic DNA contamination. The amount of total RNA was quantified by monitoring absorbance at 260 nm. To check that the genomic DNA had been eliminated, the obtained total RNA was used for the template to PCR amplify the target genes, and no PCR product was obtained. cDNA was synthesized using M-MLV RT (400 units reverse transcriptase; Invitrogen, San Diego) in a reaction mixture containing 1 μg Oligo(dT), 1 mM deooxynucleoside triphosphates, 14 units anti-RNase, and 1 μg of total RNA at 42° for 45 min, and the reaction was stopped after 5 min by incubation at 72°.

Real-time PCR analysis was performed in the DNA Engine Opticon (MJ Research, Boston, MA). The QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA) and the primers (the sequences are presented in supplemental Table S1 at http://www.genetics.org/supplemental/) resulting in ~100 bp amplicon were applied in the PCR reaction. The reaction contained 12.5 μl of 2× qPCR reaction mix and 15 pmol of primers and was run with a cycle of 50° for 2 min, 95° for 15 min, followed by 40 cycles of 95° for 15 sec, 54° for 30 sec, and 72° for 15 sec. A standard curve for each primer set was performed with 1, 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000 dilutions of the wild-type cDNA. The C_T-value, the cycle when sample fluorescence exceeds a chosen threshold above background fluorescence, was determined using the software program. The copy number of *PTR2*, *PMA1*, and *ACT1* genes in each deletion mutant strain was calculated on the basis of the standard curve. The ratio of the fold change of target genes (*PTR2* and *PMA1*) *vs.* the fold change of the internal control gene (*ACT1*) in the tested mutant strains was calculated to show upregulation or downregulation. The ratio of the fold change in the wild type was standardized as 1.0.

Ptr2p localization in deletion mutant strains: To trace the localization of Ptr2p, a Ptr2p-GFP construct was created as follows. The primers PTR2-FLAG-GFP-F and PTR2-Flag-GFP-R (see supplemental Table S1 at http://www.genetics.org/supplemental/ for the sequence) were used to PCR amplify two tandem copies of the GFP gene using pKW430 [a gift of Mary Miller, Rhodes College, Memphis, TN (Stade *et al.* 1997)] as the template. The amplified PCR product (1.5 kb) had two copies of GFP sequence with 40 bp of flanking sequence homologous to pMS2, which contained *PTR2* with its endogenous promoter and terminator sequences and both FLAG and His tags located at the C terminus. The plasmid was linearized with *Age*I at a unique restriction site located between the FLAG and His tags. The centromeric plasmid pMS4, containing Ptr2p-FLAG-GFP_2-His6 and the selectable marker *URA3*, was created by homologous recombination.

To test the localization of Ptr2p-GFP in different yeast strains, the pMS4 construct was transformed into each deletion mutant of interest and transformants were selected by growth in the absence of uracil. Yeast strains carrying pMS4 were pregrown in MM + HLK overnight and then inoculated into fresh MM + HLK at an initial cell concentration of 2×10^6 cells/ml. Cells were concentrated by centrifugation and observed at 10 hr after inoculation by fluorescence microscopy using a 470- to 490-nm excitation wavelength and 515-nm emission filter fitted to an Olympus (Lake Success, NY) microscope. Images were taken with a MicroFire camera (model S99809; Olympus). All mutants were in the log phase at the 10-hr time point as ascertained by the high proportion of cells with buds and the low density of cells under the experimental conditions used. To visualize the amount of GFP expression, each image was captured with the same exposure time. Significantly higher and lower GFP signals could be distinguished from the captured images. For those images with higher GFP signal, the images were taken with a decreased exposure time to be visualized clearly.

RESULTS

Primary screening of deletion mutants to identify strains demonstrating increased or decreased dipeptide utilization: To identify genes involved in dipeptide utilization, we screened a library of 4826 haploid, single-gene deletion strains for mutants with an altered dipeptide utilization profile. The leucine auxotrophic marker in the BY4742 strain background (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) was used to monitor how well each strain was able to utilize the dipeptide His–Leu. Under the conditions of the screen, cells could grow only if they were able to take up the dipeptide His–Leu from the medium and release leucine from the peptide intracellularly. Numerous previous studies have shown that there is no extracellular peptidase or protease in *S. cerevisiae* able to catalyze the hydrolysis of dipeptides under the growth conditions used. In this screening, any mutant strain demonstrating either enhanced or decreased growth was considered to have a deletion in a gene involved in dipeptide utilization. Since Ptr2p is the only dipeptide transporter expressed in this yeast under the conditions used for the screen, we expected that genes related to the regulation of *PTR2* expression or Ptr2p function would comprise the list of candidates. The yeast deletion mutant library was generated by the Saccharomyces Genome Deletion Project, which involved several laboratories (Winzeler *et al.* 1999). Only nonessential genes are represented in this deletion mutant collection.

In the primary screening, yeast deletion mutants were grown in a poor nitrogen medium with allantoin as the nitrogen source. These conditions are known to partially upregulate *PTR2* expression (Island *et al.* 1991). All deletion mutant strains not capable of growing in allantoin as the sole nitrogen source were necessarily excluded from this study. One hundred nine strains of the haploid collection were identified in this category. For a full list of the 109 strains see supplemental Table S2 at http://www.genetics.org/supplemental/. The rest of the deletion series, including strains known to be deficient (*ptr1*) and hyperactive (*cup9*) for dipeptide utilization, grew similarly in the MM + HLKU medium.

A screen was performed of the mutant collection in medium [MM + (H–L)HKU + Trp] containing His–Leu as the only leucine source. Tryptophan upregulates

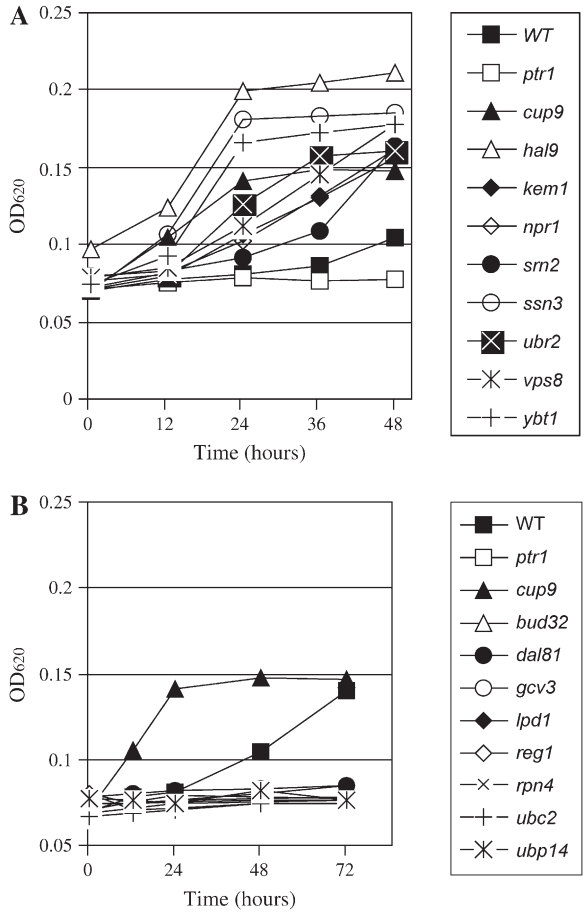

FIGURE 1.—Growth patterns of various selected strains that demonstrate increased or decreased dipeptide utilization. (A) The growth pattern of wild type (BY4742), *ptr1*, *cup9*, and eight other deletion mutant strains in MM + H(H–L)KU + Trp medium. (B) The growth pattern of wild type (BY4742), *ptr1*, *cup9*, and nine other deletion mutant strains in MM + H(H–L)KU + Trp medium.

PTR2 expression via the SPS system over the enhancement induced by growth on MM alone, thus providing the maximal dipeptide utilization phenotype (ISLAND *et al.* 1987; FORSBERG and LJUNGDAHL 2001b). The screen was also performed without tryptophan addition with identical results observed (data not shown). Two hundred seventy-eight deletion mutants were obtained from the primary screening that showed either enhanced dipeptide utilization (the deleted genes are "negative regulator genes") or decreased dipeptide utilization (the deleted genes are "positive regulator genes"). As controls for comparison of strains that show increased or decreased dipeptide utilization, *cup9* and *ptr1* mutants were used. Cup9p is a repressor of *PTR2* transcriptional expression; the *cup9* deletion mutant strain has a higher expression level of *PTR2* in comparison to that of the wild type (BYRD *et al.* 1998); therefore the hyperactive strain (*cup9*) grew better than wild type on dipeptide (Figure 1A). Ptr1p is required for degradation of Cup9p; in the *ptr1* deletion mutant strain Cup9p is stabilized (ISLAND *et al.* 1991; DU *et al.* 2002), and there is an undetectable level of *PTR2* expression preventing this strain from growing on dipeptide (Figure 1A). For clarity of presentation and to present data representative of the phenotypes among the mutants identified, we highlight in the RESULTS section 18 deletion mutant strains (*bud32*, *cup9*, *dal81*, *gcv3*, *hal9*, *kem1*, *lpd1*, *npr1*, *ptr1*, *reg1*, *rpn4*, *ssn3*, *srn2*, *ubc2*, *ubp14*, *ubr2*, *vps8*, and *ybt1*).

Growth patterns of deletion mutant strains *hal9*, *kem1*, *npr1*, *srn2*, *ssn3*, *ubr2*, *vps8*, and *ybt1* were similar to that of the *cup9* deletion mutant in that they had a shorter lag phase and a higher growth rate compared to the wild type (Figure 1A). Therefore, these deletion strains were considered to carry a deletion in a gene involved in decreasing dipeptide utilization. Deletion mutants *bud32*, *dal81*, *gcv3*, *lpd1*, *reg1*, *rpn4*, *ubc2*, and *ubp14* had a similar growth pattern to that of the *ptr1* deletion strain, which did not grow in MM + (H–L)HKU + Trp medium (Figure 1B). These deletion mutants were considered to carry a deletion in a gene involved in increasing dipeptide utilization.

Verification of candidate genes—solid media growth assay and toxic dipeptide halo assay: The candidate mutants were further tested in a solid medium growth assay and a toxic dipeptide halo assay. The deletion mutant strains were grown on solid medium plates containing MM + HLKU, MM + (H–L)HKU, or MM + (H–L)HKU + Trp. Eighteen representative deletion mutant strains with enhanced or decreased dipeptide utilization are shown in Figure 2. All the tested deletion mutant strains grew to a similar extent as compared to that of the wild type in MM + HLKU. The deletion mutants *hal9*, *kem1*, *npr1*, *srn2*, *ssn3*, *ubr2*, *vps8*, and *ybt1* had a similar growth pattern to that of the *cup9* deletion mutant strain, which grew better than wild type in both MM + (H–L)HKU + Trp (Figure 2) and MM + (H–L)HKU (data not shown). In contrast, the deletion mutants *bud32*, *dal81*, *gcv3*, *lpd1*, *reg1*, *rpn4*, *ubc2*, and *ubp14* had a similar growth pattern to that of the *ptr1* deletion mutant strain, which could not grow in MM + (H–L)HKU + Trp medium (Figure 2) or in MM + (H–L)HKU (data not shown). The growth phenotype found in the liquid growth screening of these chosen candidate mutants with respect to the regulation of dipeptide utilization was therefore confirmed in this solid medium test.

The mutants identified in the initial screen were also subjected to a toxic dipeptide assay for further verification of the peptide transport phenotype. In this assay, cells were grown on MM + HLKU and tested for their sensitivity to Ala–Eth. The halo size of each deletion mutant strain was measured and compared to that of wild type. A larger halo indicated that the deletion mutant strain was more sensitive to Ala–Eth toxic dipeptide than the wild type, while a smaller halo indicated less sensitivity. As expected, *cup9* developed a larger halo

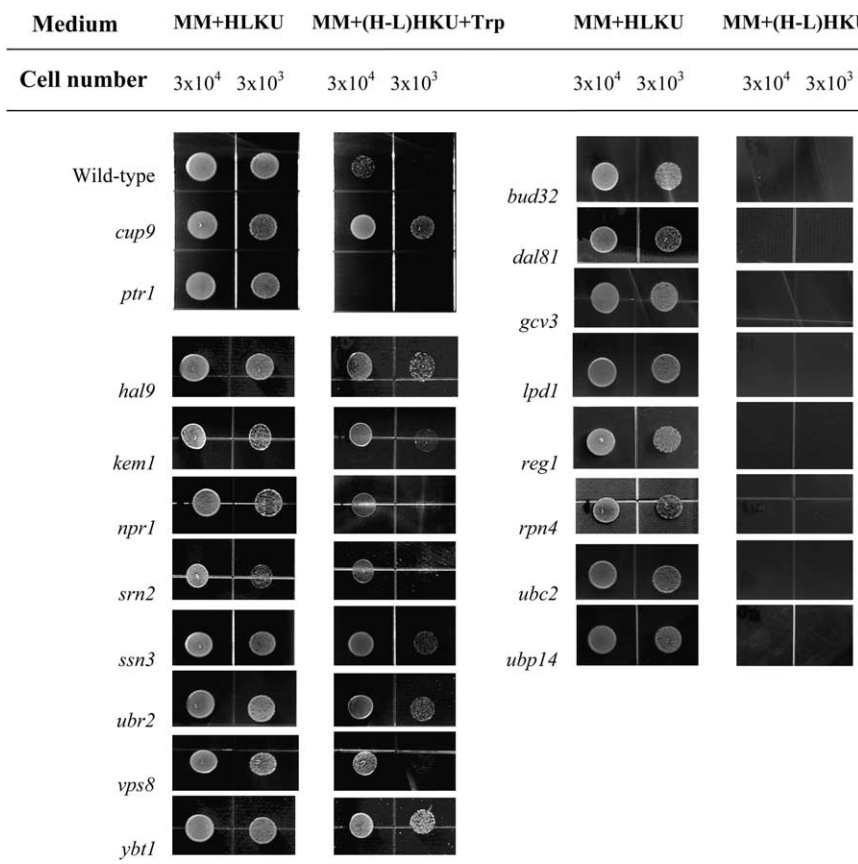

FIGURE 2.—Growth of selected strains on solid medium. Two cell dilutions, 3×10^4 cells/ml and 3×10^3 cells/ml, were tested on MM + HLKU and MM + (H–L)HKU + Trp media.

than that of wild type and *ptr1* was not sensitive to the toxic dipeptide. Similar to *cup9*, deletion strains *hal9*, *kem1*, *npr1*, *srn2*, *ssn3*, *ubr2*, *vps8*, and *ybt1* developed a larger halo than that of wild type (Table 1), indicating that the deleted genes negatively regulated dipeptide utilization. Conversely, similar to *ptr1*, deletion strains *bud32*, *dal81*, *gcv3*, *lpd1*, *reg1*, *rpn4*, *ubc2*, and *ubp14* were not sensitive to toxic dipeptide (Table 2), indicating that the deleted genes positively regulated dipeptide utilization.

TABLE 1

The response of deletion mutants with increased dipeptide utilization to Ala-Eth, NaCl, and canavanine

Gene name	Description of gene product	Toxicity of Ala–Eth (% of wild type)[a]	NaCl Sensitivity[b]	Canavanine toxicity (% of the wild type)[c]
Wild type		100		
CUP9	Specific RNA polymerase II transcription factor activity	120	—	—
HAL9	Specific RNA polymerase II transcription factor activity	131	—	—
KEM1	5′–3′ exoribonuclease activity	135	More sensitive	Less sensitive
NPR1	Serine/threonine protein kinase	129	—	Less sensitive
SRN2	Class E vacuolar sorting protein	109	More sensitive	More sensitive
SSN3	Cyclin-dependent protein kinase activity	114	—	—
UBR2	Ubiquitin-protein ligase activity	129	—	—
VPS8	Vacuolar sorting	121	More sensitive	More sensitive
YBT1	Bile acid transporter activity	156	—	—

Data are also presented in supplemental Table S3 and Figures S4, and S5 at http://www.genetics.org/supplemental/.

[a] The halo size formed by each deletion mutant was compared to the halo size of the wild-type strain by dividing the halo size of the mutant by the halo size of the wild-type strain and multiplied by 100 to get a percentage.

[b] "—" indicates that the deletion strain had no difference in its sensitivity to NaCl compared to that of the wild type. A strain that was more sensitive exhibited greater growth inhibition to 1.0 M NaCl in comparison to that shown by the wild type.

[c] "—" indicates that the deletion strain had no difference in sensitivity to canavanine compared to that of the wild type.

TABLE 2

The response of deletion mutants with decreased dipeptide utilization to Ala-Eth, NaCl, and canavanine

Gene name	Description of gene product	Toxicity of Ala–Eth (% of wild type)[a]	NaCl sensitivity[b]	Canavanine toxicity (% of the wild type)[c]
Wild type		100		
PTR1	Ubiquitin–protein ligase activity	0	—	—
BUD32	Protein serine/threonine kinase activity	0 (fuzzy halo)	More sensitive	Less sensitive
DAL81	Specific RNA polymerase II transcription factor activity	0	—	More sensitive
GCV3	Glycine dehydrogenase (decarboxylating) activity	0	—	More sensitive
LPD1	Dihydrolipoyl dehydrogenase activity	0	—	More sensitive
REG1	Protein phosphatase type 1 activity	0 (fuzzy halo)	—	More sensitive
RPN4	Transcriptional activator activity	0	—	—
UBC2	Ubiquitin-conjugating enzyme activity	0	—	—
UBP14	Ubiquitin-specific protease activity	73	—	—

These data are also presented in supplemental Table S3 and supplemental Figures S4 and S5 at http://www.genetics.org/supplemental/.

[a] The halo size formed by each deletion mutant was compared to the halo size of the wild-type strain by dividing the halo size of the mutant by the halo size of the wild-type strain and multiplied by 100 to get a percentage.

[b] "—" indicates that the deletion strain had no difference in its sensitivity to NaCl compared to that of the wild type. A strain that was more sensitive exhibited greater growth inhibition to 1.0 M NaCl in comparison to that shown by the wild type.

[c] "—" indicates that the deletion strain had no difference in sensitivity to canavanine compared to that of the wild type.

Of the 278 strains identified in the initial liquid growth screen, 103 strains showed increased or decreased peptide utilization in all three assays: liquid and solid media growth and the toxic dipeptide halo assay [supplemental Tables S3 and S4 (http://www.genetics.org/supplemental/) listed in order of biological process according to Gene Ontology annotation]. These 103 genes were considered to be the set of genes involved as either negative or positive regulators of peptide utilization as determined by the three experimental tests. As expected, 5 genes (*PTR1*, *CUP9*, *UBC2*, *DAL81/UGA35*, and *STP2*) previously known to be involved in dipeptide utilization were identified among the 103 genes, which validated the utility of the screening method. Three other genes (*SSY1*, *PTR3*, and *SSY5*), known to affect dipeptide utilization via the regulation of *PTR2* mRNA expression, were not found in the screen since they are not included in the deletion mutant library. The number of deletion mutant strains found by our methods to modulate dipeptide utilization accounted for ~2% of the entire collection. Of the 46 genes whose deletion mutant strains enhanced dipeptide utilization (supplemental Table S3), 20 encoded proteins with more than a 30% protein sequence identity to a human protein (supplemental Table S3, footnote *a*). Additionally, of the 57 genes whose deletion mutant strain decreased dipeptide utilization (supplemental Table S4), 33 encoded proteins with more than a 30% sequence identity to a human protein (supplemental Table S4, footnote *a*). According to GO annotation in the SGD, the 103 genes covered 29 of the 34 given Biological Processes GO categories. The categories of each gene are indicated in supplemental Tables S3 and S4.

Ptr2p-GFP expression and localization in the identified deletion strains: To further explore the impact of the identified 103 genes on dipeptide utilization, we measured Ptr2p expression and localization using a Ptr2p-GFP chimera. This construct (pMS4), which encoded Ptr2p-GFP under the control of its native promoter, was transformed into the 103 deletion strains, and Ptr2p-GFP in log phase cells was observed by fluorescence microscopy. The addition of the GFP tag did not affect the function of Ptr2p as demonstrated by halo and uptake assays (data not shown). Ptr2p-GFP was primarily localized to the plasma membrane in wild-type log-phase cells (Figure 3). The amount of the Ptr2p-GFP expression signal and its localization in the 103 deletion mutant strains revealed seven phenotypic patterns (Table 3).

Ptr2p-GFP expression and localization in deletion strains with increased dipeptide utilization: The Ptr2p-GFP expression signal and localization in 46 deletion strains with increased dipeptide utilization are shown in supplemental Figure S1 at http://www.genetics.org/supplemental/ (9 deletion strains and wild type are shown in Figure 3 as representative of the full set).

Strains with enhanced Ptr2p-GFP expression signal: A higher level of Ptr2p-GFP expression signal in the plasma membrane was observed in the *cup9* deletion strain as compared to the wild type (Figure 3, Table 3). The enhanced Ptr2p-GFP expression level corresponded with the phenotype of increased dipeptide utilization as reflected in growth on dipeptide in liquid and solid medium and toxicity to Ala–Eth (Figures 1 and 2, and Table 1). In addition, Ptr2p-GFP was also observed in the vacuole, possibly due to the overexpressed *PTR2* in the *cup9* strain. Similar to the *cup9* strain, the deletion of

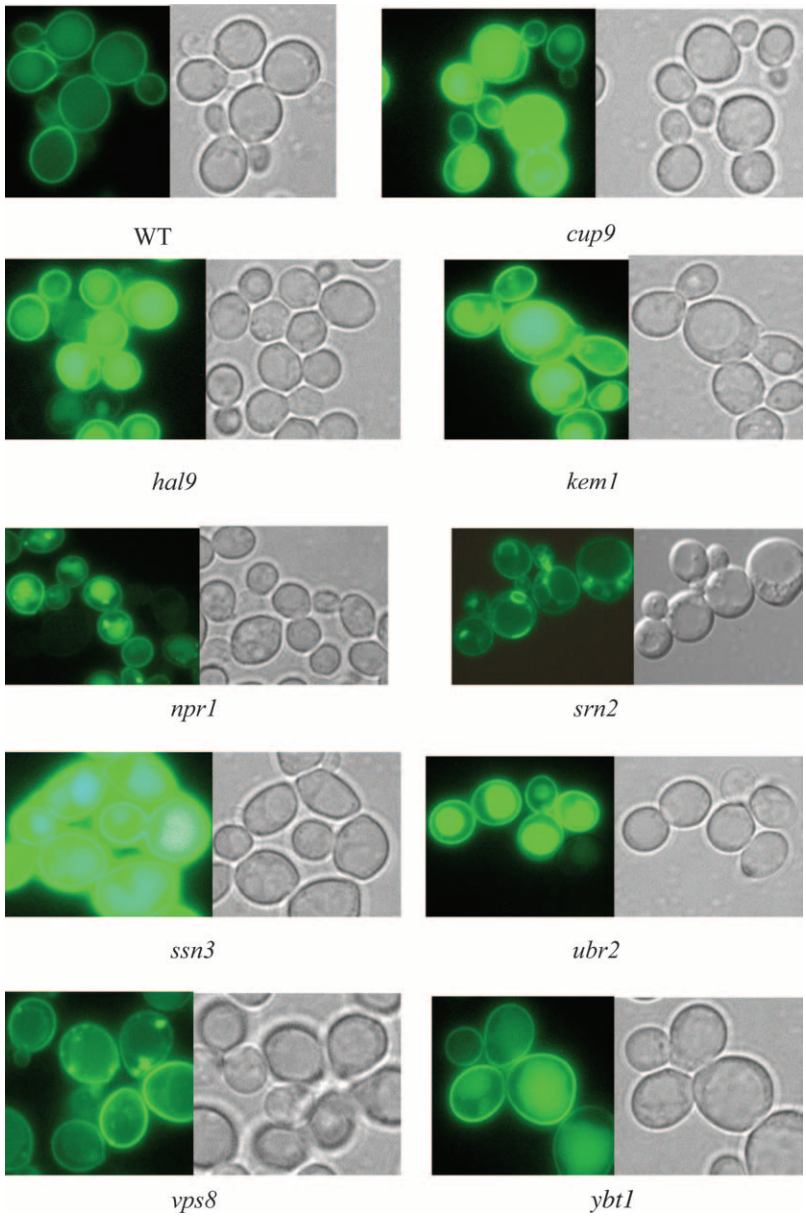

FIGURE 3.—Ptr2p-GFP expression and localization in the wild type (WT) and nine deletion strains that demonstrated increased dipeptide utilization. The right-hand side of each strain shows phase microscopy of the same field as the left-hand side, which shows the fluorescently labeled Ptr2p-GFP. Deletion strain *ssn3* showed a very high amount of GFP signal under the same exposure time as that of the wild type.

HAL9, *KEM1*, *SSN3*, and *UBR2* showed an increased Ptr2p-GFP expression signal in both the plasma membrane and the vacuole. Enhanced Ptr2p-GFP expression signal was also observed in 18 other deletion strains including four unknown gene deletion mutants, *yfr044c*, *ylr114c*, *ynl123w*, and *yor322c* (Table 3 and supplemental Figure S1 at http://www.genetics.org/supplemental/). Consistently, the increased Ptr2p-GFP expression agreed with increased sensitivity to toxic dipeptide and better growth on dipeptide substrate (supplemental Table S3 at http://www.genetics.org/supplemental/).

Strains with altered Ptr2p-GFP localization: Fourteen deletion strains with increased dipeptide utilization showed altered localization of Ptr2p-GFP (Table 3 and supplemental Figure S1 at http://www.genetics.org/supplemental/). An example of three deletion mutants, *npr1*, *srn2*, and *vps8* is shown in Figure 3. These gene products had GO annotations of transport, vesicle-mediated transport, cytokinesis, or related to protein modification. Ptr2p-GFP signal in the *srn2* and *vps8* deletion mutants was localized mainly to the plasma membrane and the endosome. In addition, Ptr2p-GFP remained in the endosome in stationary phase cells and targeting of Ptr2p-GFP to the vacuole was defective (data not shown). Similar to *srn2* and *vps8* strains, the deletion strains *eaf7*, *sac6*, *snf7*, *vps36*, and *ypl073c* exhibited Ptr2p-GFP localization to the plasma membrane and to the endosome (supplemental Figure S1 at http://www.genetics.org/supplemental/), suggesting that these gene products are involved in a similar cellular process regulating dipeptide utilization. Snf7p, Srn2p, and Vps36p are three key proteins involved in ESCRT (endosomal

TABLE 3

Ptr2p-GFP expression and localization in 103 identified strains with decreased or increased dipeptide utilization

Phenotypic category	Increased dipeptide utilization (46 strains)	Decreased dipeptide utilization (57 strains)
Strains with enhanced Ptr2p-GFP expression	*arp5*, *arp8*, ***cup9***, *csm1*, *dbr1*, *eaf3*, *hal9*, *ies6*, *kem1*, *mrps9*, *pat1*, *sfp1*, *srb8*, *ssd1*, *ssn2*, *ssn3*, *ssn8*, *tom72*, *ubr2*, *yor322c*, *ynl123w*, *yfr044c*, *ylr114c*	*asm4*, *ydr015c*, *ydr290w*
Strains with altered Ptr2p-GFP localization	*bni1*, *def1*, *eaf7*, *lst4*, *npr1*, *sac6*, *snf7*, *srn2*, *tpm1*, *vam10*, *vps8*, *vps36*, *ypl073c*, *ynl295w*	*bem4*, *bud32*, *csf1*, *etr1*, *gcv2*, *gcv3*, *hfm1*, *ilm1*, *isa1*, *isa2*, *mck1*, *mre11*, *npl3*, *shp1*, *reg1*, *rmd12*, *rps9b*, *tsa1*, *vbm1*, *ydr433w*, *yjl046w*, *ydr157w*, *yjl175w*
Strains with no apparent effect on Ptr2p-GFP localization or expression	*cik1*, *kti12*, *mlh1*, *mrp17*, *pho2*, *rim101*, *tif3*, *ybt1*, *ydr417c*	*bud28*, *bye1*, *elf1*, *hfa1*, *ira2*, *lat1*, *lpd1*, *mct1*, *oar1*, *pdb1*, *pdx1*, *prd1*, *rad23*, *spo21*, *ypl098c*
Strains with decreased Ptr2p-GFP expression	No mutant strains were found in this category.	***dal81***, *hof1*, *ipk1*, *nfu1*, *pho85*, ***ptr1***, *rpl21a*, *rpn4*, *shm2*, ***stp2***, *taf14*, *thp1*, *uba3*, *ubp14*, ***ubc2***, *ypr174c*

Ptr2p-GFP expression and localization in the underlined strains are shown in Figures 3 and 4. The underlined strains were the representatives chosen to highlight in the RESULTS section. Ptr2p-GFP expression and localization of all the listed strains are shown in supplemental Figures S1 and S3 at http://www.genetics.org/supplemental/. The strains in boldface type (***cup9***, ***dal81***, ***ptr1***, ***stp2***, and ***ubc2***) contain the deleted genes previously identified as being involved in regulation of *PTR2* transcription.

sorting complex required for transport) protein complexes required for endocytotic degradation of membrane proteins (BOWERS *et al.* 2004).

To further characterize the ESCRT protein complexes involved in Ptr2p endocytotic degradation, deletion mutant strains of other components of the ESCRT protein complexes (*vps2*, *vps20*, *vps22*, *vps23*, *vps24*, *vps25*, and *vps28*) were also transformed with the pMS4 construct. The localization of Ptr2p-GFP in these strains was similar to that in *snf7*, *srn2*, and *vps36* strains (supplemental Figure S2 at http://www.genetics.org/supplemental/). Additionally, *vps2*, *vps20*, *vps22*, *vps23*, *vps24*, *vps25*, and *vps28* strains were more sensitive to toxic dipeptide as compared to the wild type (supplemental Table S5 at http://www.genetics.org/supplemental/), confirming the role of the ESCRT protein complex in dipeptide utilization.

The deletion strain *npr1* showed an accumulation of Ptr2p-GFP signal in both endosomal vesicles and the vacuole in addition to the plasma membrane localization (Figure 3). The Ptr2p sorting process is likely regulated by Npr1p, a protein kinase involved in the regulation of vesicle transport systems (supplemental Table S3 at http://www.genetics.org/supplemental/). Similar to Ptr2p-GFP localization in the *npr1* strain, deletion strains *bni1*, *def1*, *lst4*, and *tpm1* showed Ptr2p-GFP localization to the plasma membrane and the vacuole (supplemental Figure S1 at http://www.genetics.org/supplemental/), implicating them as well as being involved in the protein trafficking of Ptr2p.

Strains with no apparent effect on Ptr2p-GFP expression or localization: The deletion strain *ybt1* showed no apparent difference in the expression level of Ptr2p-GFP signal in the cytoplasmic membrane (Figure 3), although there was an increase in the Ptr2p-GFP signal in the vacuole. In this strain dipeptide utilization was increased (Figures 1 and 2 and Table 1). In addition, seven other deletion strains, *cik1*, *kti12*, *mlh1*, *mrp17*, *pho2*, *rim101*, *tif3*, and *ydr417c* demonstrated increased dipeptide utilization with no apparent effect on Ptr2p-GFP expression in the cytoplasmic membrane (Table 3 and supplemental Figure S1 at http://www.genetics.org/supplemental/). These gene products might impact dipeptide utilization independently of Ptr2p function. For example, Ybt1p, known as an ABC transporter and localized on the vacuolar membrane, may be involved in the uptake of dipeptides into the vacuole. The deletion of this gene resulted in increased growth response and dipeptide toxicity.

Ptr2p-GFP expression and localization in deletion strains with decreased dipeptide utilization: The expression level of Ptr2p-GFP signal and localization of 9 representative strains are shown in Figure 4, and all 57 deletion strains listed in Table 3 with decreased dipeptide utilization are shown in supplemental Figure S3 at http://www.genetics.org/supplemental/.

Gene deletion enhanced the expression level of Ptr2p-GFP signal: The deletion strains *asm4*, *ydr015c*, and *ydr290w* showed an enhanced expression level of Ptr2p-GFP at the cytoplasmic membrane and the vacuole (supplemental Figure S4 at http://www.genetics.org/supplemental/). These strains showed a decreased sensitivity to toxic dipeptide, however, with no growth change on dipeptide (supplemental Table S4 at http://www.genetics.org/supplemental/). In the *asm4* strain, a fuzzy halo was observed in the toxic dipeptide halo assay, indicating a transient growth inhibition. *ASM4* encodes a component of the karyopherin docking complex of the nuclear pore (MARELLI *et al.* 1998), indicating that some

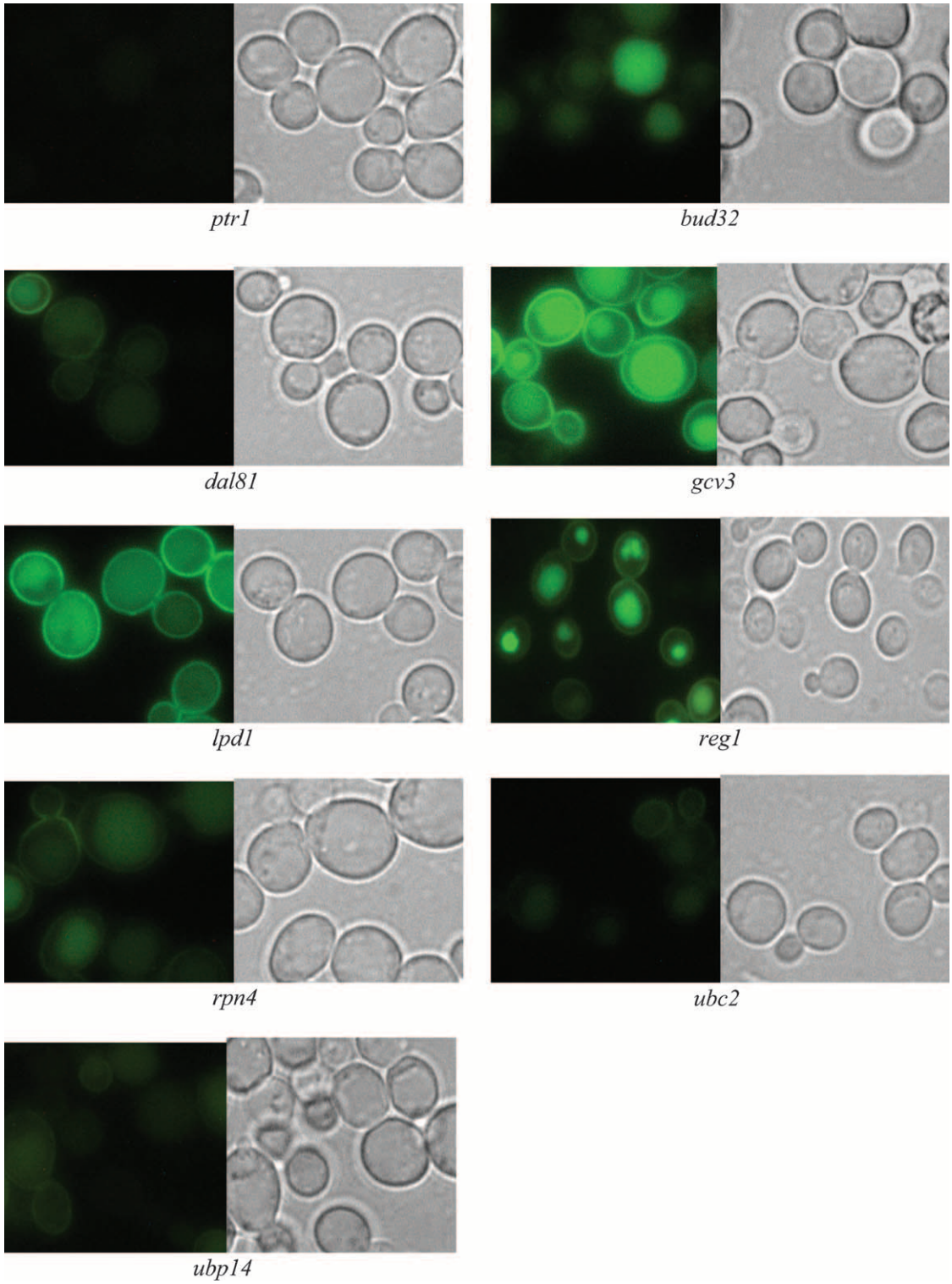

FIGURE 4.—Ptr2p-GFP expression and localization in nine selected deletion strains that demonstrated decreased dipeptide utilization. The right-hand side of each strain shows phase microscopy of the same field as the left-hand side, which shows the fluorescently labeled Ptr2p-GFP.

cellular protein involved in ethionine toxicity requires Asm4p for its full expression.

Strains with altered Ptr2p-GFP localization: Compared to the wild type, deletion strains *bud32* and *reg1* showed higher expression level of Ptr2p-GFP signal at the vacuole and less expression in the plasma membrane (*reg1*) or no visible plasma membrane expression (*bud32*) (Figure 4). In addition, deletion strain *gcv3* showed

Ptr2p-GFP localization both at the vacuole and at the plasma membrane (Figure 4). Similar to the Ptr2p-GFP localization at the vacuole in *bud32*, *reg1*, or *gcv3* strains, the deletion strains *bem4*, *csf1*, *etr1*, *gcv2*, *hfm1*, *ilm1*, *isa1*, *isa2*, *mck1*, *mre11*, *npl3*, *rps9b*, *rmd12*, *shp1*, *tsa1*, *vbm1* and four unknown gene deletion strains (*ydr157w*, *ydr433w*, *yjl046w*, and *yjl175w*) resulted in Ptr2p-GFP localization both at the vacuole and at the plasma membrane (supplemental Table S4 and supplemental Figure S3 at http://www.genetics.org/supplemental/).

Strains with no apparent effect on the expression or localization of Ptr2p-GFP: No apparent change in the expression or localization of Ptr2p-GFP was observed in the *lpd1* deletion strain (Figure 4). Similarly, Ptr2p-GFP localization and expression level was not different in the deletion strains *bye1*, *bud28*, *elf1*, *hfa1*, *ira2*, *lat1*, *mct1*, *oar1*, *pdb1*, *pdx1*, *rad23*, and *ypl098c* as compared to that in the wild type (supplemental Table S4 and supplemental Figure S3 at http://www.genetics.org/supplemental/).

Strains with decreased expression of Ptr2p-GFP: The expression level of Ptr2p-GFP signal was lower in the *ptr1*, *dal81*, *rpn4*, *ubc2*, and *ubp14* deletion strain as compared to that in the wild type (Figure 4). A decreased Ptr2p-GFP expression signal was also observed in the deletion strains *hof1*, *ipk1*, *nfu1*, *pho85*, *rpl21a*, *shm2*, *stp2*, *taf14*, *thp1*, *uba3*, and *ypr174c* (supplemental Table S4 and supplemental Figure S3 at http://www.genetics.org/supplemental/). The decreased Ptr2p-GFP expression signal in these strains was consistent with a decrease of dipeptide utilization that was previously documented (supplemental Table S4) and is consistent with the observation that Ptr1p, Stp2p, Ubc2p, and Dal81p positively regulate *PTR2* transcription (Bernard and Andre 2001; Andreasson and Ljungdahl 2002; Du *et al.* 2002).

Transcriptional regulation of *PTR2*: To explore whether strains with enhanced or reduced expression level of Ptr2p-GFP showed a change in the transcriptional regulation of *PTR2*, the fold change of *PTR2* mRNA compared to that of the wild type was measured by real-time reverse transcription PCR in the 18 representative deletion strains. In control experiments, *PTR2* mRNA expression level in the *cup9* strain was upregulated more than nine times that of the wild type (Table 4). This result also agreed with the increased *PTR2* mRNA level in Northern analysis and *lacZ* activity as compared to the wild type (data not shown). The increase in *PTR2* mRNA expression in the *cup9* strain is consistent with the increased expression level of Ptr2p-GFP signal (Figure 3). Similar to that of the *cup9* strain, *PTR2* mRNA expression level was highly upregulated in *ssn3* and *kem1*. *PTR2* mRNA expression was upregulated in *ubr2*, *hal9*, *ybt1*, *vpc8*, and *gcv3* less than twofold. In contrast to the *cup9*, *hal9*, *kem1*, *ssn3*, *ubr2*, *vps8*, and *ybt1* strains, which showed increased dipeptide utilization, the *gcv3* strain exhibited a decrease in peptide utilization (supplemental Table S4 at http://www.genetics.org/supplemental/). The apparent discrepancy between decrease in utilization and robust expression in the *gcv3* strain could result from a posttranslational alteration of Ptr2p function or the alteration of the metabolic pathway(s) of dipeptide utilization.

TABLE 4

The expression of *PTR2* and *PMA1* in various strains

Strains	*PTR2* expression[a]	*PMA1* expression[a]
ubc2	0.06	0.94
ptr1	0.07	1.08
ubp14	0.08	1.09
rpn4	0.21	1.23
npr1	0.48	1.33
dal81	0.79	1.70
reg1	0.88	1.03
lpd1	0.82	2.30
srn2	0.92	0.87
bud32	0.94	1.44
ubr2	1.23	2.95
hal9	1.37	1.93
ybt1	1.51	2.37
vps8	1.66	2.09
gcv3	1.93	1.22
ssn3	5.00	2.41
cup9	9.36	0.98
kem1	12.12	3.06

[a] Expression level measured by real-time RT–PCR analysis is shown as a ratio calculated by the fold change of the target gene (*PTR2* or *PMA1*) in the mutant compared to that of the wild type divided by the fold change of *ACT1* in the mutant compared to that of the wild type.

PTR2 mRNA expression level in the *ptr1* strain was only 7% that of the wild type (Table 4) in accordance with the known requirement of *Ptr1p* for *PTR2* transcription (Byrd *et al.* 1998; Turner *et al.* 2000). This result also agreed with the decreased *PTR2* mRNA level in Northern analysis and lacZ activity as compared to that in the wild type (data not shown). The decrease in *PTR2* mRNA expression in the *ptr1* strain is consistent with the decreased expression level of Ptr2p-GFP signal (Figure 4). Similar to that in the *ptr1* strain, *PTR2* mRNA was highly downregulated in *ubc2*, *ubp14*, and *rpn4* strains. The expression of *PTR2* mRNA in *npr1*, *dal81*, *reg1*, *lpd1*, *srn2*, and *bud32* strains was not more than twofold less than that of the wild type.

Dipeptide uptake capability: To explore whether altered dipeptide utilization was correlated directly to flux of dipeptide, the accumulation of [^{3}H]Leu–Leu dipeptide was measured in the 18 representative strains (Figure 5). In a control experiment, the accumulation of [^{3}H]Leu–Leu increased remarkably in the *cup9* strain as compared to that in the wild type (Figure 5). Similar to that in the *cup9* strain, the accumulation of [^{3}H]Leu–Leu was higher in the *hal9*, *kem1*, *npr1*, *srn2*, *ssn3*, *ubr2*, *vps8*, and *ybt1* strains as compared to that in the wild type. The increased uptake in these strains was reflected

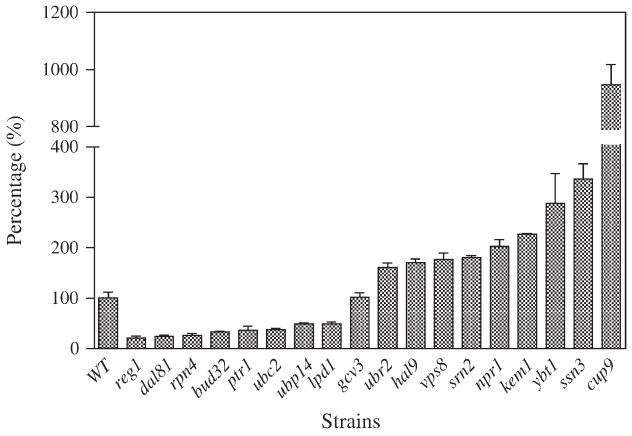

FIGURE 5.—The uptake of [^{3}H]Leu–Leu dipeptide in log phase cells of the wild type, *cup9*, and *ptr1* and the 16 deletion strains *bud32*, *dal81*, *gcv3*, *hal9*, *kem1*, *lpd1*, *npr1*, *reg1*, *rpn4*, *srn2*, *ssn3*, *ubc2*, *ubr2*, *ubp14*, *vps8*, and *ybt1*. The percentage of the accumulation of [^{3}H]Leu–Leu for each deletion mutant was calculated as [(the accumulation of [^{3}H]Leu–Leu in each mutant strain)/(the accumulation of [^{3}H]Leu–Leu in the wild type) × 100].

in their increased ability to utilize dipeptide. In contrast to the increased accumulation of dipeptide in *hal9*, *kem1*, *npr1*, *srn2*, *ssn3*, *ubr2*, *vps8*, and *ybt1* strains, the accumulation of [^{3}H]Leu–Leu in *gcv3* was not significantly different as compared to that in the wild type, suggesting that deletion of this gene did not impact the import of dipeptide even though dipeptide utilization was decreased.

The accumulation of [^{3}H]Leu–Leu in the *ptr1* strain was lower than that in the wild type. The reduced accumulation of dipeptide was consistent with the decreased expression level of *PTR2* mRNA and Ptr2p-GFP in the *ptr1* strain. Similar to that in the *ptr1* strain, the accumulation of [^{3}H]Leu–Leu was lower in the *bud32*, *dal81*, *lpd1*, *reg1*, *rpn4*, *ubc2*, and *ubp14* strains as compared to that in the wild type (Figure 5). The decreased uptake in these strains was reflected in their decreased expression of *PTR2* in *dal81*, *rpn4*, *ubc2*, and *ubp14* strains (Table 4), decreased amount of Ptr2p-GFP in the cytoplasmic membrane (Figure 4), and the alteration localization of Ptr2p in *bud32* and *reg1* strains (Figure 4). In contrast, although the transport of dipeptide was reduced in the *lpd1* strain, the expression level of Ptr2p-GFP remained similar to that in the wild type (Figure 4).

Effects of gene deletions on osmotic and canavanine sensitivity and *PMA1* expression: To determine whether gene deletions also affected general membrane properties, additional assays were performed. Osmotic sensitivity, toxicity of canavanine, and the expression level of *PMA1* encoding a proton transporter localized in the cytoplasmic membrane were examined. NaCl at 1.0 M inhibits the growth of wild-type *S. cerevisiae* by causing osmotic destabilization of the cell membrane (HOHMANN 2002). NaCl at 1.0 M significantly inhibited the growth of all strains on MM + HLKU plates. Fourteen deletion mutant strains, *cup9*, *dal81*, *gcv3*, *hal9*, *lpd1*, *npr1*, *ptr1*, *reg1*, *rpn4*, *ssn3*, *ubc2*, *ubp14*, *ubr2*, and *ybt1*, showed similar effects to NaCl as the wild type (Tables 1 and 2 and supplemental Figure S4 at http://www.genetics.org/supplemental/), suggesting that the deletion of those genes did not change the osmotic sensitivity of the deletion mutant strains. In contrast, four deletion mutant strains, *bud32*, *kem1*, *srn2*, and *vps8*, showed greater sensitivity to 1.0 M NaCl than the wild type (Tables 1 and 2 and supplemental Figure S4).

Canavanine is transported by the arginine permease and changes in canavanine toxicity have been correlated directly to arginine permease function (HAMPSEY 1997). In the canavanine toxicity assay, nine deletion mutant strains, *cup9*, *hal9*, *ptr1*, *rpn4*, *ssn3*, *ubc2*, *ubp14*, *ubr2*, and *ybt1*, did not show significant alteration in the canavanine sensitivity as compared with that of the wild type. In contrast, six deletion mutants, *dal81*, *gcv3*, *lpd1*, *reg1*, *srn2*, and *vps8*, were more sensitive to canavanine, and three deletion mutant strains, *bud32*, *kem1*, and *npr1*, were less sensitive than the wild type (Tables 1 and 2 and supplemental Figure S5 at http://www.genetics.org/supplemental/).

To examine whether the gene representative deletion mutants were also affected in the expression of other transport proteins, the expression level of *PMA1* encoding a proton transporter was measured. *PMA1* mRNA expression level in *cup9* and *ptr1* strains was similar to that of the wild type (Table 4). In contrast, *PMA1* mRNA level was upregulated more than twofold in *kem1*, *lpd1*, *ssn3*, *ubr2*, *vps8*, and *ybt1* strains and less than twofold in *bud32*, *dal81*, *gcv3*, *hal9*, *npr1*, *reg1*, *rpn4*, *srn2*, *ubc2*, and *ubp14* strains (Table 4). Overall, these changes in expression levels of *PMA1* in the various mutant strains did not correlate with the changes in expression in *PTR2* except for upregulation of both genes in the *ssn3* and *kem1* strains.

DISCUSSION

We have systematically screened a haploid, single-gene, deletion mutant library and identified 103 genes involved in dipeptide utilization. To our knowledge, this is the first such screen for a membrane transport system. *PTR2* expression is known to be regulated by amino acids via the SPS protein complex and by dipeptides themselves (Figure 6). Import of di/tripeptides containing basic or bulky hydrophobic N-terminal residues (N-end rule peptides) induces *PTR2* by reducing cellular levels of the *PTR2* repressor Cup9p (BYRD *et al.* 1998; TURNER *et al.* 2000; DU *et al.* 2002). In the SPS complex regulatory pathway a few downstream proteins, such as *STP2* and components of the SCF^{Grr1} ubiquitin ligase complex (Skp1p, Hrt1p, Cdc34p, and Cdc53p), are involved in the Ssy1p-induced signal transduction pathway via proteolytic processing of Stp2p (BERNARD and

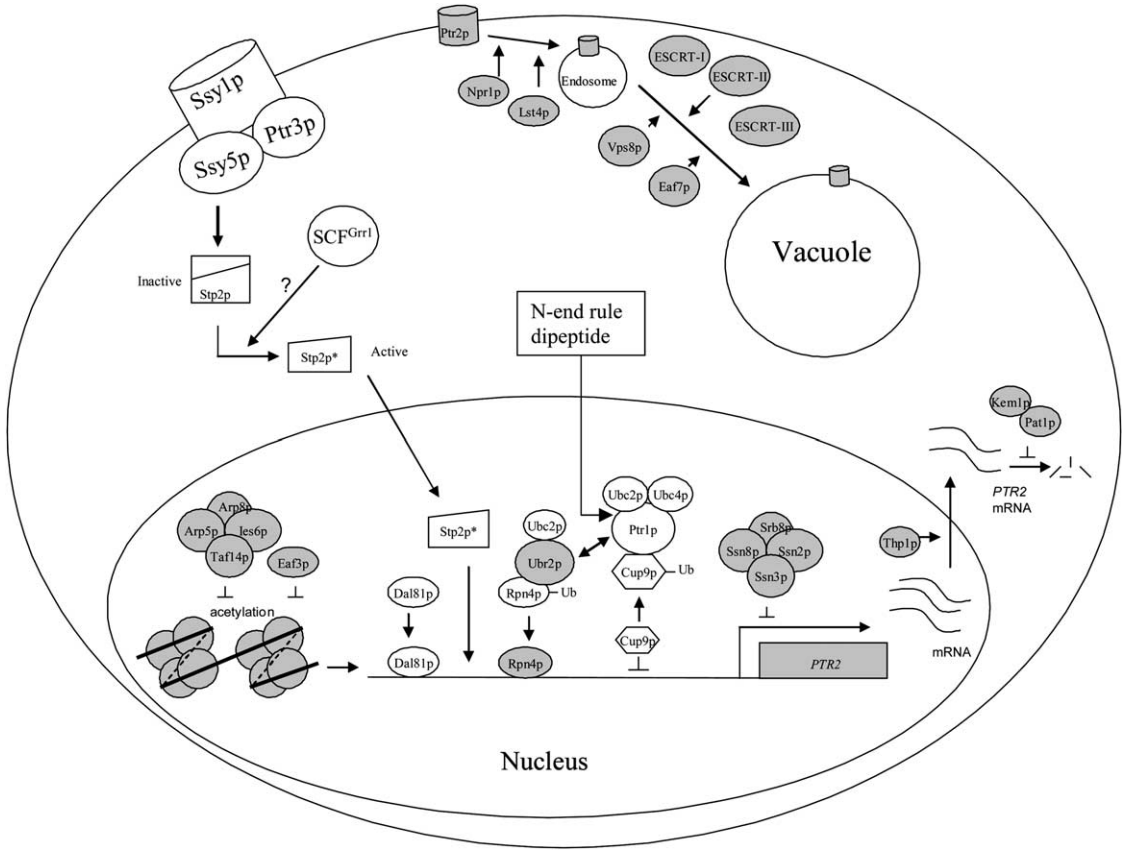

Figure 6.—A hypothetical model for the regulation of dipeptide utilization in *S. cerevisiae*. The proteins with clear background represent those that have been reported previously to be involved in *PTR2* regulation. The shaded background represents those proteins that have been determined in this study. The SPS protein complex (Ssy1p-Ptr3p-Ssy5p) is an amino acid sensor, which leads to a cleavage of the N-terminal of Stp2p. The activated form of Stp2p is localized to the nucleus and regulates *PTR2* expression. In addition, Dal81p synergistically regulates *PTR2* expression together with Stp2p. Other proteins, Rpn4p, a positive regulator, and Cup9p, a negative regulator, bind at the *PTR2* promoter region and are also involved in *PTR2* transcription. The stability of Rpn4p is modulated by Ubr2p and Ubc2p and the stability of Cup9p by Ptr1p, Ubc2p, and Ubc4p via the ubiquitination pathway. Binding of N-end rule dipeptides to Ptr1p/Ubr1p results in ubiquitin-mediated degradation of Cup9p. Ubr2p competes with Ptr1p for the degradation of Cup9p. Arp5p, Arp8p, Ies6p, and Taf14p (components of the INO80 complex) together with Eaf3p possibly negatively regulate *PTR2* transcription via repressing the acetylation of histones in the *PTR2* coding region. The transcription of *PTR2* is negatively regulated by a RNA polymerase mediator protein complex, which includes Ssn2p, Ssn3p, Ssn8p, and Srb8p. Thp1p might be involved in the translocation of *PTR2* mRNA from the nucleus to the cytosol. The interaction between Kem1p and Pat1p is potentially involved in the degradation of *PTR2* mRNA. Dipeptide utilization is also modulated by the Ptr2p trafficking system; Srn2p, Vps36p, and Snf7p represent components of ESCRT-I, -II, and -III, together with Vps8p and Eaf7p, which appear to regulate Ptr2p internalization from the endosome to the vacuole. In addition, Npr1 and Lst4p are also involved in Ptr2p trafficking and affect the retention of Ptr2p in the plasma membrane.

Andre 2001; Andreasson and Ljungdahl 2002). Due to the complex nature of signal transduction pathways, such as the well-studied pheromone-mediated mitogen-activated protein (MAP) kinase pathway in yeast (Gustin *et al.* 1998), we expected that other regulatory proteins would be identified in this screen.

The 103 genes identified in the screen cover a number of different biological processes based on the Gene Ontology annotation. It is clear from the broad range of mutants identified that genes involved in both direct and indirect regulation of peptide utilization were discovered. Such a global array of genes supports the interconnectivity of networks involved in regulating biological processes (Lee *et al.* 2002; Chen *et al.* 2003), but such a heterogeneous collection of interacting genes has not been identified previously as specifically regulating membrane transport systems.

Genes that regulate *PTR2* transcription: In this study we have considerably expanded the identification of genes involved in *PTR2* transcriptional regulation. The deletion of these genes results in the alteration of dipeptide utilization.

First, we have shown that dipeptide utilization can be affected by gene products involved in the modification of the nucleosome, namely Arp5p, Arp8p, Ies6p, and Taf14p. These proteins belong to the components of the chromatin remodeling INO80 protein complex that carries ATPase activity, DNA binding, and nucleosome

mobilization (Sanders *et al.* 2002) and that preferentially interacts with histones H3 and H4 (Shen *et al.* 2003). Another histone- modification-related gene product, Eaf3p, was also identified in our screen. Eaf3p is a component of the NuA4 histone acetyltransferase complex that maintains the acetylation of histones H3 and H4 (Reid *et al.* 2004). It is possible that these chromatin remodeling proteins negatively regulate *PTR2* transcription via the modification of histones. A model representing the potential involvement of these proteins and others is shown (Figure 6).

Second, four gene products with transcription factor activity, Cup9p, Dal81p, Stp2p, and Rpn4p were shown to regulate *PTR2* expression and affected dipeptide utilization. Cup9p binds the region between −488 and −897 upstream of the *PTR2* start codon (Byrd *et al.* 1998), while Rpn4p may bind between −610 and −603 due to the existence of the Rpn4p binding consensus sequence (5′-GGTGGAAA-3′) present at this location (Mannhaupt *et al.* 1999). Cup9p is a repressor of *PTR2*, whereas Rpn4p is a "positive regulator" as reflected in the observation that the *cup9* deletion strain showed an increase in peptide utilization and the *rpn4* strain has decreased peptide utilization. Subsequent to amino acid induction via the SPS system and its conversion into the active form after proteolytically processing and translocation into the nucleus (Andreasson and Ljungdahl 2002, 2004), the active form of Stp2p (truncated at the N terminus) binds to the upstream activation sequence (UAS) of the promoter region of *BAP2* and *BAP3* (de Boer *et al.* 2000; Nielsen *et al.* 2001). Because *BAP2*, *BAP3*, and *PTR2* are simultaneously regulated in response to amino acid induction via the SPS-Stp2p system, the active form of Stp2p likely also binds to the *PTR2* promoter region and regulates its transcription. Dal81p is a transcriptional activator essential for *PTR2* expression under the induction by the SPS signal pathway as well (Bernard and Andre 2001). Dal81p acts synergistically with Stp1p to regulate the transcription of *AGP1* in response to the amino acid induction, and the sequence 5′-CGGC-3′ of a UAS element is important for *AGP1* transcriptional regulation (Abdel-Sater *et al.* 2004). Since the *PTR2* promoter region has this consensus sequence and because Dal81p was identified as important for *PTR2* transcription and dipeptide utilization in our screening, we speculate that Dal81p and Stp2p act synergistically in *PTR2* transcription in a similar manner.

Third, other gene products identified in this screen such as Ubr2p, Ptr1p, and Ubc2p regulate *PTR2* expression by modulating the stability of Rpn4p and Cup9p (Figure 6). Cup9p stability is regulated by Ptr1p, Ubc2p, and Ubc4p (Du *et al.* 2002). Ubr2p is associated with Rpn4p to regulate proteasome gene expression through the ubiquitination pathway (Wang *et al.* 2004). In addition, Ubr2p has high amino acid sequence similarity to Ptr1p, presumably allowing a competition with Ptr1p for the stabilization of Cup9p (Bartel *et al.* 1990). Therefore, Ubr2p probably regulates *PTR2* transcription by impacting the stability of both Rpn4p and Cup9p (Figure 6).

Fourth, we identified one other gene product (*SSN3*) involved in *PTR2* transcription that is known to be general transcription factor, which also affected *PMA1* expression. Ssn3p/Srb10p, Ssn8p/Srb11p, Ssn2p/Srb9p, and Srb8p have been identified as a cyclin-dependent serine/threonine protein kinase complex that functions as a mediator of RNA polymerase (Kornberg 2005). This complex is also involved in the glucose signaling pathway (Balciunas and Ronne 1995; Kuchin *et al.* 1995). Deletion of these genes leads to derepression of a wide variety of downstream genes, including *GAL* and *SUC*, which are important for galactose and raffinose metabolism, respectively (Myer and Young 1998; Kuchin *et al.* 2000). The deletion of *SSN3* resulted in a change of *PMA1* and *PTR2* expression level.

Finally, we identified gene products involved in *PTR2* transcription by means that are not clear. For example, *UBP14* was important for *PTR2* expression since deletion of these genes significantly decreased *PTR2* mRNA level (Table 4), and the strain carrying deletion of this gene exhibited decreased peptide utilization. Ubp14p has ubiquitin-specific protease activity (supplemental Table S4 at http://www.genetics.org/supplemental/). It is probably modulating *PTR2* transcription via the ubiquitination pathway. In addition, the deletion of *UBP14* had no effect on the expression of *PMA1* or on sensitivity to NaCl and canavanine. Further experiments are needed to provide some insight into whether the gene product acts directly or indirectly to control *PTR2* expression.

Genes involved in *PTR2* mRNA maturation: Gene products (Kem1p, Pat1p, and Thp1p) involved in post-transcriptional regulation impacting the stability and transport of *PTR2* mRNA also affected dipeptide utilization (Figure 6). Both Kem1p and Pat1p belong to an mRNA decay protein complex (Bonnerot *et al.* 2000; Bouveret *et al.* 2000). *PTR2* mRNA was highly upregulated in the kem1 strain (Table 4). It is likely that *PTR2* mRNA was more stable in *kem1* and *pat1* strains, which resulted in more Ptr2p being synthesized, leading to enhanced Ptr2p-GFP expression (Figure 3) and increased dipeptide utilization (Table 1 and Figures 1 and 2). The deletion of *KEM1* resulted in also an increased expression level of *PMA1* and changes in sensitivity to NaCl and canavanine (Table 1). It is not surprising that the deletion of this gene affected several phenotypes related to membrane function, since a deletion in this gene would impact gene expression of many different genes. Another gene shown to impact dipeptide utilization was Thp1p, which is involved in mRNA export from the nucleus to the cytoplasm (Fischer *et al.* 2002). The *thp1* strain probably has a defect in transporting *PTR2* mRNA into the cytoplasm for protein

synthesis. Therefore this mutant demonstrated a decrease in peptide utilization.

Genes that regulate Ptr2p trafficking: This screen revealed that dipeptide utilization is regulated by the trafficking system. Deletion of components of ESCRT-I, ESCRT-II, and ESCRT-III protein complexes showed a defect in Ptr2p-GFP trafficking from the endosome to the vacuole (Figure 6 and supplemental Figure S2 at http://www.genetics.org/supplemental/). These protein complexes are essential for sorting ubiquitinylated membrane proteins from the plasma membrane to the multivesicular body and to the vacuole for further degradation (Raiborg *et al.* 2003). Ptr2p has been shown to be ubiquitinylated (Hitchcock *et al.* 2003). Strains *srn2*, *vps36*, and *snf7* demonstrated increased dipeptide utilization perhaps due to an increased residence time of Ptr2p at the cytoplasmic membrane. In addition, the *eaf7* and *vps8* strains showed Ptr2p localization similar to that of the ESCRT protein mutants. Surprisingly, Eaf7p is annotated as a component of the NuA4 HAT complex, which is an essential histone acetyltransferase complex that acetylates the N-terminal tails of histones H4 and H2A (Krogan *et al.* 2004). It is possible that Eaf7p has dual functions or indirectly affects Ptr2p localization through other proteins. Both *srn2* and *vps8* strains showed an altered sensitivity to NaCl and canavanine. It has been reported that the osmotic sensitivity changes if vacuolar development is affected (Hampsey 1997). Thus a disruption of the protein-sorting process that affects the vacuole could lead to alteration of osmotic sensitivity.

Deletion mutants (*npr1*, *lst4*) with increased dipeptide utilization and mutants (*bud32*, *reg1*) with decreased utilization showed an accumulation of Ptr2p-GFP in vesicles. *NPR1* encodes a Ser/Thr protein kinase, involved in post-translational control of Gap1p. Npr1p is required for Gap1p to be targeted to the plasma membrane, and an *npr1* deletion mutant loses Gap1p function by sorting Gap1p from the Golgi into the vacuole, bypassing the plasma membrane (De Craene *et al.* 2001). Similarly, mutations in *LST4* reduced the function of Galp1p and other amino acid permeases by the interruption of sorting of these transporters to the cell surface (Roberg *et al.* 1997). Our observations indicated that *NPR1* and *LST4* regulate Ptr2p differently from the way they regulate Gap1p. It is not clear how Lst4p and Npr1p regulate Ptr2p in a manner opposite to that of their regulation of Gap1p. Bud32p is a Ser/Thr protein kinase involved in polar bud site selection and changing the vacuolar morphology (Bonangelino *et al.* 2002). Reg1p is a regulatory subunit for protein phosphatase Glc7p involved in the repression of many glucose-regulated genes and vesicular trafficking (Cui *et al.* 2004). The involvement of Bud32p and Reg1p in dipeptide utilization reflects their roles in Ptr2p trafficking.

Genes that regulate dipeptide utilization independent of Ptr2p: The alteration of dipeptide utilization also results from Ptr2p-independent metabolic processes as indicated by the observation of no apparent phenotypic change in expression or localization of Ptr2p-GFP in a number of mutants (Table 3). Mutation in genes encoding a variety of cellular metabolic processes (supplemental Figure S6 at http://www.genetics.org/supplemental/) (Nagarajan and Storms 1997; Schneider *et al.* 1997; Stoops *et al.* 1997), such as the conversion of pyruvate into acetyl-CoA by pyruvate dehydrogenase complex (Pdx1p, Lpd1, Pdb1p, and Lat1p), the conversion of acetyl-CoA to malonyl-CoA by Hfa1p, fatty acid synthesis by fatty acid synthase complex (Oar1p, Mct1p, and Etr1p), glycine degradation (Gcv2p, Gcv3p, and Lpd1p), and a peptidase activity (Prd1p), resulted in a decrease of dipeptide utilization (Table 3). In these mutants the impairment of the dipeptide utilization appeared to be independent of Ptr2p transport activity since Ptr2p-GFP could be observed at the plasma membrane (supplemental Figure S3 at http://www.genetics.org/supplemental/). The assay of [^{3}H]Leu–Leu uptake in *gcv3* strains suggested that the import of dipeptide was still maintained at a wild-type level, which showed that Ptr2p was functional (Figure 5), indicating that the decrease of dipeptide utilization resulted from the inability of the mutant to release amino acid from accumulated dipeptide or from a metabolic block in using the released amino acid for protein biosynthesis. Prd1p is a metalloendoproteinase (Buchler *et al.* 1994). The deletion mutant might be impaired in the degradation of intracellularly transported dipeptide, which would result in a decrease of dipeptide utilization.

Deletion strains *cik1*, *kti12*, *mlh1*, *mrp17*, *pho2*, *rim101*, *tif3*, *ybt1*, and *ydr417c* showed no apparent effect on Ptr2p-GFP localization or expression but demonstrated increased dipeptide utilization. The utilization of dipeptide in these strains appears to involve Ptr2p-independent cellular processes as well. For example, Ybt1p belongs to the ABC transporter family, exhibiting ATP-dependent bile acid transport (Ortiz *et al.* 1997; Paulsen *et al.* 1998). As expected for an ABC transporter, the *ybt1* mutant was not only more sensitive to the toxic dipeptide Ala–Eth, but also this strain was more sensitive to ethionine and Lys–Ala–Eth (data not shown). We speculate that this protein might be involved in the transport of dipeptide into the vacuole. In the deletion mutant the defect in the uptake of dipeptide into the vacuole would lead to an increase of dipeptide availability in the cytoplasm. It is also possible that the deletion of a gene might result in a derepression or change in function of other transport systems that may mediate dipeptide utilization in the mutant without alteration of *PTR2* expression or Ptr2p function. We are currently working on unraveling the involvement of these metabolic genes in dipeptide utilization.

Unknown genes that regulate dipeptide utilization: Fifteen unknown gene deletion mutant strains were

identified in this screen, including 7 strains exhibiting increased and 8 strains showing decreased dipeptide utilization (Table 3). Deletion mutants *yor322c*, *ynl123w*, *yfr044c*, and *ylr114c* showed enhanced expression level of Ptr2p-GFP signal compared to that of wild type, and *ypr174c* had reduced expression of Ptr2p-GFP; these results were consistent with dipeptide utilization in these strains, suggesting that these unknown genes products are involved in the regulation of *PTR2* expression. Deletion mutants *ynl295w*, *ypl073c*, *yjl175w*, *ydr157w*, and *ydr433w* showed accumulation of Ptr2p-GFP signal in the vesicles or the vacuole, suggesting that these unknown gene products are involved in Ptr2p trafficking and localization. Similar to the expression level of Ptr2p-GFP in the *asm4* strain, enhanced Ptr2p-GFP expression was observed in the deletion mutant strains of unknown function, *ydr015c* and *ydr290w*. These two mutants, however, showed a decreased sensitivity to toxic dipeptide and no growth change on dipeptide; it is not clear how these genes regulate dipeptide utilization.

Overall, this investigation has provided a list of genes involved directly and indirectly in the utilization of dipeptides in yeast. In our screen, 53 genes encoding proteins with >30% identity to human genes have been identified (see supplemental Tables S3 and S4, footnote *a*, at http://www.genetics.org/supplemental/). To date no genes have been reported in the regulation of the human *PTR2* homologs PEPT1 and PEPT2 (Daniel 2004). Further investigation of these human genes may promote understanding of the regulation of dipeptide utilization in mammalian systems. This study represents the first such global analysis of a membrane transport process and as such provides a rich starting ground for future investigations.

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LITERATURE CITED

Abdel-Sater, F., I. Iraqui, A. Urrestarazu and B. Andre, 2004 The external amino acid signaling pathway promotes activation of Stp1 and Uga35/Dal81 transcription factors for induction of the AGP1 gene in *Saccharomyces cerevisiae*. Genetics **166:** 1727–1739.

Andreasson, C., and P. O. Ljungdahl, 2002 Receptor-mediated endoproteolytic activation of two transcription factors in yeast. Genes Dev. **16:** 3158–3172.

Andreasson, C., and P. O. Ljungdahl, 2004 The N-terminal regulatory domain of Stp1p is modular and, fused to an artificial transcription factor, confers full Ssy1p-Ptr3p-Ssy5p sensor control. Mol. Cell. Biol. **24:** 7503–7513.

Aouida, M., N. Page, A. Leduc, M. Peter and D. Ramotar, 2004 A genome-wide screen in Saccharomyces cerevisiae reveals altered transport as a mechanism of resistance to the anticancer drug bleomycin. Cancer Res. **64:** 1102–1109.

Askree, S. H., T. Yehuda, S. Smolikov, R. Gurevich, J. Hawk *et al.*, 2004 A genome-wide screen for Saccharomyces cerevisiae deletion mutants that affect telomere length. Proc. Natl. Acad. Sci. USA **101:** 8658–8663.

Baetz, K., L. McHardy, K. Gable, T. Tarling, D. Reberioux *et al.*, 2004 Yeast genome-wide drug-induced haploinsufficiency screen to determine drug mode of action. Proc. Natl. Acad. Sci. USA **101:** 4525–4530.

Balciunas, D., and H. Ronne, 1995 Three subunits of the RNA polymerase II mediator complex are involved in glucose repression. Nucleic Acids Res. **23:** 4421–4425.

Barnes, D., W. Lai, M. Breslav, F. Naider and J. M. Becker, 1998 PTR3, a novel gene mediating amino acid-inducible regulation of peptide transport in Saccharomyces cerevisiae. Mol. Microbiol. **29:** 297–310.

Bartel, B., I. Wunning and A. Varshavsky, 1990 The recognition component of the N-end rule pathway. EMBO J. **9:** 3179–3189.

Bernard, F., and B. Andre, 2001 Ubiquitin and the SCF(Grr1) ubiquitin ligase complex are involved in the signalling pathway activated by external amino acids in Saccharomyces cerevisiae. FEBS Lett. **496:** 81–85.

Bonangelino, C. J., E. M. Chavez and J. S. Bonifacino, 2002 Genomic screen for vacuolar protein sorting genes in Saccharomyces cerevisiae. Mol. Biol. Cell **13:** 2486–2501.

Bonnerot, C., R. Boeck and B. Lapeyre, 2000 The two proteins Pat1p (Mrt1p) and Spb8p interact in vivo, are required for mRNA decay, and are functionally linked to Pab1p. Mol. Cell. Biol. **20:** 5939–5946.

Bouveret, E., G. Rigaut, A. Shevchenko, M. Wilm and B. Seraphin, 2000 A Sm-like protein complex that participates in mRNA degradation. EMBO J. **19:** 1661–1671.

Bowers, K., J. Lottridge, S. B. Helliwell, L. M. Goldthwaite, J. P. Luzio *et al.*, 2004 Protein-protein interactions of ESCRT complexes in the yeast Saccharomyces cerevisiae. Traffic **5:** 194–210.

Buchler, M., U. Tisljar and D. H. Wolf, 1994 Proteinase yscD (oligopeptidase yscD). Structure, function and relationship of the yeast enzyme with mammalian thimet oligopeptidase (metalloendopeptidase, EP 24.15). Eur. J. Biochem. **219:** 627–639.

Byrd, C., G. C. Turner and A. Varshavsky, 1998 The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. EMBO J. **17:** 269–277.

Chen, Y., Y. Liu, K. M. Goldstein, J. M. Becker, Y. Xu *et al.*, 2003 A computational study on the signal transduction pathway for amino acid and peptide transport in yeast: bridging the gap between high-throughput data and traditional biology. Appl. Genomics Proteomics **2:** 43–50.

Christie, K. R., S. Weng, R. Balakrishnan, M. C. Costanzo, K. Dolinski *et al.*, 2004 Saccharomyces Genome Database (SGD) provides tools to identify and analyze sequences from Saccharomyces cerevisiae and related sequences from other organisms. Nucleic Acids Res. **32:** D311–314.

Cui, D. Y., C. R. Brown and H. L. Chiang, 2004 The type 1 phosphatase Reg1p-Glc7p is required for the glucose-induced degradation of fructose-1,6-bisphosphatase in the vacuole. J. Biol. Chem. **279:** 9713–9724.

Daniel, H., 2004 Molecular and integrative physiology of intestinal peptide transport. Annu. Rev. Physiol. **66:** 361–384.

de Boer, M., P. S. Nielsen, J. P. Bebelman, H. Heerikhuizen, H. A. Andersen *et al.*, 2000 Stp1p, Stp2p and Abf1p are involved in regulation of expression of the amino acid transporter gene BAP3 of Saccharomyces cerevisiae. Nucleic Acids Res. **28:** 974–981.

De Craene, J. O., O. Soetens and B. Andre, 2001 The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease. J. Biol. Chem. **276:** 43939–43948.

Du, F., F. Navarro-Garcia, Z. Xia, T. Tasaki and A. Varshavsky, 2002 Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. Proc. Natl. Acad. Sci. USA **99:** 14110–14115.

Fei, Y. J., Y. Kanai, S. Nussberger, V. Ganapathy, F. H. Leibach *et al.*, 1994 Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature **368:** 563–566.

Fischer, T., K. Strasser, A. Racz, S. Rodriguez-Navarro, M. Oppizzi *et al.*, 2002 The mRNA export machinery requires the novel Sac3p-Thp1p complex to dock at the nucleoplasmic entrance of the nuclear pores. EMBO J. **21:** 5843–5852.

Forsberg, H., and P. O. Ljungdahl, 2001a Genetic and biochemical analysis of the yeast plasma membrane Ssy1p-Ptr3p-Ssy5p sensor of extracellular amino acids. Mol. Cell. Biol. **21:** 814–826.

Forsberg, H., and P. O. Ljungdahl, 2001b Sensors of extracellular nutrients in Saccharomyces cerevisiae. Curr. Genet. **40:** 91–109.

Forsberg, H., C. F. Gilstring, A. Zargari, P. Martinez and P. O. Ljungdahl, 2001a The role of the yeast plasma membrane SPS nutrient sensor in the metabolic response to extracellular amino acids. Mol. Microbiol. **42:** 215–228.

Forsberg, H., M. Hammar, C. Andreasson, A. Moliner and P. O. Ljungdahl, 2001b Suppressors of *ssy1* and *ptr3* null mutations define novel amino acid sensor-independent genes in *Saccharomyces cerevisiae*. Genetics **158:** 973–988.

Gonzales, T., and J. Robert-Baudouy, 1996 Bacterial aminopeptidases: properties and functions. FEMS Microbiol. Rev. **18:** 319–344.

Gustin, M. C., J. Albertyn, M. Alexander and K. Davenport, 1998 MAP kinase pathways in the yeast Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. **62:** 1264–1300.

Hampsey, M., 1997 A review of phenotypes in Saccharomyces cerevisiae. Yeast **13:** 1099–1133.

Hauser, M., V. Narita, A. M. Donhardt, F. Naider and J. M. Becker, 2001 Multiplicity and regulation of genes encoding peptide transporters in Saccharomyces cerevisiae. Mol. Membr. Biol. **18:** 105–112.

Herrera-Ruiz, D., and G. T. Knipp, 2003 Current perspectives on established and putative mammalian oligopeptide transporters. J. Pharm. Sci. **92:** 691–714.

Hitchcock, A. L., K. Auld, S. P. Gygi and P. A. Silver, 2003 A subset of membrane-associated proteins is ubiquitinated in response to mutations in the endoplasmic reticulum degradation machinery. Proc. Natl. Acad. Sci. USA **100:** 12735–12740.

Hodges, P. E., A. H. McKee, B. P. Davis, W. E. Payne and J. I. Garrels, 1999 The Yeast Proteome Database (YPD): a model for the organization and presentation of genome-wide functional data. Nucleic Acids Res. **27:** 69–73.

Hoffman, G. A., T. R. Garrison and H. G. Dohlman, 2002 Analysis of RGS proteins in Saccharomyces cerevisiae. Methods Enzymol. **344:** 617–631.

Hohmann, S., 2002 Osmotic stress signaling and osmoadaptation in yeasts. Microbiol. Mol. Biol. Rev. **66:** 300–372.

Iraqui, I., S. Vissers, F. Bernard, J. O. de Craene, E. Boles *et al.*, 1999 Amino acid signaling in Saccharomyces cerevisiae: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. Mol. Cell. Biol. **19:** 989–1001.

Island, M. D., F. Naider and J. M. Becker, 1987 Regulation of dipeptide transport in Saccharomyces cerevisiae by micromolar amino acid concentrations. J. Bacteriol. **169:** 2132–2136.

Island, M. D., J. R. Perry, F. Naider and J. M. Becker, 1991 Isolation and characterization of S. cerevisiae mutants deficient in amino acid-inducible peptide transport. Curr. Genet. **20:** 457–463.

Kornberg, R. D., 2005 Mediator and the mechanism of transcriptional activation. Trends Biochem. Sci. **30:** 235–239.

Krogan, N. J., K. Baetz, M. C. Keogh, N. Datta, C. Sawa *et al.*, 2004 Regulation of chromosome stability by the histone H2A variant Htz1, the Swr1 chromatin remodeling complex, and the histone acetyltransferase NuA4. Proc. Natl. Acad. Sci. USA **101:** 13513–13518.

Kuchin, S., P. Yeghiayan and M. Carlson, 1995 Cyclin-dependent protein kinase and cyclin homologs SSN3 and SSN8 contribute to transcriptional control in yeast. Proc. Natl. Acad. Sci. USA **92:** 4006–4010.

Kuchin, S., I. Treich and M. Carlson, 2000 A regulatory shortcut between the Snf1 protein kinase and RNA polymerase II holoenzyme. Proc. Natl. Acad. Sci. USA **97:** 7916–7920.

Lee, T. I., N. J. Rinaldi, F. Robert, D. T. Odom, Z. Bar-Joseph *et al.*, 2002 Transcriptional regulatory networks in Saccharomyces cerevisiae. Science **298:** 799–804.

Lee, V. H., 2000 Membrane transporters. Eur. J. Pharm. Sci. **11**(Suppl 2): S41–S50.

Liu, W., R. Liang, S. Ramamoorthy, Y. J. Fei, M. E. Ganapathy *et al.*, 1995 Molecular cloning of PEPT 2, a new member of the H+/peptide cotransporter family, from human kidney. Biochim. Biophys. Acta **1235:** 461–466.

Lubkowitz, M. A., L. Hauser, M. Breslav, F. Naider and J. M. Becker, 1997 An oligopeptide transport gene from Candida albicans. Microbiology **143**(2): 387–396.

Mannhaupt, G., R. Schnall, V. Karpov, I. Vetter and H. Feldmann, 1999 Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. FEBS Lett. **450:** 27–34.

Marelli, M., J. D. Aitchison and R. W. Wozniak, 1998 Specific binding of the karyopherin Kap121p to a subunit of the nuclear pore complex containing Nup53p, Nup59p, and Nup170p. J. Cell Biol. **143:** 1813–1830.

Marston, A. L., W. H. Tham, H. Shah and A. Amon, 2004 A genome-wide screen identifies genes required for centromeric cohesion. Science **303:** 1367–1370.

Marzluf, G. A., 1997 Genetic regulation of nitrogen metabolism in the fungi. Microbiol. Mol. Biol. Rev. **61:** 17–32.

Miyake, T., M. Kanayama, H. Sammoto and B. Ono, 2002 A novel cis-acting cysteine-responsive regulatory element of the gene for the high-affinity glutathione transporter of Saccharomyces cerevisiae. Mol. Genet. Genomics **266:** 1004–1011.

Myer, V. E., and R. A. Young, 1998 RNA polymerase II holoenzymes and subcomplexes. J. Biol. Chem. **273:** 27757–27760.

Nagarajan, L., and R. K. Storms, 1997 Molecular characterization of GCV3, the Saccharomyces cerevisiae gene coding for the glycine cleavage system hydrogen carrier protein. J. Biol. Chem. **272:** 4444–4450.

Nielsen, P. S., B. van den Hazel, T. Didion, M. de Boer, M. Jorgensen *et al.*, 2001 Transcriptional regulation of the Saccharomyces cerevisiae amino acid permease gene BAP2. Mol. Gen. Genet. **264:** 613–622.

Ortiz, D. F., M. V. St. Pierre, A. Abdulmessih and I. M. Arias, 1997 A yeast ATP-binding cassette-type protein mediating ATP-dependent bile acid transport. J. Biol. Chem. **272:** 15358–15365.

Page, N., M. Gerard-Vincent, P. Menard, M. Beaulieu, M. Azuma *et al.*, 2003 A *Saccharomyces cerevisiae* genomewide mutant screen for altered sensitivity to K1 killer toxin. Genetics **163:** 875–894.

Paulsen, I. T., M. K. Sliwinski, B. Nelissen, A. Goffeau and M. H. Saier, Jr., 1998 Unified inventory of established and putative transporters encoded within the complete genome of Saccharomyces cerevisiae. FEBS Lett. **430:** 116–125.

Perry, J. R., M. A. Basrai, H. Y. Steiner, F. Naider and J. M. Becker, 1994 Isolation and characterization of a Saccharomyces cerevisiae peptide transport gene. Mol. Cell. Biol. **14:** 104–115.

Poulsen, P., B. Wu, R. F. Gaber, K. Ottow, H. A. Andersen *et al.*, 2005 Amino acid sensing by Ssy1. Biochem. Soc. Trans. **33:** 261–264.

Pritchard, G. G., and T. Coolbear, 1993 The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. **12:** 179–206.

Raiborg, C., T. E. Rusten and H. Stenmark, 2003 Protein sorting into multivesicular endosomes. Curr. Opin. Cell Biol. **15:** 446–455.

Reid, J. L., Z. Moqtaderi and K. Struhl, 2004 Eaf3 regulates the global pattern of histone acetylation in Saccharomyces cerevisiae. Mol. Cell. Biol. **24:** 757–764.

Roberg, K. J., S. Bickel, N. Rowley and C. A. Kaiser, 1997 Control of amino acid permease sorting in the late secretory pathway of *Saccharomyces cerevisiae* by SEC13, LST4, LST7 and LST8. Genetics **147:** 1569–1584.

Sanders, S. L., J. Jennings, A. Canutescu, A. J. Link and P. A. Weil, 2002 Proteomics of the eukaryotic transcription machinery: identification of proteins associated with components of yeast TFIID by multidimensional mass spectrometry. Mol. Cell. Biol. **22:** 4723–4738.

Scherens, B., and A. Goffeau, 2004 The uses of genome-wide yeast mutant collections. Genome Biol. **5:** 229.

Schneider, R., B. Brors, F. Burger, S. Camrath and H. Weiss, 1997 Two genes of the putative mitochondrial fatty acid synthase in the genome of Saccharomyces cerevisiae. Curr. Genet. **32:** 384–388.

Shen, X., R. Ranallo, E. Choi and C. Wu, 2003 Involvement of actin-related proteins in ATP-dependent chromatin remodeling. Mol. Cell **12:** 147–155.

Stade, K., C. S. Ford, C. Guthrie and K. Weis, 1997 Exportin 1 (Crm1p) is an essential nuclear export factor. Cell **90:** 1041–1050.

Steiner, H. Y., F. Naider and J. M. Becker, 1995 The PTR family: a new group of peptide transporters. Mol. Microbiol. **16:** 825–834.

Steinmetz, L. M., C. Scharfe, A. M. Deutschbauer, D. Mokranjac, Z. S. Herman *et al.*, 2002 Systematic screen for human disease genes in yeast. Nat. Genet. **31:** 400–404.

Stoops, J. K., R. H. Cheng, M. A. Yazdi, C. Y. Maeng, J. P. Schroeter *et al.*, 1997 On the unique structural organization of the Saccharomyces cerevisiae pyruvate dehydrogenase complex. J. Biol. Chem. **272:** 5757–5764.

Turner, G. C., F. Du and A. Varshavsky, 2000 Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. Nature **405:** 579–583.

Wang, L., X. Mao, D. Ju and Y. Xie, 2004 Rpn4 is a physiological substrate of the Ubr2 ubiquitin ligase. J. Biol. Chem. **279:** 55218–55223.

Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson *et al.*, 1999 Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science **285:** 901–906.

Xie, Y., and A. Varshavsky, 1999 The E2–E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. EMBO J. **18:** 6832–6844.

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