Role of PUG1 in Inducible Porphyrin and Heme Transport in *Saccharomyces cerevisiae*\(^7\)

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Unlike pathogenic fungi, the budding yeast *Saccharomyces cerevisiae* is not efficient at using heme as a nutritional source of iron. Here we report that for this yeast, heme uptake is induced under conditions of heme starvation. Heme synthesis requires oxygen, and yeast grown anaerobically exhibited an increased uptake of hemin. Similarly, a strain lacking amionolevulinate synthase exhibited a sixfold increase in heme uptake when grown without 2-aminolevulinic acid. We used microarray analysis of cells grown under reduced oxygen tension or reduced intracellular heme conditions to identify candidate genes involved in heme uptake. Surprisingly, overexpression of *PUG1* (protoporphyrin uptake gene 1) resulted in reduced utilization of exogenous heme by a heme-deficient strain and, conversely, increased the utilization of protoporphyrin IX. *Pug1p* was localized to the plasma membrane by indirect immunofluorescence and subcellular fractionation. Strains overexpressing *PUG1* exhibited decreased accumulation of \[^{55}\text{Fe}\text{]hemin}\] but increased accumulation of protoporphyrin IX compared to the wild-type strain. To measure the effect of *PUG1* overexpression on intracellular heme pools, we used a *CYC1-lacZ* reporter, which is activated in the presence of heme, and we monitored the activity of a heme-containing metalloreductase, Fre1p, expressed from a constitutive promoter. The data from these experiments were consistent with a role for *Pug1p* in inducible protoporphyrin IX influx and heme efflux.  

Exogenous heme is an important nutritional source of iron in many organisms, from prokaryotes to higher eukaryotes. In humans, intestinal absorption of heme iron is significantly higher than that of nonheme iron (23). Heme is an essential nutrient in free-living worms such as *Caenorhabditis elegans* and parasitic helminths, because these organisms do not synthesize heme de novo and rely exclusively on exogenous sources of heme (31).

There is virtually no free iron available inside a human host, and iron-withholding systems constitute an important aspect of the innate immune system (7). Pathogenic bacteria and fungi actively utilize heme iron during infection, because heme is the most abundant source of iron in the human host. For *Staphylococcus aureus*, heme is used in preference to transferrin as a nutritional iron source (40). Pathogenic bacteria have developed two main strategies to compete with the host for heme iron. In gram-negative organisms, transport of heme occurs across the outer membrane through specific receptors that depend on the TonB/ExbB/ExbD complex (50). Also, some species of gram-negative bacteria secrete hemophores, small proteins that bind heme and heme proteins to facilitate the uptake of heme (46). Gram-positive bacteria, such as *S. aureus*, express two proteins associated with the cell surface, IsdA and IsdC, that bind and transport heme, respectively, across the cell wall before it is taken up at the plasma membrane by a transporter of the ATP-binding cassette (ABC) family (21).

Although significant progress has been made in studying heme uptake in bacteria, less is known about this process in eukaryotes. The dimorphic fungus *Candida albicans* is part of the commensal flora of humans and is also an opportunistic pathogen associated with mucocutaneous infections. Systemic infections with *C. albicans* can be lethal in immunocompromised patients. *Candida* spp. excrete a hemolytic factor to assist in the release of heme and heme proteins from erythrocytes (20). Heme uptake in this species is induced under iron deficiency and requires the heme oxygenase activity of *C. albicans* Hmx1p to release iron from heme (26, 35). A heme-binding protein, Rtb5p, is expressed on the cell surface and is involved in heme iron utilization (47), but no high-affinity heme transporter has been identified.

In eukaryotes, heme synthesis starts and ends in the mitochondria, while intermediate steps occur in the cytoplasm. The mechanisms by which heme and porphyrins traffic across cellular membranes are largely unknown. Heme and porphyrins are reactive and potentially toxic compounds, and their cellular levels are tightly regulated. Recently, the human ABCB6 transporter was shown to be involved in the uptake of porphyrins into mitochondria (17), while other authors have found that the transporter also functions at the plasma membrane to block porphyrin accumulation (25). FLVCR1, first identified as the receptor for feline leukemia virus, is a transporter that has heme export activity. It is required for erythroid cell differentiation and may protect cells from heme toxicity (30). Other studies have reported that the human multidrug transporter ABCG2 exports heme and protoporphyrin IX (PPIX) (44). Other transporters may facilitate the uptake of heme into cells. Human HCP1 is an intestinal high-affinity folate transporter, which was initially identified as a heme carrier (29, 37).

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The FLC family of endoplasmic reticulum proteins from yeasts and fungi is essential for the transport of FAD into this compartment. Overexpression of C. albicans Flc1p and Saccharomyces cerevisiae Flc1p and -2p promotes heme uptake in yeasts (28).

Although C. albicans can efficiently use heme as a nutritional source of iron, S. cerevisiae does not efficiently use exogenous heme to meet the cell’s requirement for iron and does not take up heme in response to iron deficiency (28, 48). Here we report that, in S. cerevisiae, uptake systems for heme and porphyrin are induced under heme starvation and hypoxia. We used microarrays to identify transcripts that are selectively induced under heme starvation and hypoxia. We used a canonical heme-starvation protocol, where cultures were grown in heme-deficient medium for 8–16 h, harvested by centrifugation, and washed twice with water. The cultures were resuspended in fresh glucoseminimal medium and harvested again after 16 h.

**MATERIALS AND METHODS**

**Strains and media.** S. cerevisiae strains were constructed in YPH499, BY4742, and BY4741 (Table 1). PCR-mediated gene disruption was used to generate gene deletions, and all strains were tested for correct genome integration by PCR. For deletion of PUG1, strain BY4741 was transformed with a PCR product amplified from genomic DNA isolated from the YER185W haploid deletion mutant (MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100). This study

**TABLE 1. List of strains used in this study**

| Strain name | Short genotype | Genetic background | Relevant genotype | Source |
|-------------|----------------|--------------------|-------------------|--------|
| DY1457      | hem1Δ(6D)      | W303               | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY110      | hem1Δ fet3Δ   | DY1457             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY111      | fre2Δ         | DY1457             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| YPH499      |               | YPH499             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| CPY119      | fet3Δ         | YPH499             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY101      | hem1Δ         | YPH499             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY102      | hem1Δ fre2Δ   | YPH499             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY103      | PUG1-myc      | YPH499             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| BY4742      |               | BY4742             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY104      | hem1Δ         | BY4742             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY105      | hem1Δ         | BY4742             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY106      | hem1Δ         | BY4742             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY107      | hem1Δ pug1Δ   | BY4742             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY108      | hem1Δ hem15Δ  | BY4742             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |
| OPY109      | hem1Δ hem15Δ  | BY4742             | MATa ura3-52 leu2-3,112 trp1-1 his3-11 ade2 can1-100 | 4      |

**TABLE 2. List of primers used in this study**

| Primer name | Sequence (5’ to 3’) |
|-------------|---------------------|
| YER185W-250-F | CTCCATACAGGATGAAGTGTTC |
| YER185W-230-R | CACCCGTCCGTCACTCTCA |
| HEM1-D-F | GTAACAGTTTTAAGATCGT |
| HEM1-D-R | CTCTAAATGGCACTATT |
| HEM15-DEL-F | GTTAAATCTATAACTATCATTA |
| HEM15-DEL-R | GGATGAATGCCTTTATCAGTA |
| YER185W-3HA-F2 | TCTTCCGATCCGATCTCCGAT |
| YER185W-3HA-R1 | GAGGAGTGTGTTCAAGTGTTATTA |
| YER185W-3HA-R | GGATTGCCGTTTGAATTA |
| YER185W-F | GGTTAATCTTAAATCACAACAAACAA |
| YER185W-R | ATCCGATATCGATCAGGTCAG |
| YER185W-MYC-F | ATCCGATATCGATCAGGTCAG |
| YER185W-MYC-R | ATCCGATATCGATCAGGTCAG |
| YER213C-F | GTCTTCTGAGAT |
| YER213C-R | TCTTCTGAGAT |
| FRE1-F | ATCCGATATCGATCAGGTCAG |
| FRE1-R | ATCCGATATCGATCAGGTCAG |
| PUG1-230-F | CACACAAAGCTTATGCGTT |
| PUG1-250-R | ATCCGATATCGATCAGGTCAG |
| RTA1-474-F2 | TCTTCTGAGAT |
| RTA1-540-R | TCTTCTGAGAT |
| UPC2-F2 | ATCCGATATCGATCAGGTCAG |
| B7 18S-1114F | GTATGGTCGCAAGGCTGAAAC |
| UPC2-R | GTATGGTCGCAAGGCTGAAAC |
| YER185W-210-F | GTATGGTCGCAAGGCTGAAAC |
| YER185W-230-R | GTATGGTCGCAAGGCTGAAAC |
| YER185W-250-F | GTATGGTCGCAAGGCTGAAAC |
| YER185W-3HA-F2 | GTATGGTCGCAAGGCTGAAAC |
| YER185W-3HA-R1 | GTATGGTCGCAAGGCTGAAAC |
| YER185W-F | GTATGGTCGCAAGGCTGAAAC |
| YER185W-R | GTATGGTCGCAAGGCTGAAAC |
| YER185W-MYC-F | GTATGGTCGCAAGGCTGAAAC |
| YER185W-MYC-R | GTATGGTCGCAAGGCTGAAAC |
| YER213C-F | GTCTTCTGAGAT |
| YER213C-R | TCTTCTGAGAT |
| FRE1-F | GTATGGTCGCAAGGCTGAAAC |
| FRE1-R | GTATGGTCGCAAGGCTGAAAC |
| PUG1-230-F | GTATGGTCGCAAGGCTGAAAC |
| PUG1-250-R | GTATGGTCGCAAGGCTGAAAC |
| RTA1-474-F2 | GTATGGTCGCAAGGCTGAAAC |
| RTA1-540-R | GTATGGTCGCAAGGCTGAAAC |
| UPC2-F2 | GTATGGTCGCAAGGCTGAAAC |
| B7 18S-1114F | GTATGGTCGCAAGGCTGAAAC |
| B8 18S-1294R | GTATGGTCGCAAGGCTGAAAC |
deletion mutant (MATaMATα ura3 met-15 his3-1 leu2-1 his3 ΔCANMXHEM1) (Open Biosystems) using the same primers. Genomic-resistant-clones were selected on YPD plates supplemented with G418 and 250 μg ALA. The fre1Δ fre2Δ strain was constructed as described elsewhere (8). To construct OPY102 (hem1Δ fre1Δ fre2Δ), HEM1 was deleted using the hem1Δ CANMX cassette as described above. To construct OPY112 (hem1Δ fre1Δ fre2Δ P62G1-FRE1), plasmid YlpDCE51-P62G1-FRE1 was linearized with Stul and integrated into the ADE2 locus of OPY102. Transformants were selected on synthetic complete (SC) medium lacking adenine and supplemented with 250 μg ALA. To delete HEM5, the hem1Δ S. cerevisiae deletion cassette was amplified from plasmid pFA6a-His3MX6 as a template and primers YER185W-F and YER185W-HA-R. Transformants were selected on medium lacking histidine and supplemented with 40 μM hemin. The PUG1-myc strain expressing 13 copies of the Myc tag on the carboxyl terminus was constructed by epitope tagging as described elsewhere (19). The tagging cassette was amplified by PCR using pFA6a-13Myc-kanMX6 as a template and primers YER185W-HA-F and YER185W-HA-R. All constructs and strains generated were verified by extensive PCR analysis and selection on the appropriate media.

Rich medium (YPD) and SC defined medium were prepared as described elsewhere (38). Genomic-resistant clones were selected on YPD plates containing 80 mg/liter G418 (Invertrogen). Defined iron media were prepared as described elsewhere (27) using yeast nitrogen base without iron and 1 mM ferrozine, an iron chelator, in addition to the indicated heme supplement.

Plasmids. To make plasmid pPUG1-Leu, the open reading frame (ORF) of PUG1 was amplified by PCR from plasmid pBG1800-YER185WS (Yeast ORF Collection; Open Biosystems) using primers YER185W-F and YER185W-R, and in vivo recombinant in yeast was used to clone the PUG1 insert into pYX22 digested with EcoRI. Subsequently, to make plasmid pPUG1-Ura, pPUG1-Leu was digested with SacI and pPUG1-Ura was digested with SacI, and the PUG1-containing segments were ligated into pYX212 digested with the same restriction enzymes. To make plasmid pPUG1-myc, genomic DNA from OPY103, containing a genomic copy of PUG1-13myc, was used as a template to amplify PUG1-myc using primers YeR185W-MYC-F and YeR185W-R. The PCR product was cut with EcoRI and HindIII and ligated into pYX212 digested with HindIII and SmaI and made to pRTA1. To make plasmid YlpDCE51-P62G1-FRE1, the FRE1 ORF was amplified from pBG1800-FRE1 (Open Biosystems) using primers FRE1-F and FRE1-R. The PCR product and vector YIPDCE1 (42) were digested with SacI and BamHI and then ligated to yield YlpDCE51-P62G1-FRE1. pCYC1-LaCZ was a kind gift from A. Hinnebusch.

Western blotting. For Western blot experiments, cells were disrupted with glass beads in a buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1% sodium dodecyl sulfate (SDS), 8 M urea, 0.01% bromophenol blue, and the following protease inhibitors: phenylmethylsulfonyl fluoride (1 mM), benzamidine (4 mM), leupeptin (2 μg/ml), and pepstatin (1 μg/ml). Cell lysates were heated at 65°C and centrifuged for 2 min at 13,000 rpm prior to SDS-polyacrylamide gel electrophoretic analysis. Subcellular fractionation was performed as described elsewhere (42), followed by the following treatments. Cells were sonicated with glass beads, and unbroken cells were removed by centrifugation at 500 × g for 2 min. Cell lysates were applied to the top of 20 to 60% continuous sucrose gradients. Samples were centrifuged at 28,500 × g for 17 h, and 0.9-ml fractions were collected. Western blotting was performed using a 1:10,000 dilution of the anti-Myc antibody 9E10 (Covance) as the primary antibody, followed by a 1:1,000 dilution of a Cy3-conjugated donkey anti-rabbit antibody. Antibodies to Dpm1p, porin, and Vps10p were purchased from Molecular Probes and used according to the manufacturer’s manual. Anti-Pnak1p antibody (Santa Cruz Biotechnology, Inc.) was used at a 1:5,000 dilution. Antibodies were detected by using enhanced chemiluminescence (Amersham Biosciences) or fluorescence imaging (Typhoon, GE).

Immunofluorescence. Strain OPY106 (hem1Δ) was transformed with pPUG1-myc, transformants were grown to mid-log phase in SC (–Ura) selective medium, and the cells were prepared for immunofluorescence microscopy as described elsewhere (51). Transport assays. [55Fe]Hemin uptake was measured as described elsewhere (36) with the following modifications. Washed cells were suspended in phosphate-buffered saline containing 5% glucose, 0.05% Tween 20, and 0.5% bovine serum albumin and then preincubated for 25 min at 30°C. [55Fe]Hemin was added at the indicated concentrations to 0.1 ml of the cell suspension with a final uptake density of 600 nm [OD600] of 4.0 and was incubated for 30 min at 30°C or in an ice-H2O bath. To stop the reaction, cells were briefly centrifuged at high speed. The cells were washed 4 times with 1.2 ml of buffer without glucose. Accumulation of [55Fe]Hemin was measured by scintillation counting. [55Fe] Hemin uptake was reported as the difference in [55Fe]hemin accumulation at 30°C and 0°C. [55Fe]Hemin was synthesized as described elsewhere (9) from PPIX (Porphyrin Products, Logan, UT) and [55FeCl3 (Perkin-Elmer Life Sciences). PPIX uptake was measured in phosphate-buffered saline containing 5% glucose, 0.05% Tween 20, and 1% bovine serum albumin, in which cells had been preincubated for 25 min at 30°C. PPIX was added at a final concentration of 25 μM to 0.08 ml of the cell suspension with a final OD600 of 4.0 and was incubated at 30°C. Cells were applied on the top of a cushion of 0.3 ml phosphate-buffered saline containing 5% glucose, 0.05% Tween 20, and 10% bovine serum albumin in elongated microcentrifuge tubes and were centrifuged 5 min at 2,500 rpm and 4°C. The bottoms of the tubes were cut, and samples were resuspended in 0.5 ml of 0.1 M Tris-HCl (pH 7.4) and 0.5% Triton X-100 (Sigma). The PPIX content was measured fluorometrically at an excitation wavelength of 387 nm and an emission wavelength of 635 nm on an ISS FC fluorescent spectrophotometer.

Assays. β-Galactosidase assays (2) and ferredoxin assays (6) were performed as described elsewhere.

Microarray and real-time PCR (RT-PCR) analysis. Strain OPY104 was grown for 6 h in YPD medium supplemented with 250 μg ALA. Cells were washed three times with water to remove excess ALA. Cells were then inoculated in duplicate into 15 ml cultures of YPD medium with or without ALA. Cultures were induced for 16 h before 5 ml of cells was harvested and washed three times in water. The density of the culture at the time of harvest, expressed as the OD600, was approximately 0.5. BY4742 was first grown for 6 h in YPD medium, then inoculated into 15 ml of YPD medium, and finally grown under normoxic or hypoxic conditions for 12 h. To create hypoxic conditions, cultures were incubated in an anaerobic chamber using a BBL-GasPak (BD Biosciences). Total RNA was extracted from the cells using Trizol reagent (Invitrogen) with subsequent DNase treatment (Ambion) and RNA purification using an RNAeasy kit (Qiagen). Microarray analyses were performed using Yeast Genome S98 arrays (Affymetrix), by the Microarray Facility of the Genomics Core Laboratory of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. Details of cDNA synthesis, hybridization, and data analysis are available at http://genomics.niddk.nih.gov/.

RNA samples submitted for microarray analysis were analyzed by quantitative RT-PCR using the relative quantitation method. cDNA was synthesized using SuperScriptII reverse transcriptase (Invitrogen) according to the manufacturer’s protocol in a volume of 25 μl and was subsequently diluted to 200 μl. The 7500 Real Time PCR system (Applied Biosystems) was used for RT-PCR analysis. Reactions were performed in triplicate in a 96-well plate format. A 20-μl volume of reaction mixture contained 1 μl of template cDNA, 0.3 μM gene-specific primers (for PUG1, primers PUG1-210-F2 and PUG1-292-R2; for RTA1, primers RTA1-474-F2 and RTA1-540-R2; for UPC2, primers UPC2-F2 and UPC2-R2), and 5 μl of Sybr green PCR master mix (Applied Biosystems). The amplification program included enzyme activation for 2 min at 50°C; 10 min at 95°C; 40 cycles of denaturation for 15 s at 95°C; and annealing and extension for 1 min at 60°C. The Ct (cycle threshold) for each gene was determined using the automated threshold analysis function of the instrument and normalized to the Ct of 18S RNA. The differences in gene expression were indicated by the ∆∆Ct values, which were calculated as (∆Ct without ALA) – (∆Ct with ALA) for the hem1Δ strain and (∆Ct in hypoxia) – (∆Ct in normoxia) for the wild type strain. The fold change in gene expression was calculated as 2^–∆∆Ct.

Microarray data accession number. The full microarray data set has been deposited with Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE5914.

RESULTS

Inducible heme uptake in S. cerevisiae. Mutant strains of S. cerevisiae that lack high-affinity iron uptake grow poorly on media containing hemin as the sole source of iron (48) (Fig. 1A), indicating that iron-deficient yeast cells do not take up sufficient quantities of heme to meet their nutritional requirement for iron. However, mutant strains that are deficient in heme biosynthesis can grow well on media supplemented with hemin, indicating that heme-deficient yeast cells can take up sufficient quantities of heme to meet their nutritional requirement for heme. We questioned whether this discrepancy in heme uptake was due to the presence of an inducible heme uptake system. HEM1 encodes ALA synthase, which catalyzes the first step of heme biosynthesis. We deleted HEM1 in strains
carrying either wild-type or deletion alleles of \( FET3 \), the ferrodoxidase component of the high-affinity ferrous transport complex (43). When grown on media lacking iron and either heme or heme precursors, the \( hem1 \Delta \) \( fet3 \Delta \) strain is both heme and iron deficient. While all strains grew well on medium supplemented with both iron and ALA, and only the wild-type strain grew well on medium lacking both iron and hemin or ALA (data not shown), the \( fet3 \Delta \) strain grew very slowly on iron-poor medium supplemented with hemin (Fig. 1A). Yet deletion of \( HEM1 \) in the \( fet3 \Delta \) strain improved growth on iron-poor medium supplemented with hemin, suggesting that deletion of \( HEM1 \) increased the uptake of hemin by the \( fet3 \Delta \) strain.

To test this observation further, we measured the uptake of \([^{55}\text{Fe}]\)hemin by a \( hem1 \Delta \) strain when the level of heme biosynthesis was regulated by supplementation with different amounts of ALA. Cells grown in the absence of ALA accumulated sixfold more \([^{55}\text{Fe}]\)hemin than the same strain sufficiently supplemented with ALA (Fig. 1B, left). Because heme biosynthesis requires oxygen, and cells grown under hypoxic conditions develop heme deficiency, we compared the levels of heme uptake in wild-type cells grown under normoxia and hypoxia (Fig. 1B, right). Like the \( hem1 \Delta \) strain, wild-type cells grown under reduced oxygen tension accumulated 5 times more \([^{55}\text{Fe}]\)hemin than cells grown in room air. Thus, \emph{S. cerevisiae} expresses a heme uptake system that is inducible under conditions of heme starvation. This heme uptake could not be explained as simple diffusion, since depletion of cellular energy with the metabolic poisons potassium fluoride and sodium azide inhibited the induced \([^{55}\text{Fe}]\)hemin uptake by 50 to 67% (Fig. 1C). We examined the kinetics of heme uptake in the \( hem1 \Delta \) strain and found that the transport process was nonsaturable at concentrations as high as 50 \( \mu \text{M} \) (Fig. 1D); thus, uptake through a single transporter with Michaelis-Menten kinetics did not explain the observed uptake. A likely explanation of these data is that the uptake process was genetically complex and that multiple transporters, some energy independent, or an internalization process such as endocytosis was contributing to uptake. Nevertheless, these data indicated that yeast expresses inducible uptake systems for heme and that cells accumulate heme through an energy-dependent process.

**Identification of candidate genes for heme transport.** To identify transporters involved in heme uptake, we analyzed gene expression in response to heme deficiency or oxygen deficiency by using microarrays. We first grew the \( hem1 \Delta \) strain in room air in medium with or without ALA and the wild-type strain in either room air or a hypoxic chamber; then we prepared RNA for microarray analysis. We confirmed that heme uptake was induced four- to sixfold in strains grown under both conditions. Transcripts that were expressed at >2-fold higher levels under both heme-deficient and oxygen-deficient conditions were examined for the presence of predicted transmembrane domains. Because previously identified transporters typically contain >2 membrane-spanning domains, we selected genes induced under heme deficiency and hypoxia with >2 transmembrane domains (Table 3). This set of genes was predicted to include all transporters and other multipass integral membrane proteins that are transcriptionally induced under heme and oxygen deficiency. In agreement with previous analyses of anaerobically grown cells, numerous transporters involved in the uptake of sterols, sugars, and amino acids were induced under heme deficiency (18, 45). Numerous other transporters and putative transporters were induced, the roles of which in anaerobic growth were much less clear.

**FIG. 1.** Inducible hemin uptake by the yeast \emph{S. cerevisiae}. (A) Blockage of heme biosynthesis improved the utilization of hemin as an iron source in a \( fet3 \Delta \) strain. The \emph{S. cerevisiae} congenic strains DY1457 (wild type [WT]), DY1457 \emph{hem1 \Delta} (\( hem1 \Delta \)), and OPY110 (\( hem1 \Delta \) \( fet3 \Delta \)), and OPY111 (\( fet3 \Delta \)) were grown overnight on iron-free SC medium supplemented with \( 1 \text{mM} \) ferrozine and then plated in serial dilutions on the same medium supplemented with \( 10 \mu \text{M} \) hemin (\(-\text{Fe}, +\text{Hemin}\)) or on iron-replete medium supplemented with \( 250 \mu \text{M} \) ALA (\(+\text{Fe}, +\text{ALA}\)). (B) Hypoxia or heme starvation induced heme uptake. A \([^{55}\text{Fe}]\)hemin uptake assay was performed on OPY104 (\( hem1 \Delta \)) grown overnight in YPD medium with or without \( 250 \mu \text{M} \) ALA (left) and on BY4742 (WT) grown overnight in YPD medium under normoxic or hypoxic conditions (right). (C) Energy dependence of heme uptake. OPY104 (\( hem1 \Delta \)) was pregrown in YPD medium supplemented with \( 250 \mu \text{M} \) ALA and then incubated overnight in YPD medium without ALA. Cells were incubated with or without \( 100 \text{mM} \) \( \text{NaNO}_2 \) and \( 100 \text{mM} \) \( \text{KF} \) (inhibitors) for 25 min prior to the addition of \([^{55}\text{Fe}]\)hemin. (D) Hemin uptake is nonsaturable. OPY104 (\( hem1 \Delta \)) was grown with or without ALA as described above, and the uptake of \([^{55}\text{Fe}]\)hemin was measured at the indicated concentrations. Error bars, standard errors. Duplicate samples were used, and experiments were repeated at least three times; data from a representative experiment are shown.
TABLE 3. Multipass integral membrane proteins identified by microarray analysis

| Gene       | Name | No. of TMD | Fold change in expression in: | Function                                      |
|------------|------|------------|-------------------------------|-----------------------------------------------|
|            |      |            | WT strain (hypoxia vs normoxia) | hem1Δ strain (without ALA vs with ALA)       |
|            |      |            | Expt 1 | Expt 2 | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| Transport  | Sterols |          |        |        |        |        |        |        |
| YOR011W    | AUS1 | 13         | 22.5   | 22.3   | 6.2    | 6.1    |        |        |
| YIL013C    | PDR11| 12         | 8.1     | 6.4     | 3.9    | 4.9    |        |        |
| Sugars     |              |          |        |        |        |        |        |        |
| YHR096C    | HXT5 | 11         | 49.1   | 36.1   | 14.1   | 28.0   |        |        |
| YJR160C    | MPH2 | 12         | 2.3    | 4.0     | 3.3    | 2.5    |        |        |
| YDL194W    | SNF3 | 12         | 1.9    | 2.3     | 2.1    | 1.5    |        |        |
| YDL138W    | RGT2 | 12         | 3.4    | 3.5     | 3.6    | 3.2    |        |        |
| YPL189W    | GUP2 | 10         | 2.3    | 2.6     | 3.0    | 2.3    |        |        |
| YFL054C    |      | 6          | 2.8    | 2.0     | 2.8    | 2.4    |        |        |
| Allantoin   |              |          |        |        |        |        |        |        |
| YAL067C    | AUS1 | 12         | 21.1   | 23.2   | 8.8    | 8.8    |        |        |
| YJR152W    | DAL5 | 12         | 8.5    | 8.2     | 9.9    | 5.8    |        |        |
| YLL055W    |      | 10         | 4.2    | 4.5     | 5.9    | 6.3    |        |        |
| Cations and anions |              |          |        |        |        |        |        |        |
| YBR296C    | PHO89| 10         | 7.4    | 8.6     | 4.1    | 4.0    |        |        |
| YCL038C    | PHO87| 12         | 3.3    | 3.8     | 1.9    | 1.2    |        |        |
| YLR092W    | SUL2 | 8          | 6.2    | 6.8     | 1.7    | 1.6    |        |        |
| YNR002C    | ATO2 | 5          | 8.0    | 9.4     | 3.2    | 4.1    |        |        |
| YDR039C    | MEP1 | 11         | 2.4    | 1.6     | 4.8    | 3.1    |        |        |
| YBR295W    | IZH2 | 7          | 5.4    | 5.4     | 1.9    | 1.6    |        |        |
| YLR023C    | IZH3 | 7          | 2.0    | 2.4     | 2.2    | 2.2    |        |        |
| YOL002C    | IZH4 | 7          | 3.9    | 3.7     | 2.0    | 1.9    |        |        |
| YGR121C    | ZRT1 | 8          | 2.4    | 3.3     | 2.3    | 2.4    |        |        |
| YOL101C    | FRE8 | 5          | 2.7    | 3.7     | 5.5    | 3.9    |        |        |
| YBR295W    | PCA1 | 7          | 5.9    | 7.4     | 6.9    | 6.5    |        |        |
| YJL094C    | FET4 | 7          | 2.0    | 2.4     | 2.7    | 2.1    |        |        |
| YJL094C    | TRK2 | 10         | 2.1    | 2.1     | 3.4    | 3.2    |        |        |
| YJL094C    | KHA1 | 12         | 2.0    | 2.4     | 3.3    | 4.4    |        |        |
| YML132W    | COS3 | 4          | 1.9    | 1.7     | 2.1    | 2.6    |        |        |
| YDR038C    | ENA5 | 10         | 1.8    | 1.9     | 3.1    | 1.3    |        |        |
| YCR037C    | CCH1 | 22         | 3.1    | 3.5     | 4.4    | 3.7    |        |        |
| YGL006W    | PMC1 | 9          | 2.9    | 2.7     | 1.7    | 2.8    |        |        |
| Vitamins   |      |            |        |        |        |        |        |        |
| YOR306C    | MCH5 | 12         | 5.2    | 7.2     | 2.3    | 2.0    |        |        |
| YOL119C    | MCH4 | 12         | 2.1    | 2.6     | 1.7    | 1.9    |        |        |
| YNL125C    | ESBP6| 11         | 2.9    | 3.2     | 2.0    | 2.0    |        |        |
| YPL221W    | FLC1 | 11         | 2.5    | 3.0     | 2.2    | 1.8    |        |        |
| Amino acids |              |          |        |        |        |        |        |        |
| YFL055W    | AGP3 | 12         | 3.3    | 2.6     | 3.1    | 1.8    |        |        |
| YPL274W    | SAM3 | 11         | 2.8    | 3.2     | 1.7    | 2.6    |        |        |
| YCL025C    | AGP1 | 12         | 2.5    | 2.5     | 1.9    | 1.5    |        |        |
| YMR088C    | VBA1 | 12         | 2.1    | 2.4     | 2.5    | 2.5    |        |        |

Continued on following page
| Gene         | Name | No. of TMD | WT strain (hypoxia vs normoxia) | hem1Δ strain (without ALA vs with ALA) | Function* |
|--------------|------|-----------|---------------------------------|---------------------------------------|-----------|
|              |      |           | Exp1   | Exp2   | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| YKR039W      | GAP1 | 12        | 2.0    | 2.7    | 3.0    | 2.9    |        |        |
| YDR160W      | SSY1 | 12        | 2.2    | 2.3    | 1.9    | 1.7    |        |        |
| YKL209C      | STE6 | 10        | 3.0    | 3.3    | 3.3    | 2.9    |        |        |
| YMR056C      | AAC1 | 4         | 2.7    | 3.2    | 5.5    | 5.4    |        |        |
| YMR162C      | DNF3 | 8         | 2.2    | 2.8    | 2.2    | 2.2    |        |        |

**Other substances**

| Gene         | Name | No. of TMD | WT strain (hypoxia vs normoxia) | hem1Δ strain (without ALA vs with ALA) | Function* |
|--------------|------|-----------|---------------------------------|---------------------------------------|-----------|
|              |      |           | Exp1   | Exp2   | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| YKL209C      | STE6 | 10        | 3.0    | 3.3    | 3.3    | 2.9    |        |        |
| YMR056C      | AAC1 | 4         | 2.7    | 3.2    | 5.5    | 5.4    |        |        |
| YMR162C      | DNF3 | 8         | 2.2    | 2.8    | 2.2    | 2.2    |        |        |

**Resistance to xenobiotics**

| Gene         | Name | No. of TMD | WT strain (hypoxia vs normoxia) | hem1Δ strain (without ALA vs with ALA) | Function* |
|--------------|------|-----------|---------------------------------|---------------------------------------|-----------|
|              |      |           | Exp1   | Exp2   | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| YOR328W      | PDR10| 11        | 4.7    | 5.2    | 4.9    | 4.6    |        |        |
| YPR198W      | SGE1 | 14        | 3.4    | 3.6    | 2.8    | 2.7    |        |        |
| YBR008C      | FLR1 | 12        | 2.8    | 3.3    | 2.1    | 2.0    |        |        |
| YBR043C      | QDR3 | 12        | 2.7    | 3.7    | 2.9    | 2.4    |        |        |
| YGR213c      | RTA1 | 7         | 18.2   | 18.8   | 14.8   | 15.0   |        |        |

**Other cellular processes**

| Gene         | Name | No. of TMD | WT strain (hypoxia vs normoxia) | hem1Δ strain (without ALA vs with ALA) | Function* |
|--------------|------|-----------|---------------------------------|---------------------------------------|-----------|
|              |      |           | Exp1   | Exp2   | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| YDL149W      | ATG9 | 6         | 1.8    | 2.1    | 2.4    | 2.1    |        |        |
| YCL025C      | ATG22| 10        | 4.6    | 5.2    | 3.7    | 2.0    |        |        |
| YKR053C      | YSR3 | 5         | 46.6   | 49.3   | 13.5   | 12.5   |        |        |
| YGR032W      | GSC2 | 14        | 15.8   | 12.5   | 3.4    | 5.3    |        |        |
| YOR034C      | AKR2 | 7         | 9.3    | 11.0   | 6.1    | 5.1    |        |        |
| YNR041C      | COQ2 | 5         | 3.9    | 3.9    | 2.7    | 2.6    |        |        |
| YCR048W      | ARE1 | 9         | 3.9    | 4.4    | 2.3    | 2.2    |        |        |
| YMR306W      | FKS3 | 18        | 3.3    | 3.4    | 6.7    | 7.4    |        |        |
| YOR213W      | FKS3 | 18        | 3.3    | 3.4    | 6.7    | 7.4    |        |        |

**Uncharacterized genes**

| Gene         | Name | No. of TMD | WT strain (hypoxia vs normoxia) | hem1Δ strain (without ALA vs with ALA) | Function* |
|--------------|------|-----------|---------------------------------|---------------------------------------|-----------|
|              |      |           | Exp1   | Exp2   | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| YGR131W      |      | 4         | 26.4   | 26.5   | 6.5    | 10.8   |        |        |
| YER185W      | PUG1 | 7         | 6.5    | 6.0    | 7.8    | 4.4    |        |        |
| YLR046C      |      | 6         | 3.7    | 4.4    | 3.5    | 2.9    |        |        |
| YMR034C      |      | 10        | 5.6    | 8.2    | 5.0    | 5.7    |        |        |
| YJR116W      |      | 5         | 5.1    | 6.1    | 3.7    | 4.0    |        |        |
| YOL084W      | PHM7 | 10        | 4.6    | 4.8    | 8.1    | 9.0    |        |        |
| YDR018C      |      | 4         | 4.4    | 6.0    | 3.8    | 3.7    |        |        |
| YBR051W      | YRO2 | 7         | 3.4    | 3.3    | 3.5    | 4.1    |        |        |
| YBR051W      |      | 12        | 3.2    | 4.0    | 5.9    | 6.9    |        |        |
| YBR051W      |      | 12        | 3.2    | 4.0    | 5.9    | 6.9    |        |        |
| YJR161C      | COS5 | 4         | 3.0    | 2.2    | 2.2    | 3.4    |        |        |

Continued on following page
TABLE 3—Continued

| Gene        | Name | No. of TMD | WT strain (hypoxia vs normoxia) | hem1Δ strain (without ALA vs with ALA) | Functionb |
|-------------|------|------------|---------------------------------|---------------------------------------|------------|
| YGR295C     | COS6 | 4          | 2.7 2.5                         | 2.6 2.4                               | Protein of unknown function |
| YDL248W     | COS7 | 4          | 3.5 1.4                         | 3.0 3.5                               | Putative protein of unknown function |
| YHL048W     | COS8 | 3          | 2.0 1.5                         | 2.2 2.6                               | Nuclear membrane protein |
| YLR152C     |      | 9          | 2.9 3.8                         | 3.1 2.9                               | Putative protein of unknown function |
| YDL206W     |      | 12         | 2.7 3.3                         | 2.5 2.4                               | Putative protein of unknown function |
| YNL095C     |      | 9          | 2.6 3.0                         | 3.0 3.4                               | Putative protein of unknown function |
| YGR149W     |      | 7          | 2.5 2.1                         | 2.4 3.0                               | Putative protein of unknown function |
| YMR052C-A   |      | 3          | 2.5 3.0                         | 2.6 3.3                               | Protein of unknown function |
| YOR092W     | ECM3 | 8          | 2.4 2.5                         | 2.0 1.7                               | Protein of unknown function |
| YOR292C     |      | 5          | 2.4 2.8                         | 1.9 1.6                               | Protein of unknown function |
| YOR291W     |      | 11         | 2.4 2.9                         | 2.0 1.7                               | Protein of unknown function |
| YDR107C     |      | 9          | 2.3 2.5                         | 2.7 –1.1                              | Protein of unknown function |
| YOL075C     |      | 13         | 2.2 2.4                         | 3.1 3.4                               | Protein of unknown function |
| YBR235W     |      | 10         | 2.1 2.4                         | 2.2 1.8                               | Protein of unknown function |
| YFL034W     |      | 4          | 2.1 1.9                         | 2.0 2.0                               | Protein of unknown function |
| YPR114W     |      | 5          | 2.1 2.4                         | 1.7 1.7                               | Protein of unknown function |
| YLR241W     |      | 11         | 2.1 2.2                         | 2.1 2.1                               | Protein of unknown function |
| YLR426W     |      | 3          | 2.1 2.2                         | 2.3 2.2                               | Protein of unknown function |
| YLL222C     | FMP45| 4          | 2.0 1.7                         | 3.8 2.9                               | Protein of unknown function |
| YBR241C     |      | 11         | 1.9 2.4                         | 2.7 2.2                               | Protein of unknown function |
| YDR387C     |      | 12         | 1.9 1.9                         | 3.0 2.9                               | Protein of unknown function |
| YDL133W     |      | 4          | 1.9 2.3                         | 2.4 2.2                               | Protein of unknown function |
| YGR045C     |      | 3          | 1.8 1.7                         | 2.7 2.1                               | Protein of unknown function |
| YDL199C     |      | 11         | 1.8 2.3                         | 4.3 4.6                               | Protein of unknown function |
| YNL115C     |      | 5          | 1.7 1.8                         | 2.9 3.3                               | Protein of unknown function |
| YKR106W     |      | 12         | 6.7 7.5                         | 3.1 3.3                               | Protein of unknown function |
| Q0010       |      | 3          | 3.8 2.4                         | 5.1 6.6                               | Dubious mitochondrial ORF |
| YOL047C     |      | 4          | 1.7 2.7                         | 6.3 7.1                               | Protein of unknown function |
| YMR040W     |      | 3          | 4.2 5.4                         | 6.5 7.6                               | Protein of unknown function |
| YGR049W     | SCM4 | 4          | 5.1 4.6                         | 2.7 2.7                               | Potential regulatory effector of CDC4 function |

a TMD, transmembrane domains; WT, wild type; MDR, multidrug resistance; CoA, coenzyme A.
b Gene function was assigned according to the Saccharomyces Genome Database (http://www.yeastgenome.org/).

We evaluated candidate heme transport genes either by direct measurement of heme uptake in the deletion strains (39 strains tested) or by cloning the ORFs into a high-copy-number vector under the control of a strong promoter, transforming the resulting plasmids into the fet3Δ strain, and testing the transformants for growth on an iron-poor medium containing hemin as the iron source (12 ORFs tested). We found no single gene that accounted for the majority of high-affinity heme uptake. Although the latter assay was designed to detect genes that enhanced growth on hemin, we found that overexpression of YER185W resulted in significantly reduced growth of the fet3Δ strain on this medium (Fig. 2A). Although growth was improved when the medium was supplemented with higher concentrations of hemin, the inhibitory effect of YER185W persisted, suggesting that overexpression of YER185W affected the uptake or use of heme. We termed this gene PUG1, for protoporphyrin uptake gene 1.

PUG1 belongs to a gene family that includes RTA1 (44% identical) and RTMI (62% identical). Overexpression of RTA1 confers resistance to 7-aminocolesterol, a drug that leads to the production of toxic analogues of oxysterols in yeast (41). RTMI overexpression confers resistance to molasses and is present in multiple copies in industrial strains of yeast but is not present in strains derived from S. cerevisiae S288C (22). A more distantly related paralogue, RSBI (24% identical), encodes an efflux pump for sphingoid long-chain bases (15). Each of these genes is predicted to contain seven transmembrane domains, with the amino terminus predicted to be extracytosolic and the carboxyl terminal cytosolic. We confirmed that the mRNA levels of PUG1 and RTA1 were significantly increased under oxygen and heme deficiency by using quantitative real-time PCR (Fig. 2B). UPC2, a gene known to be induced under oxygen deficiency (1, 5), was included in order to confirm the depletion of oxygen and heme in the cultures. To determine whether protein levels of Pug1p were also regulated by oxygen and heme, we constructed a strain containing 13 copies of the Myc tag at the carboxyl terminus of PUG1. Western blot experiments confirmed that the abundance of Pug1p was increased under hypoxic conditions (Fig. 2C, lanes 1 to 3). Under denaturing conditions, Pug1-myc was largely detected as species of approximately 60 kDa, although its predicted molecular size is 33.7 kDa. However, when Pug1-myc was overexpressed from a plasmid, an additional band of approximate 35 kDa appeared (Fig. 2C, lanes 4 and 5). These data raised the possibility that Pug1p might form a dimer or might be present as an oligomeric complex within cells.
Alteration of porphyrin utilization in Pug1p-overexpressing strains. We further tested the effects of Pug1p overexpression in the hem1/[H9004] strain, which both exhibits induction of heme uptake systems and is dependent on porphyrin uptake for growth. Like the effects on the growth of the fet3/[H9004] strain, overexpression of Pug1p in the hem1/[H9004] strain inhibited growth on media containing both lower and higher concentrations of hemin (Fig. 3A). More hemin led to more growth in all strains, suggesting that the inhibitory effect of Pug1p overexpression was due to insufficient heme rather than to the conversion of heme to a toxic metabolite.

The heme deficiency of a hem1/[H9004] strain can be rescued with intermediates of heme biosynthesis in addition to heme, and we tested whether Pug1p overexpression also affected the utilization of PPIX, the immediate precursor of heme, and ALA (Fig. 3B and C). Surprisingly, overexpression of Pug1p in the hem1/[H9004] strain had the opposite effect on growth when the medium contained PPIX, with Pug1p overexpression leading to enhanced growth on PPIX. Overexpression of the paralogue Rta1p had no effect on the growth of the hem1/[H9004] strain on either hemin or PPIX, indicating that these effects were specific to Pug1p. Overexpression of these genes also had no effect on the utilization of hemin as an iron source and heme-dependent regulation of PUG1 expression.
on growth when the medium contained ALA, indicating that Pug1p primarily inhibited the utilization of exogenous hemin rather than that of endogenously produced heme.

Zinc can be incorporated into PPIX instead of iron to form ZnPPIX. Although ZnPPIX cannot functionally substitute for heme in heme proteins, it is structurally similar to heme and can be transported by some heme transporters (17, 23). We found that an excess of ZnPPIX also rescued the growth defect of the *hem1Δ* *pPUG1* strain on hemin, suggesting that both metalloporphyrins were substrates for Pug1p (Fig. 3D). Taken together, these data indicated that Pug1p could affect the utilization of both PPIX and heme and that it might affect the transport of both protoporphyrins.

**Plasma membrane localization of Pug1.** The function of a putative transport protein is determined in part by its subcellular localization. Because Pug1p could not be detected by indirect immunofluorescence in the strain containing Myc epitopes integrated into the *PUG1* locus, we transformed yeast with *pPUG1-myc* and examined the localization of Pug1p by indirect immunofluorescence (Fig. 4A). We confirmed that Pug1p-myc was functional by overexpressing Pug1p-myc and reproducing the growth inhibition on hemin. Pug1p was detected predominantly at the periphery of the cell in a pattern that indicated plasma membrane localization. Because overexpression of integral membrane proteins can lead to mislocalization, we also examined the localization of Pug1p by subcellular fractionation of a strain carrying the chromosomally tagged Pug1p-myc. The *PUG1-myc* strain was grown under hypoxic conditions, and cell lysates were separated on sucrose density gradients. Fractions were subjected to Western blot analysis using antibodies directed against the Myc epitope and resident proteins of membrane-bound organelles (Fig. 4B). Pug1p-myc was detected primarily in fractions 9 to 11 and cosedimented with Pma1p, a resident protein of the plasma membrane. Pug1p-myc did not substantially colocalize with resident proteins of other organelles, including Pho8p (vacuole), Pep12p (late endosomes), Dpm1p (endoplasmic reticulum), Vps10p (late Golgi complex), and porin (mitochondria). We also confirmed by subcellular fractionation that when Pug1p-myc was overexpressed from a plasmid, the protein entirely cosedimented with the plasma membrane protein Pma1p and showed no localization to other organelles (Fig. 4C). These data indicated that the phenotypes associated with Pug1p overexpression were due to augmented activities on the plasma membrane and not to incorrect intracellular trafficking of heme or PPIX due to mislocalization of Pug1p to intracellular membranes.

**Accumulation of hemin and PPIX in the *PUG1*-overexpressing strain.** To determine whether Pug1p participates in the transport of hemin and PPIX, we measured uptake activity by *PUG1* deletion strains or strains overexpressing *PUG1*. To avoid the possibility that increased expression of *RTA1* could compensate for the absence of *PUG1*, we also deleted *RTA1* in the *pug1Δ* strain. We then deleted *HEMI* in the *pug1Δ rtalΔ* strain, and we transformed the resulting *hem1Δ* *pug1Δ rtalΔ* strain with plasmids overexpressing *PUG1* or *RTA1* or with the empty parent vector. Strains were grown for 12 h in the absence of ALA to induce heme uptake, and washed cells were incubated in the presence of [55Fe]hemin prior to washing and scintillation counting. The *hem1Δ* and *hem1Δ* *pug1Δ* *rtalΔ* strains transformed with empty plasmids showed similar rates of [55Fe]hemin accumulation (Fig. 5), indicating that these genes are not required for heme uptake. However, the overexpression of *PUG1* in the *hem1Δ* *pug1Δ* *rtalΔ* strain led to a 33% inhibition of [55Fe]hemin accumulation (Fig. 5). Although...
we could not reliably measure heme efflux in these strains, these data indicated that overexpression of Pug1p interfered with the intracellular accumulation of heme.

Although PPIX has been shown to rescue the heme deficiency of heme biosynthetic mutants (11), the uptake of PPIX into yeast cells has not been characterized. We developed an uptake assay based on the inherent fluorescence of PPIX. Because the fluorescence of PPIX is lost when the compound is converted into heme, we performed these assays with a hem15Δ strain, which lacks ferrochelatase activity and therefore does not convert intracellular PPIX into heme. Because the hem15Δ strain can accumulate endogenously produced fluorescent porphyrin intermediates, HEM1 was also deleted in the hem15Δ strain to produce a hem1Δ hem15Δ strain, which synthesizes no porphyrin intermediates. The hem1Δ hem15Δ strain was grown in medium with or without heme supplementation, cells were washed and incubated in PPIX, and the fluorescence of crude cell lysates was measured. In a manner similar to the uptake of heme, the uptake of PPIX was 47-fold higher in cells grown without heme supplementation (Fig. 6A), indicating that both heme and PPIX uptake systems were induced by heme deficiency. Heme-deficient cells incubated at 0°C also accumulated PPIX, indicating that surface binding of PPIX was also induced. The kinetics of PPIX uptake were different from those of heme. Cells incubated with PPIX exhibited a very rapid initial uptake that reached a plateau after approximately 20 min (Fig. 6B). Depletion of cellular ATP with sodium fluoride and potassium azide did not inhibit the uptake of PPIX. These data suggested that cells accumulated PPIX through a process of facilitated diffusion rather than active transport.

We measured the contribution of Pug1p to PPIX uptake in a hem1Δ hem15Δ strain that expressed endogenous levels of Pug1p and Rta1p or in a hem1Δ hem15Δ strain in which PUG1 and RTA1 were also deleted. Both strains were transformed with the plasmid overexpressing Pug1p or with the empty parent vector and were grown in medium without heme prior to incubation with PPIX (Fig. 6C). When Pug1p was not overexpressed, no significant difference in PPIX uptake was observed between the hem1Δ hem15Δ strain and the hem1Δ hem15Δ strain that also lacked PUG1 and RTA1, indicating that PUG1 and RTA1 do not encode the major PPIX uptake systems of yeast. However, overexpression of Pug1p led to a significant increase in PPIX uptake in both strains, indicating that PPIX was a transport substrate of Pug1p.

Increased utilization of PPIX and decreased utilization of heme in cells overexpressing Pug1p. If Pug1p overexpression stimulated significant uptake of PPIX and efflux of heme, then
cells overexpressing Pug1p would be predicted to demonstrate alterations in the activity of heme-dependent processes. To confirm that Pug1p overexpression affected intracellular heme status, we employed two different approaches. First, we used a CYC1-lacZ reporter in which the β-galactosidase coding sequences are under the control of the CYC1 promoter, which is activated by the heme-dependent transcription factor Hap1p and reflects the regulatory pools of intracellular heme (12, 13). The hem1Δ strain containing the CYC1-lacZ reporter was transformed with pPug1 or the empty parent vector and grown in media supplemented with different concentrations of ALA, PPIX, or hemin (Fig. 7A). When the strain transformed with vector was supplemented with increasing concentrations of ALA or PPIX, the β-galactosidase activity increased in a dose-dependent manner. When cells transformed with pPug1 or vector were grown on ALA, both strains exhibited similar levels of β-galactosidase activity. However, cells that overexpressed Pug1p exhibited more than twofold higher reporter activity than vector-transformed cells when they were grown in medium supplemented with PPIX. These data confirmed that Pug1p overexpression stimulated the accumulation of PPIX and its subsequent conversion to heme.

Surprisingly, when both strains were grown in medium containing heme, reporter activity was uniformly low. Growth rates for these strains were similar whether the medium was supplemented with ALA, PPIX, or heme (data not shown), indicating that the uptake and utilization of heme was sufficient to meet the metabolic needs of the cell. These data indicated that the regulatory pool of heme differed depending on whether the source of heme was endogenous (synthesized from ALA or PPIX) or exogenous (hemin). One possible explanation for this observation is that newly synthesized heme may be bound by heme chaperones as it exits the mitochondria, and exogenous hemin entering the cell from the plasma membrane may not have access to the same chaperones. Again, deletion of PUG1 and RTA1 did not result in significant changes in CYC1-lacZ activity (data not shown), confirming that these genes do not encode the major PPIX uptake activity.

Because the CYC1-lacZ reporter did not respond to exogenously derived hemin, we developed an assay based on the activity of a heme-dependent enzyme, Fre1p. FRE1 and FRE2 encode the two major metalloreductases responsible for plasma membrane ferric reductase activity in yeast (6, 10). We constructed a strain with deletions of FRE1, FRE2, and HEM1 and placed the FRE1 coding sequences under the control of the constitutively active promoter PGK1. The resulting strain had only one major cell surface reductase, which was constitutively expressed independently of cellular iron or copper levels. When the hem1Δ fre1Δ fre2Δ PGK1-FRE1 strain was grown on different concentrations of ALA, PPIX, and heme, the ferric reductase activity was proportional to the concentration of supplemental ALA, PPIX, or heme. Activity was low on minimal concentrations and gradually increased with the addition of more compound to the medium (Fig. 7B). These experiments confirmed that the ferric reductase activity in the hem1Δ fre1Δ fre2Δ PGK1-FRE1 strain reflected the metabolically available pool of heme. We then transformed this strain with pPUG1 or the empty parent vector and measured ferric reductase activity after growth on medium supplemented with ALA, PPIX, or heme (Fig. 7C). Overexpression of Pug1p had...
opposing effects on ferric reductase activity: for cells grown in low concentrations of PPIX, Pug1p overexpression led to higher levels of reductase activity, while for cells grown in hemin, Pug1p overexpression led to lower levels of reductase activity. Pug1p overexpression also led to a slight increase in reductase activity in cells grown in ALA, raising the possibility that Pug1p may have affected ALA accumulation as well. Taken together, these data indicate that Pug1p overexpression led to the expansion of intracellular heme pools when cells were grown in PPIX but to the contraction of heme pools when cells were grown in hemin. These data suggest that Pug1p may act as a transporter, facilitating the uptake of PPIX and the efflux of hemin.

DISCUSSION

Previous studies have indicated that S. cerevisiae does not take up heme in response to iron deficiency and thus that heme is a poor nutritional source of iron for this yeast (35, 48). Here we confirm that the uptake of hemin and its precursor, PPIX, is very low in cells with adequate biosynthesis of heme, but under conditions of reduced heme biosynthesis, a marked increase in the uptake of both heme and PPIX occurs, indicating that inducible uptake systems for these compounds are expressed in yeast. These uptake systems are distinct, however. Heme uptake was energy dependent and nonsaturable, which indicated that heme is taken up through an active transport process facilitated by a primary or secondary transporter(s), such as an ABC transporter or major facilitator superfamily (MFS) transporter, respectively. In contrast, PPIX uptake was energy independent and rapidly reached a plateau, with the level of intracellular accumulation proportional to the extra-cellular concentration of PPIX. These observations suggest that PPIX comes into the cell by a process of facilitated diffusion via a carrier or channel-type transporter(s). Deletion of PUG1 and RTA1 did not affect the uptake of heme or PPIX, indicating that other loci encode the majority of these uptake activities.

Significant amounts of PPIX were associated with heme-deficient cells incubated at 0°C but not with heme-sufficient (ALA-supplemented) cells, raising the possibility that PPIX binding proteins on the cell surface or in the cell wall are induced during heme-deficient growth. Alternatively, some PPIX may infiltrate the plasma membrane of the cell by simple diffusion. Other investigators have noted that dramatic changes in the transcription of cell wall proteins occur during anaerobic growth, and cell wall proteins of the DAN/TIR and seripauperin families are among the genes most strongly induced by anaerobiosis (1, 18, 45).

Pug1p is a member of a family of fungal proteins involved in resistance to xenobiotics and endobiotics, and these proteins may constitute a family of efflux channels or pumps that are induced under conditions of enhanced uptake and dysregulated biosynthesis of intracellular small molecules. RTA1 was identified in a screen for genes that, when overexpressed, confer resistance to 7-aminocaproic acid, a drug that is a substrate for enzymes in the ergosterol biosynthetic pathway (41). Treatment of cells with 7-aminocaproic acid results in the accumulation of aberrant sterols that are toxic to the cell, and overexpression of Rta1p reduces the accumulation of at least one of the aberrant sterols. Deletion of RTA1 also rendered cells more sensitive to 7-aminocaproic acid. RTA1 was induced under heme and oxygen deficiency, conditions that also trigger increased expression of genes involved in sterol biosynthesis and uptake. Because ergosterol biosynthesis is an oxygen- and heme-dependent process, deficiencies lead to the accumulation of intermediates, such as squalene. Expression of Rta1p under hypoxic conditions may allow the cell to excrete the toxic by-products of the defective ergosterol biosynthetic pathway.

RTM1 was identified in a similar screen for genes that increase resistance to molasses (22). Molasses is a complex mixture used in fermentation with industrial strains of yeast, and sensitivity to molasses is thought to be due to the presence of toxic xenobiotics in the mixture. Industrial strains of yeast exhibit variable sensitivity to molasses and a variable number of copies of the RTM1 locus. Resistance to molasses was roughly correlated with the copy number of RTM1. Finally, RSB1 was isolated as a high-copy-number gene that rescued the sphingoid long-chain base sensitivity of a sphingolipid biosynthetic mutant (15). Rsb1p was subsequently shown to be a plasma membrane protein that facilitated the energy-dependent efflux of dihydroxyphospho-sine and other long-chain bases. Rsb1p expression is induced in response to the loss of the mitochondrial genome, which also leads to upregulation of sphingolipid biosynthesis, again suggesting that this efflux pump may contribute to sphingolipid homeostasis (24).

Here we have shown that overexpression of Pug1p resulted in enhanced accumulation and utilization of PPIX as well as reduced accumulation and utilization of exogenously acquired hemin. Pug1p was expressed exclusively on the plasma membrane, may function as a dimer or higher-order oligomer, and was shown to bind hemin. The simplest explanation for these observations is that, like other members of this family, Pug1p functions as a transporter, facilitating the influx of PPIX and the efflux of metalloporphyrins. ZnPPIX could block the growth-inhibitory effects of Pug1p overexpression on hemin medium (Fig. 3D), suggesting that ZnPPIX could compete with hemin for transport through Pug1p. Hypoxia induces PPIX and heme uptake systems, which could lead to excess accumulation of porphyrins. Pug1p may facilitate the excretion of excess porphyrins under these conditions. Because the heme biosynthetic pathway contains two oxygen-dependent enzymes, Hem13p and Hem14p, hypoxic and anaerobically grown cells accumulate porphyrin biosynthetic intermediates (34), some of which are excreted, and Pug1p may have a role in the efflux of endogenously produced porphyrin intermediates as well as in Pug1p and ZnPPIX. Hypoxia also leads to the expression of many other transporters, leaving the cells vulnerable to xenobiotics that enter as low-specificity substrates of these transporters. Both Pug1p and Rta1p may have additional roles in the excretion of other xenobiotics.

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