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Role of PUG1 in Inducible Porphyrin and Heme Transport in *Saccharomyces cerevisiae*▽

Olga Protchenko, Minoo Shakoury-Elizeh, Patricia Keane, Joshua Storey, Rachel Androphy, and Caroline C. Philpott*

Genetics and Metabolism Section, Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bldg. 10, Rm. 9B-16, Bethesda, Maryland 20892

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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 aminolevulinate synthase exhibited a sixfold increase in hemin 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}Fe]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, Rbt5p, 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).

* Corresponding author. Mailing address: Genetics and Metabolism Section, Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bldg. 10, Rm. 9B-16, Bethesda, MD 20892. Phone: (301) 435-4018. Fax: (301) 402-0491. E-mail: carolinep@intra.niddk.nih.gov.

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TABLE 1. List of strains used in this study

Strain name	Short genotype	Genetic background	Relevant genotype	Source
DY1457		W303	*MAT*α *ura3-52 leu2-3,112 trp1-1 his3-11 ade6 can1-100*	4
	*hem1*Δ(6D)	DY1457	*hem1*::*LEU2 trp1-1 his3-11 ura3-52 can1-100*	4
OPY110	*hem1*Δ *fet3*Δ	DY1457	*hem1*::*LEU2 fet3*::*HIS3*	This study
OPY111	*fet3*Δ	DY1457	*fet3*::*URA3*	This study
YPH499			*MAT***a** *ura3-52 lys2-801*(amber) *ade2-101*(ocher) *trp1-63 his3-200 leu2-1*	40
CPY119	*fet3*Δ	YPH499	*fet3*::*HIS3*	44
OPY101	*hem1*Δ	YPH499	*hem1*::*LEU2*	This study
	*fre1*Δ *fre2*Δ	YPH499	*fre1*::*LEU2 fre2*::*HIS3*	8
OPY102	*hem1*Δ *fre1*Δ *fre2*Δ	YPH499	*hem1*Δ::KanMX *fre1*::*LEU2 fre2*::*HIS3*	This study
OPY112	*hem1*Δ *fre1*Δ *fre2*Δ *PGK-FRE1*	YPH499	*hem1*Δ::KanMX *fre1*::*LEU2 fre2*::*HIS3 ade2-1*::*ADE2* pPGK-FRE1	This study
OPY103	*PUG1-myc*	YPH499	*PUG1-13myc* KanMX6	This study
BY4742			*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	3
OPY104	*hem1*Δ	BY4742	*hem1*::*LEU2*	This study
BY4741			*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	3
OPY105	*hem1*::KanMX	BY4741	*hem1*Δ::KanMX	This study
OPY106	*hem1*Δ	BY4741	*hem1*::*LEU2*	This study
OPY107	*hem1*Δ *pug1*Δ *rta1*Δ	BY4741	*hem1*::*LEU2 pug1*Δ::KanMX *rta1*Δ::KanMX	This study
OPY108	*hem1*Δ *hem15*Δ	BY4741	*hem1*::*LEU2 hem15*Δ::*HIS3*	This study
OPY109	*hem1*Δ *hem15*Δ *pug1*Δ *rta1*Δ	BY4741	*hem1*::*LEU2 hem15*Δ::*HIS3 pug1*Δ::KanMX *rta1*Δ::KanMX	This study

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 were selectively induced during oxygen and heme deficiency. One of these, termed *PUG1* (protoporphyrin uptake gene 1), affected the accumulation of exogenous heme and porphyrin. Pug1 protein was localized to the plasma membrane, and overexpression had opposing effects on the utilization of heme and PPIX. We propose that Pug1p enhances the uptake of PPIX and the efflux of heme at the plasma membrane.

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 (*MAT***a** *ura3 met-15 his3-1 leu2-1* ymr185w::KanMX) (ATCC) using primers YER185W-250-F and YER185W-230-R (Table 2). Geneticin-resistant clones were selected on yeast extract-peptone-dextrose (YPD) plates containing G418. To combine deletions of *PUG1* and *RTA1*, BY4741 *pug1*Δ was crossed with BY4742 *rta1*Δ. Auxotrophic meiotic segregants were selected on medium lacking methionine and lysine and were checked by PCR for the correct genotype of *pug1*Δ *rta1*Δ. Strain CPY119 (*fet3*Δ) was constructed as described elsewhere (43). To make strains OPY111 (*fet3*Δ) and OPY110 (*fet3*Δ *hem1*Δ), *FET3* was replaced with *URA3* as described elsewhere (16). For deletion of *HEM1*, yeast was transformed with a PCR product amplified from genomic DNA isolated from the *hem1*Δ(6D) strain using primers HEM1-D-F and HEM1-D-R. Transformants were selected on medium lacking leucine and supplemented with 250 μM aminolevulinic acid (ALA). To construct OPY105 (*hem1*Δ::KanMX), the deletion cassette was amplified from genomic DNA of the *hem1*Δ/*HEM1* diploid

TABLE 2. List of primers used in this study

Primer	Sequence (5′ to 3′)
YER185W-250-F	CTCCATACAGGATGAAGTGTTTC
YER185W-230-R	ATCTTTCTCAACGAGCTAC
HEM1-D-F	CACCCTGCTCTGCTGTCTCTCA
HEM1-D-R	GCTACAGTTTAACCATTGCAG
HEM15-DEL-F	CGTTTAAAGAATGCTTTCCAGAACA ATCCGTACACAAGGTTCCTTCCTA CTCACTATAGGGCGAATTGG
HEM15-DEL-R	AGAATATACTGATATTGAGATTGTG GGATGAATGGCCCTTATCAAGTA GCTAAAGGGAACAAAAGCTGG
YER185W-3HA-F2	TTTCGGCAACGTATTCGATGTTATA AAGGAGTGTCAAACGTTAAGTAA TCGGATCCCCGGGTTAATTAA
YER185W-3HA-R1	TAAAATTGATTGGCTGCACTACATA GAAATCAAAATTTGATATATATTA CGATGAATTCGAGCTCGTTT
YER185W-F	GCTTAAATCTATAACTACAAAAAAC ACATACAGGAATTCATGTCCACA ACTGATTCGGG
YER185W-R	CTCGAGATATCATGCGTAGTCAGGC ACATCATACGGATACCCGGGCTA ATTACTTAACGTTTGAC
YER185W-MYC-F	CACACAGAATTCATGTCCACAACTG ATTCGGGGTTC
YER185W-MYC-R	CACACAAAGCTTTCAATTCAAGTCT TCTTCTGAGAT
YGR213C-F	CACACAAAGCTTATGGCAAAAGAC GGCTTCGAGT
YGR213C-R	CACACACCCGGGTTATTCGCTCTTT GAAGCGAGATCTTC
FRE1-F	CACACAGAGCTCATGGTTAGAACCC GTGTATTA
FRE1-R	CACACAGGATCCTTACCATGTAAAA CTTTCTT
PUG1–210-F2	GCAACTAAAAATCACACCCACAGT
PUG1–292-R2	TCCCGATATACCCGACAGCTT
RTA1–474-F2	TGGTGGTGCTATGATGTCTAAAGTG
RTA1–540-R2	CAAACCAGCTGTAACGAGATGAGA
UPC2-F2	CTCTCAAAATGGCACTATT
UPC2-R2	GTTGGAACGCACCCATATTT
B7 18S-1114F	GTATGGTCGCAAGGCTGAAAC
B8 18S-1294R	CACCAACTAAGAACGGCCATG

deletion mutant (*MAT***a**/*MAT*α *ura3 met-15 his3-1 leu2-1 hem1*Δ::KanMX/*HEM1*) (Open Biosystems) using the same primers. Geneticin-resistant clones were selected on YPD plates supplemented with G418 and 250 μM ALA. The *fre1*Δ *fre2*Δ strain was constructed as described elsewhere (8). To construct OPY102 (*hem1*Δ *fre1*Δ *fre2*Δ), *HEM1* was deleted using the *hem1*Δ::KanMX cassette as described above. To construct OPY112 (*hem1*Δ *fre1*Δ *fre2*Δ *PGK1-FRE1*), plasmid YIpDCE51-PGK1-FRE1 was linearized with StuI and integrated into the *ADE2* locus of OPY102. Transformants were selected on synthetic complete (SC) medium lacking adenine and supplemented with 250 μM ALA. To delete *HEM15*, the *hem15*Δ::*HIS3* deletion cassette was amplified from plasmid pFA6a-HIS3MX6 (19) using primers HEM15-DEL-F and HEM15-DEL-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-3HA-F2 and YER185W-3HA-R1. 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). Geneticin-resistant clones were selected on YPD plates containing 80 mg/liter G418 (Invitrogen). 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 hemin supplement.

Plasmids. To make plasmid pPUG1-Leu, the open reading frame (ORF) of *PUG1* was amplified by PCR from plasmid pBG1800-YER185W (Yeast ORF Collection; Open Biosystems) using primers YER185W-F and YER185W-R, and in vivo recombination in yeast was used to clone the *PUG1* insert into pYX242 cut with EcoRI. Subsequently, to make plasmid pPUG1-Ura, pPUG1-Leu was cut with EcoRI and SacI, and the *PUG1*-containing insert was ligated into pYX212 cut 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 the same restriction enzymes. *RTA1* was amplified from pBG1800-YGR213c (Open Biosystems) using primers YGR213C-F and YGR213C-R and then ligated into pYX212 digested with HindIII and SmaI to make pRTA1. To make plasmid YIpDCE51-PGK1-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 YIpDCE51-PGK1-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), 10 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 (14) with the following modifications. Cells were disrupted 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 1:1,000 dilution of the anti-c-Myc antibody 9E10 (Covance) as the primary antibody, followed by a 1:1,000 dilution of a Cy3-conjugated donkey anti-mouse antibody. Antibodies to Dpm1p, porin, and Vps10p were purchased from Molecular Probes and used according to the manufacturer's manual. Anti-Pma1p 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 (*hem1*Δ) 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. [^{55}Fe]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. [^{55}Fe]hemin was added at the indicated concentrations to 0.1 ml of the cell suspension with a final optical density at 600 nm (OD_{600}) of 4.0 and was incubated for 30 min at 30°C or in an ice-H_2O 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 [^{55}Fe]hemin was measured by scintillation counting. [^{55}Fe]hemin uptake was reported as the difference in [^{55}Fe]hemin accumulation at 30°C and 0°C. [^{55}Fe]hemin was synthesized as described elsewhere (9) from PPIX (Porphyrin Products, Logan, UT) and $^{55}FeCl_3$ (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 OD_{600} 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 PC fluorescent spectrophotometer.

Assays. β-Galactosidase assays (2) and ferrireductase 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 μM 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 OD_{600}, 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 RNeasy 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 C_T (cycle threshold) for each gene was determined using the automated threshold analysis function of the instrument and normalized to the C_T of 18S RNA. The differences in gene expression were indicated by the $\Delta\Delta C_T$ values, which were calculated as (ΔC_T without ALA) − (ΔC_T with ALA) for the *hem1*Δ strain and (ΔC_T in hypoxia) − (ΔC_T in normoxia) for the wild type strain. The fold change in gene expression was calculated as $2^{-\Delta\Delta CT}$.

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 GSE9514.

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

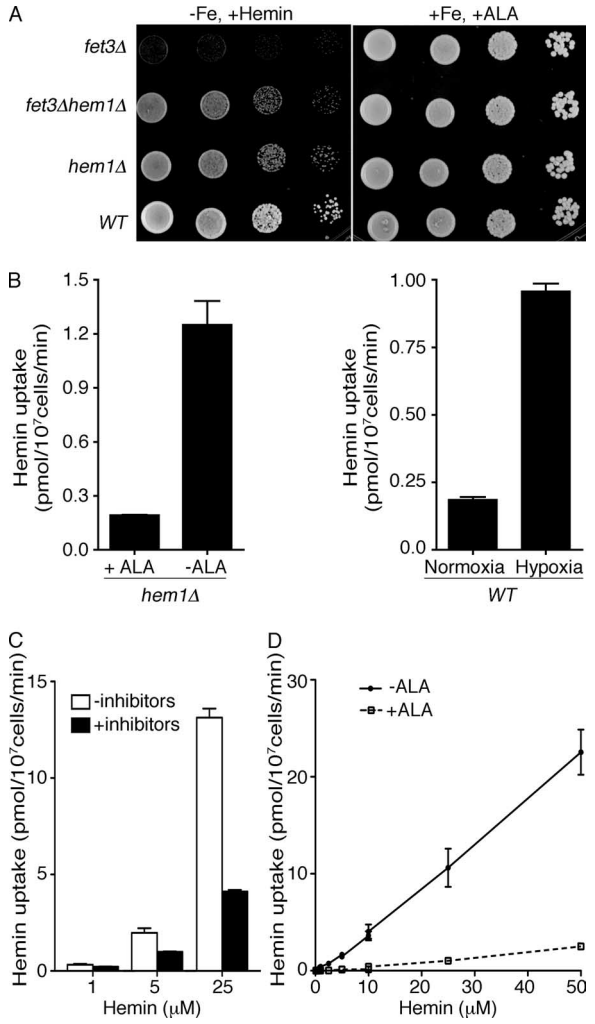

FIG. 1. Inducible hemin uptake by the yeast *S. cerevisiae*. (A) Blockage of heme biosynthesis improved the utilization of hemin as an iron source in a *fet3*Δ strain. The *S. cerevisiae* congenic strains DY1457 (wild type [WT]), DY1457 *hem1*Δ(6D) (*hem1*Δ), OPY110 (*hem1*Δ *fet3*Δ), and OPY111 (*fet3*Δ) were grown overnight on iron-free SC medium supplemented with 1 mM ferrozine and then plated in serial dilutions on the same medium supplemented with 10 μM hemin (−Fe, +Hemin) or on iron-replete medium supplemented with 250 μM ALA (+Fe, +ALA). (B) Hypoxia or heme starvation induced heme uptake. A [^{55}Fe]hemin uptake assay was performed on OPY104 (*hem1*Δ) grown overnight in YPD medium with or without 250 μM 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*Δ) was pregrown in YPD medium supplemented with 250 μM ALA and then incubated overnight in YPD medium without ALA. Cells were incubated with or without 100 mM NaN_3 and 100 mM KF (inhibitors) for 25 min prior to the addition of [^{55}Fe]hemin. (D) Hemin uptake is nonsaturable. OPY104 (*hem1*Δ) was grown with or without ALA as described above, and the uptake of [^{55}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.

carrying either wild-type or deletion alleles of *FET3*, the ferroxidase component of the high-affinity ferrous transport complex (43). When grown on media lacking iron and either heme or heme precursors, the *hem1*Δ *fet3*Δ 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*Δ strain grew very slowly on iron-poor medium supplemented with hemin (Fig. 1A). Yet deletion of *HEM1* in the *fet3*Δ strain improved growth on iron-poor medium supplemented with hemin, suggesting that deletion of *HEM1* increased the uptake of hemin by the *fet3*Δ strain.

To test this observation further, we measured the uptake of [^{55}Fe]hemin by a *hem1*Δ 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}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*Δ strain, wild-type cells grown under reduced oxygen tension accumulated 5 times more [^{55}Fe]hemin than cells grown in room air. Thus, *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}Fe]hemin uptake by 50 to 67% (Fig. 1C). We examined the kinetics of heme uptake in the *hem1*Δ strain and found that the transport process was nonsaturable at concentrations as high as 50 μM (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*Δ 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.

TABLE 3. Multipass integral membrane proteins identified by microarray analysis[a]

Gene	Name	No. of TMD	Fold change in expression in: WT strain (hypoxia vs normoxia) Expt 1	WT strain (hypoxia vs normoxia) Expt 2	*hem1*Δ strain (without ALA vs with ALA) Expt 1	*hem1*Δ strain (without ALA vs with ALA) Expt 2	Function[b]
Transport							
Sterols							
YOR011W	*AUS1*	13	22.5	22.3	6.2	6.1	ABC transporter involved in uptake of sterols and anaerobic growth
YIL013C	*PDR11*	12	8.1	6.4	3.9	4.9	ABC transporter involved in uptake of sterols and MDR
Sugars							
YHR096C	*HXT5*	11	49.1	36.1	14.1	28.0	Hexose transporter
YJR160C	*MPH2*	12	2.3	4.0	3.3	2.5	Alpha-glucoside permease
YDL194W	*SNF3*	12	1.9	2.3	2.1	1.5	Plasma membrane glucose sensor that regulates glucose transport
YDL138W	*RGT2*	12	3.4	3.5	3.6	3.2	Plasma membrane glucose receptor, highly similar to Snf3p
YPL189W	*GUP2*	10	2.3	2.6	3.0	2.3	Membrane protein with a possible role in proton symport of glycerol
YFL054C		6	2.8	2.0	2.8	2.4	Similar to the glycerol channel-like protein Fps1p
Allantoin							
YAL067C	*SEO1*	12	21.1	23.2	8.8	8.8	MFS transporter, member of allantoin permease family
YJR152W	*DAL5*	12	8.5	8.2	9.9	5.8	MFS transporter, allantoin permease
YLL055W		10	4.2	4.5	5.9	6.3	Cysteine-specific MFS transporter, similar to Dal5p
Cations and anions							
YBR296C	*PHO89*	10	7.4	8.6	4.1	4.0	Na^+/P_i cotransporter
YCL038C	*PHO87*	12	3.3	3.8	1.9	1.2	Low-affinity inorganic phosphate transporter
YLR092W	*SUL2*	8	6.2	6.8	1.7	1.6	High-affinity sulfate permease
YNR002C	*ATO2*	5	8.0	9.4	3.2	4.1	Putative transmembrane protein involved in export of ammonia
YDR039C	*MEP1*	11	2.4	1.6	4.8	3.1	Ammonium permease
YBR295W	*IZH2*	7	5.4	5.4	1.9	1.6	Membrane protein involved in zinc metabolism
YLR023C	*IZH3*	7	2.0	2.4	2.2	2.2	Membrane protein involved in zinc metabolism
YOL002C	*IZH4*	7	3.9	3.7	2.0	1.9	Membrane protein involved in zinc metabolism
YGR121C	*ZRT1*	8	2.4	3.3	2.3	2.4	High-affinity zinc transporter of the plasma membrane
YOL101C	*FRE8*	5	2.7	3.7	5.5	3.9	Similar to iron/copper reductases
YBR295W	*PCA1*	7	5.9	7.4	6.9	6.5	P-type metal-transporting ATPase with a role in copper and iron homeostasis
YJL094C	*FET4*	7	2.0	2.4	2.7	2.1	Low-affinity Fe(II) transporter of the plasma membrane
YJL094C	*TRK2*	10	2.1	2.1	3.4	3.2	Component of the Trk1p-Trk2p potassium transport system
YJL094C	*KHA1*	12	2.0	2.4	3.3	4.4	Putative K^+/H^+ antiporter
YML132W	*COS3*	4	1.9	1.7	2.1	2.6	Salt resistance; enhances the antiporter function of Nha1p
YDR038C	*ENA5*	10	1.8	1.9	3.1	1.3	Protein with similarity to P-type ATPase sodium pumps
YCR037C	*CCH1*	22	3.1	3.5	4.4	3.7	Voltage-gated calcium channel involved in calcium influx
YGL006W	*PMC1*	9	2.9	2.7	1.7	2.8	Vacuolar Ca^{2+} ATPase involved in depleting cytosol of Ca^{2+} ions
Vitamins							
YOR306C	*MCH5*	12	5.2	7.2	2.3	2.0	Plasma membrane riboflavin transporter, similar to monocarboxylate permeases
YOL119C	*MCH4*	12	2.1	2.6	1.7	1.9	Similar to monocarboxylate permeases
YNL125C	*ESBP6*	11	2.9	3.2	2.0	2.0	Similar to monocarboxylate permeases; involved in transport of lactate, pyruvate, acetate
YPL221W	*FLC1*	11	2.5	3.0	2.2	1.8	Endoplasmic reticulum FAD transporter
Amino acids							
YFL055W	*AGP3*	12	3.3	2.6	3.1	1.8	Low-affinity amino acid permease
YPL274W	*SAM3*	11	2.8	3.2	1.7	2.6	High-affinity *S*-adenosylmethionine permease
YCL025C	*AGP1*	12	2.5	2.5	1.9	1.5	Low-affinity amino acid permease with broad substrate range
YMR088C	*VBA1*	12	2.1	2.4	2.5	2.5	Permease of basic amino acids in the vacuolar membrane

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TABLE 3—*Continued*

Gene	Name	No. of TMD	Fold change in expression in: WT strain (hypoxia vs normoxia) Expt 1	WT strain (hypoxia vs normoxia) Expt 2	*hem1*Δ strain (without ALA vs with ALA) Expt 1	*hem1*Δ strain (without ALA vs with ALA) Expt 2	Function[b]
YKR039W	*GAP1*	12	2.0	2.7	3.0	2.9	General amino acid permease
YDR160W	*SSY1*	12	2.2	2.3	1.9	1.7	Component of the SPS plasma membrane amino acid sensor system
Other substances							
YKL209C	*STE6*	10	3.0	3.3	3.3	2.9	ABC transporter required for the export of **a**-factor
YMR056C	*AAC1*	4	2.7	3.2	5.5	5.4	Mitochondrial inner membrane ADP/ATP translocator
YMR162C	*DNF3*	8	2.2	2.8	2.2	2.2	P-type ATPase; translocation of aminophospholipid within post-Golgi secretory vesicles
Resistance to xenobiotics							
YOR328W	PDR10	11	4.7	5.2	4.9	4.6	ABC membrane pump involved in pleiotropic drug resistance
YPR198W	*SGE1*	14	3.4	3.6	2.8	2.7	Membrane-associated multidrug transporter
YBR008C	*FLR1*	12	2.8	3.3	2.1	2.0	Plasma membrane multidrug transporter of MFS
YBR043C	*QDR3*	12	2.7	3.7	2.9	2.4	Plasma membrane multidrug transporter of MFS
YGR213c	*RTA1*	7	18.2	18.8	14.8	15.0	Protein involved in 7-aminocholesterol resistance
Other cellular processes							
YDL149W	*ATG9*	6	1.8	2.1	2.4	2.1	Formation of autophagic vesicles
YCL025C	*ATG22*	10	4.6	5.2	3.7	2.0	Breakdown of autophagic vesicles in the vacuole during autophagy
YKR053C	*YSR3*	5	46.6	49.3	13.5	12.5	Dihydrosphingosine 1-phosphate phosphatase
YGR032W	*GSC2*	14	15.8	12.5	3.4	5.3	Catalytic subunit of 1,3-β-glucan synthase
YOR034C	*AKR2*	7	9.3	11.0	6.1	5.1	Ankyrin repeat-containing protein similar to Akr1p
YNR041C	*COQ2*	5	3.9	3.9	2.7	2.6	*para*-Hydroxybenzoate:polyprenyl transferase
YCR048W	*ARE1*	9	3.9	4.4	2.3	2.2	Acyl-CoA:sterol acyltransferase isozyme of Are2p
YMR306W	*FKS3*	18	3.3	3.4	6.7	7.4	Similarity to 1,3-β-D-glucan synthase catalytic subunit Fks1p
YOR321W	*PMT3*	10	3.2	3.1	2.1	2.2	Protein *O*-mannosyltransferase
YOR303W	*CPA1*	3	2.8	3.2	2.4	2.4	Small subunit of carbamoyl phosphate synthetase
YJL142C	*YAK1*	3	2.8	3.8	6.5	5.2	Serine-threonine protein kinase
YLR450W	*HMG2*	7	2.4	2.8	2.1	1.8	One of two isozymes of HMG-CoA reductase
Q0065	*COX1*	6	2.2	1.0	2.5	4.6	Subunit I of cytochrome *c* oxidase
YOR030W	*DFG16*	6	2.0	1.9	2.0	1.5	Protein involved in invasive growth upon nitrogen starvation
YPL057C	*SUR1*	3	2.0	1.7	2.1	1.6	Probable catalytic subunit of a mannosylinositol phosphorylceramide synthase
YPL006W	*NCR1*	14	1.9	2.3	2.4	1.6	Vacuolar membrane protein, involved in sphingolipid metabolism
YGL126W	*SCS3*	5	1.9	2.0	1.8	1.6	Protein required for inositol prototrophy
YLR246W	*ERF2*	4	1.7	1.9	1.9	2.5	Subunit of a palmitoyltransferase
YNL279W	*PRM1*	3	2.1	2.8	2.4	2.9	Protein involved in membrane fusion during mating
Uncharacterized genes							
YGR131W		4	26.4	26.5	6.5	10.8	Putative membrane protein of unknown function
YER185W	*PUG1*	7	6.5	6.0	7.8	4.4	Putative protein of unknown function (porphyrin transporter [this study])
YLR046C		6	3.7	4.4	3.5	2.9	Putative membrane protein of unknown function
YMR034C		10	5.6	8.2	5.0	5.7	Putative membrane protein of unknown function
YJR116W		5	5.1	6.1	3.7	4.0	Putative protein of unknown function
YOL084W	*PHM7*	10	4.6	4.8	8.1	9.0	Protein of unknown function; expression is regulated by phosphate levels
YDR018C		4	4.4	6.0	3.8	3.7	Putative protein of unknown function
YBR054W	*YRO2*	7	3.4	3.3	3.5	4.1	Putative plasma membrane protein of unknown function
YBR054W		12	3.2	4.0	5.9	6.9	Putative protein of unknown function
YBR302C	*COS2*	4	2.6	1.3	1.9	2.5	Putative protein of unknown function
YJR161C	*COS5*	4	3.0	2.2	2.2	3.4	Putative protein of unknown function

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TABLE 3—*Continued*

Gene	Name	No. of TMD	Fold change in expression in: WT strain (hypoxia vs normoxia) Expt 1	WT strain (hypoxia vs normoxia) Expt 2	*hem1*Δ strain (without ALA vs with ALA) Expt 1	*hem1*Δ strain (without ALA vs with ALA) Expt 2	Function[b]
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	Dubious ORF
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
YDL222C	*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 *fet3*Δ 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 *fet3*Δ 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 hemin. We termed this gene *PUG1*, for protoporphyrin uptake gene 1.

PUG1 belongs to a gene family that includes *RTA1* (44% identical) and *RTM1* (62% identical). Overexpression of *RTA1* confers resistance to 7-aminocholesterol, a drug that leads to the production of toxic analogues of oxysterols in yeast (41). *RTM1* 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, *RSB1* (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 terminus 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 approximately 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.

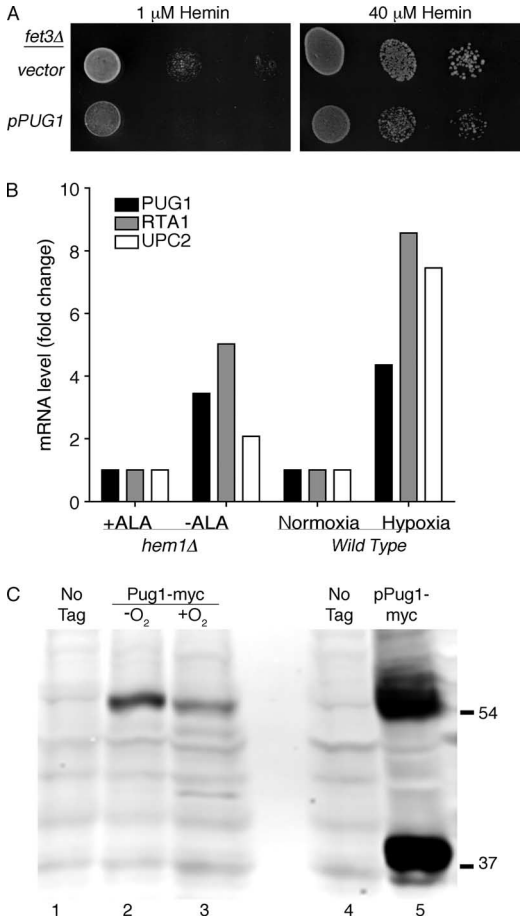

FIG. 2. Negative effect of *PUG1* overexpression on the utilization of hemin as an iron source and heme-dependent regulation of *PUG1* expression. (A) Impaired utilization of hemin as an iron source in a *fet3*Δ strain overexpressing Pug1p. CPY119 (*fet3*Δ) carrying the empty vector pYX242 or plasmid pPUG1-Leu, which expresses *PUG1* from the *TPI1* promoter, was grown overnight on iron-depleted SC medium supplemented with 1 mM ferrozine and then plated in serial dilutions on the same medium supplemented with 1 μM or 40 μM hemin. Plates were grown for 3 days at 30°C. (B) Increases in *PUG1* and *RTA1* mRNA levels under conditions of heme or oxygen deficiency. OPY104 (*hem1*Δ) was grown for 16 h with or without 250 μM ALA, and BY4742 (wild type) was grown in room air or under hypoxic conditions for 12 h. Cells were collected and washed, and RNA was isolated for microarray analysis. Aliquots of RNA were further analyzed by RT-PCR. Transcript levels were normalized to 18S RNA levels and were expressed relative to the transcript levels in cells grown in ALA (*hem1*Δ strain) or in normoxia (wild-type strain). (C) Induction of Pug1p expression by hypoxia. OPY103, which carries a genomic copy of *PUG1-myc* (lanes 2 and 3), and the untagged parent strain, YPH499 (lane 1), were grown overnight in YPD medium under hypoxic (lane 2) or normoxic conditions (lane 3). BY4742 was transformed with pYX212 (lane 4) or plasmid pPUG1-myc (lane 5) and grown overnight in SC medium. Pug1p-myc was detected by Western blotting with primary antibody 9E-10 and a Cy3-conjugated secondary antibody. Molecular mass standards (in kilodaltons) are indicated.

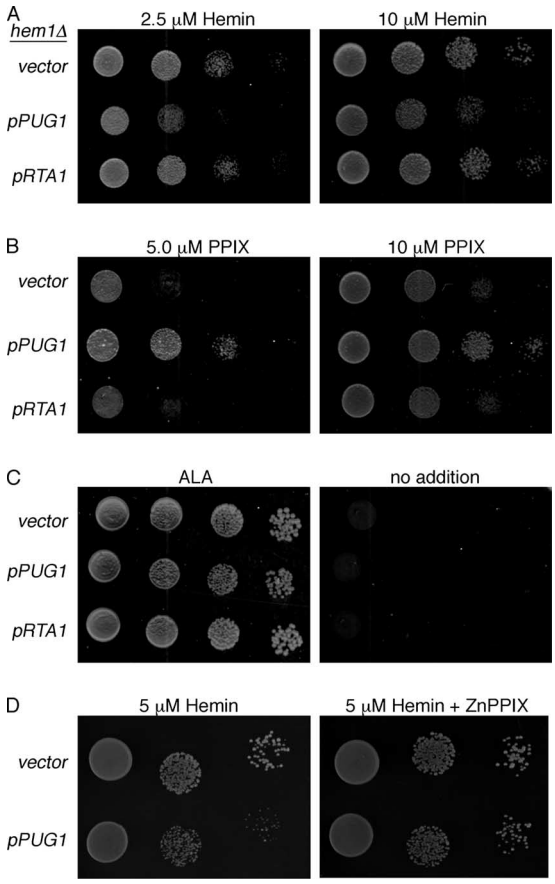

FIG. 3. (A to C) Opposing effects of *PUG1* overexpression on the utilization of hemin and PPIX. OPY101 (*hem1*Δ) was transformed with the empty vector pPUG1-Ura or with pRTA1. Strains were grown overnight in SC medium without ALA and were then plated in serial dilutions on medium supplemented with the indicated concentrations of hemin (A), PPIX (B), or ALA (C, left) or without supplementation (C, right). (D) Improved utilization of hemin in the presence of ZnPPIX in the *PUG1*-overexpressing strain. OPY101 (*hem1*Δ) was transformed with an empty vector or pPUG1-Ura, grown as described for panels A to C, and then plated in serial dilutions onto SC medium supplemented either with hemin alone or with hemin plus 25 μM ZnPPIX. Plates were grown for 3 days at 30°C.

Alteration of porphyrin utilization in Pug1p-overexpressing strains. We further tested the effects of Pug1p overexpression in the *hem1*Δ 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*Δ strain, overexpression of Pug1p in the *hem1*Δ 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*Δ 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*Δ 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*Δ strain on either hemin or PPIX, indicating that these effects were specific to Pug1p. Overexpression of these genes also had no effect

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 *hem1*Δ *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 *pug1*Δ strain. We then deleted *HEM1* in the *pug1*Δ *rta1*Δ strain, and we transformed the resulting *hem1*Δ *pug1*Δ *rta1*Δ 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 [^{55}Fe]hemin prior to washing and scintillation counting. The *hem1*Δ and *hem1*Δ *pug1*Δ *rta1*Δ strains transformed with empty plasmids showed similar rates of [^{55}Fe]hemin accumulation (Fig. 5), indicating that these genes are not required for heme uptake. However, the overexpression of *PUG1* in the *hem1*Δ *pug1*Δ *rta1*Δ strain led to a 33% inhibition of [^{55}Fe]hemin accumulation (Fig. 5). Although

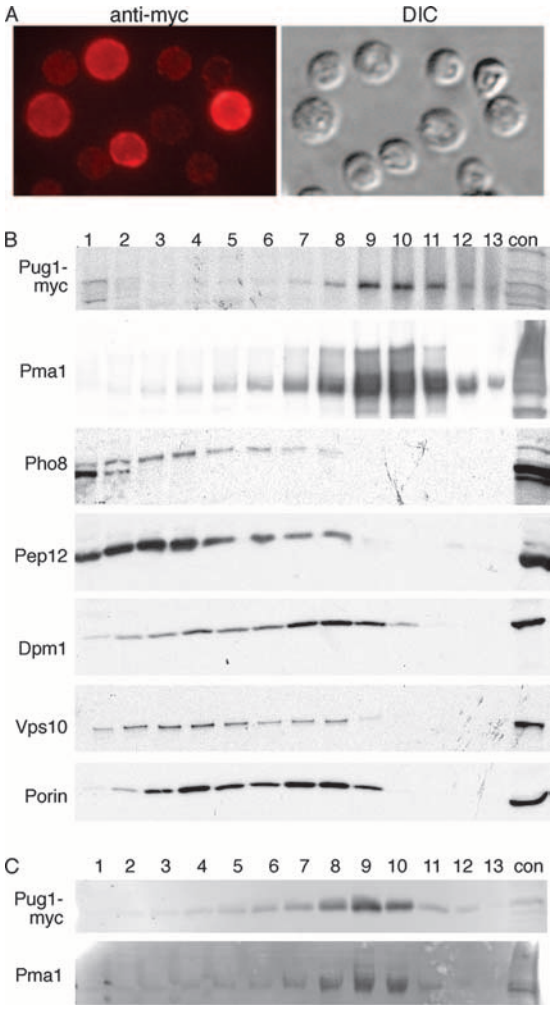

FIG. 4. Localization of Pug1p-myc to the plasma membrane. (A) Detection of Pug1p on the plasma membrane by immunofluorescence. OPY106 (*hem1*Δ) was transformed with pPUG1-myc, grown in medium supplemented with 20 μM ALA, and subjected to indirect immunofluorescence microscopy. 9E-10 was used as the primary antibody, and a Cy3-conjugated donkey anti-mouse antibody was used as the secondary antibody. (Left) Epifluorescence; (right) differential interference contrast (DIC). (B) Cosedimentation of Pug1p-myc with the plasma membrane protein Pma1p. OPY103, which carries a genomic copy of *PUG1-myc*, was grown overnight in YPD medium under hypoxic conditions to induce expression of Pug1p-myc. Cells were lysed with glass beads, and cell extracts were fractionated on 20-to-60% (wt/wt) sucrose gradients. The fractions were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting using 9E10 to detect Pug1p-myc and monoclonal antibodies directed against yeast proteins Pma1p (plasma membrane), Pho8p (vacuole), Pep12p (late endosomes), Dpm1p (endoplasmic reticulum), Vps10p (late Golgi complex), and porin (mitochondria). (C) Exclusive localization of Pug1p to the plasma membrane when overexpressed. OPY104 (*hem1*Δ) transformed with pPUG1-myc was grown as for panel A and subjected to subcellular fractionation and Western blotting as for panel B.

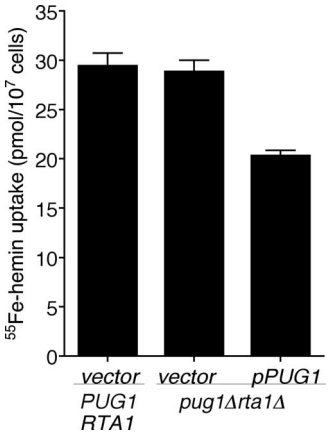

FIG. 5. Impaired [^{55}Fe]hemin uptake in a *PUG1*-overexpressing strain. Strains OPY106 (*hem1*Δ) and OPY107 (*hem1*Δ *pug1*Δ *rta1*Δ) were transformed with an empty vector or pPUG1-Ura. Strains were grown in SC medium supplemented with 250 μM ALA for 6 h and then incubated without ALA for 12 h. The [^{55}Fe]hemin uptake assay was performed as described above, with cells incubated in hemin for 60 min. Assays were performed in duplicate and repeated three times. Data from a representative experiment are shown.

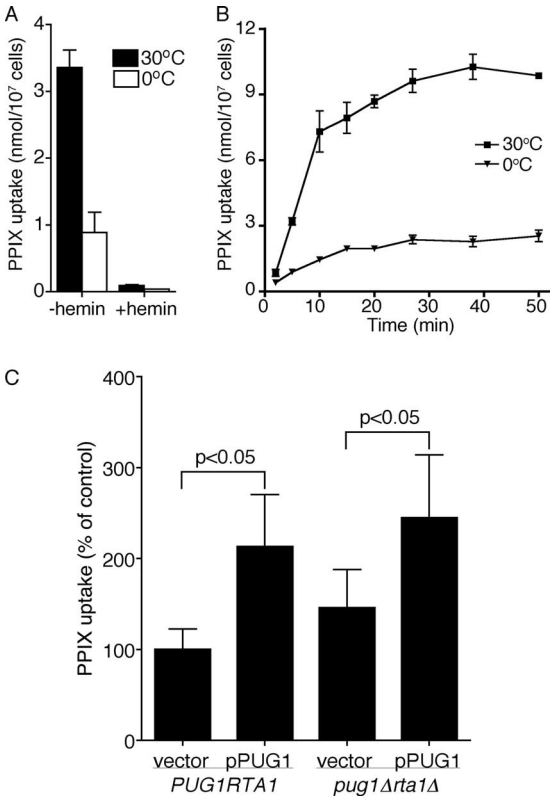

FIG. 6. PPIX uptake in strains overexpressing Pug1p. (A) Inducible PPIX uptake. OPY108 (*hem1*Δ *hem15*Δ) transformed with pYX212 was pregrown in medium supplemented with 10 μM hemin for 6 h. Cells were harvested, washed with SC medium containing 200 mM sodium arginate (pH 7.4), and incubated in the same medium with or without 10 μM hemin for 12 h. The PPIX uptake assay was performed as described above, with cells incubated in PPIX for 20 min. (B) Time course of PPIX uptake in a *hem1*Δ *hem15*Δ strain. OPY108 (*hem1*Δ *hem15*Δ) transformed with pYX212 was depleted of heme as described for panel A and then assayed for PPIX uptake. Experiments were repeated at least twice in duplicate; data from a representative experiment are shown. (C) Increased accumulation of PPIX in strains overexpressing Pug1p. OPY108 (*hem1*Δ *hem15*Δ) and OPY109 (*hem1*Δ *hem15*Δ Δ*pug1*Δ *rta1*Δ) were transformed with an empty vector or pPUG1-Ura. Strains were grown in SC medium supplemented with 10 μM hemin for 6 h and then incubated without hemin for 16 h. The assay for PPIX uptake was performed as described above, with cells incubated for 20 min. Experiments were performed 8 times, data from all experiments were pooled, and analysis of variance was performed using the Friedman test with Dunn's posttest using Prizm 4 (GraphPad). Error bars, standard errors. *P* values of <0.05 are indicated.

we could not reliably measure heme efflux in these strains, these data indicated that overexpression of Pug1p interfered with the intracellular accumulation of hemin.

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 *hem15*Δ strain, which lacks ferrochelatase activity and therefore does not convert intracellular PPIX into heme. Because the *hem15*Δ strain can accumulate endogenously produced fluorescent porphyrin intermediates, *HEM1* was also deleted in the *hem15*Δ strain to produce a *hem1*Δ *hem15*Δ strain, which synthesizes no porphyrin intermediates. The *hem1*Δ *hem15*Δ strain was grown in medium with or without hemin 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 hemin. 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 *hem1*Δ *hem15*Δ strain that expressed endogenous levels of Pug1p and Rta1p or in a *hem1*Δ *hem15*Δ 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 *hem1*Δ *hem15*Δ strain and the *hem1*Δ *hem15*Δ 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 *hem1*Δ 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 *hem1*Δ *fre1*Δ *fre2*Δ *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 *hem1*Δ *fre1*Δ *fre2*Δ *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

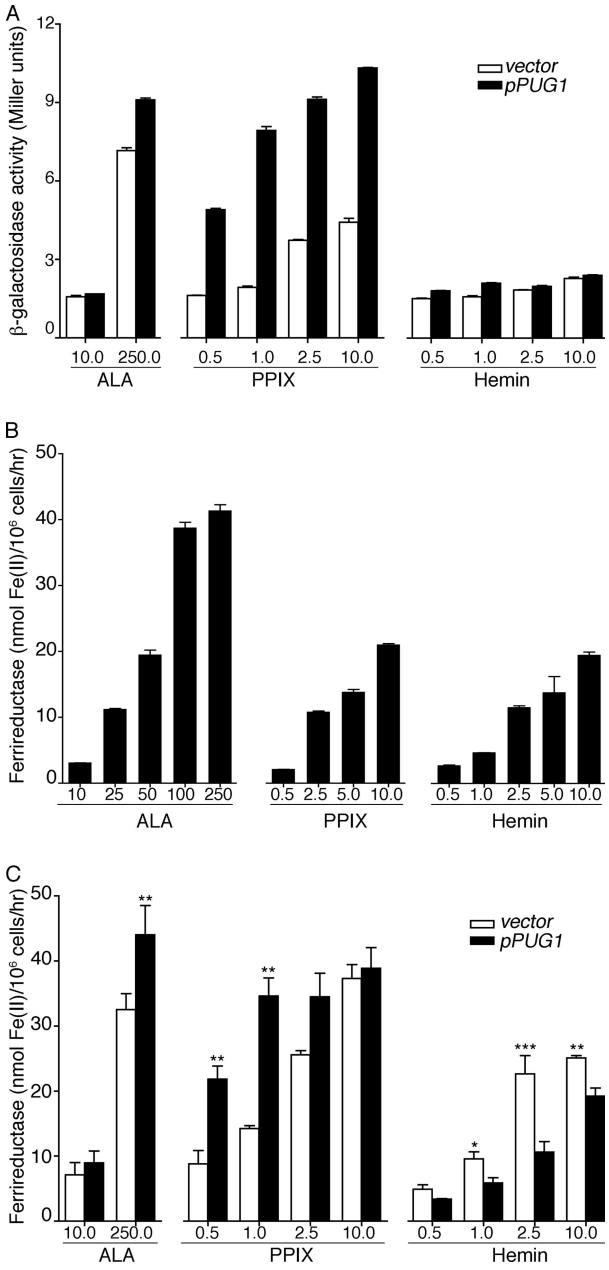

FIG. 7. Effects of Pug1p overexpression on internal utilization of exogenous hemin and PPIX. (A) Increased activation of *CYC1-lacZ* after growth in PPIX of a strain overexpressing Pug1p. OPY105 (*hem1*Δ) was transformed with pCYC1-LacZ and pYX242 or pPUG1-Leu. Strains were incubated in SC medium supplemented with 250 μM ALA for 6 h. Cells were washed twice with SC medium without ALA and inoculated for overnight growth in SC medium supplemented with the indicated concentrations of ALA, PPIX, or hemin. The experiment was repeated twice, and data from a representative experiment are shown. (B) Ferric reductase activity as an indicator of intracellular heme levels in the *PGK1-FRE1* strain. OPY102 (*hem1*Δ *fre1*Δ *fre2*Δ *PGK1-FRE1*) was incubated in SC medium supplemented with 100 μM ALA for 6 h. Cells were collected, washed, and grown in SC medium supplemented with the indicated concentrations of ALA, hemin, or PPIX for 16 h. Cells were collected and washed, and ferric reductase activity was measured. (C) Effects of Pug1p overexpression on the ferrireductase activity of the *PGK1-FRE1* strain. OPY102 (*hem1*Δ *fre1*Δ *fre2*Δ *PGK1-FRE1*) was transformed with the parent vector or pPUG1-Ura and grown as for panel B; then ferric reductase activity was measured. Experiments were performed 4 times, and data from all experiments were pooled. Variance was analyzed using the paired *t* test (Prizm 4; GraphPad). *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

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 extracellular 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-aminocholesterol, a drug that is a substrate for enzymes in the ergosterol biosynthetic pathway (41). Treatment of cells with 7-aminocholesterol 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-aminocholesterol. *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 dihydrosphingosine 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 hemin 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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