Phosphate Starvation in Fungi Induces the Replacement of Phosphatidylcholine with the Phosphorus-Free Betaine Lipid Diacylglyceryl-N,N,N-Trimethylhomoserine

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Diacylglyceryl-N,N,N-trimethylhomoserine (DGTS) is a phosphorus-free betaine-lipid analog of phosphatidylcholine (PtdCho) synthesized by many soil bacteria, algae, and nonvascular plants. Synthesis of DGTS and other phosphorus-free lipids in bacteria occurs in response to phosphorus (P) deprivation and results in the replacement of phospholipids by nonphosphorous lipids. The genes encoding DGTS biosynthetic enzymes have previously been identified and characterized in bacteria and the alga *Chlamydomonas reinhardtii*. We now report that many fungal genomes, including those of plant and animal pathogens, encode the enzymatic machinery for DGTS biosynthesis, and that fungi synthesize DGTS during P limitation. This finding demonstrates that replacement of phospholipids by nonphosphorous lipids is a strategy used in divergent eukaryotic lineages for the conservation of P under P-limiting conditions. Mutants of *Neurospora crassa* were used to show that DGTS synthase encoded by the *BTA1* gene is solely responsible for DGTS biosynthesis and is under the control of the fungal phosphorus deprivation regulon, mediated by the NUC-1/Pho4p transcription factor. Furthermore, we describe the rational reengineering of lipid metabolism in the yeast *Saccharomyces cerevisiae*, such that PtdCho is completely replaced by DGTS, and demonstrate that essential processes of membrane biogenesis and organelle assembly are functional and support growth in the engineered strain.

Phosphorus (P) in the form of inorganic phosphate (P_i) is a limiting nutrient in many environments, including freshwater ecosystems and certain soil types. As a consequence, many organisms have evolved biochemical strategies to cope with this condition, and the regulation of P_i starvation-responsive genes has been studied extensively, especially in the fungi *Neurospora crassa* (1–3) and *Saccharomyces cerevisiae* (4–6). Phospholipids constitute a major sink for cellular P_i and some groups of organisms have evolved the ability to replace phospholipids with nonphosphorous lipids when P_i is scarce (7). The shift to P-free lipids reduces the amount of P_i required for cellular homeostasis and growth, and phospholipid replacement has been observed in numerous P_i-limited bacteria and plants (8–12). For example, in seed plants such as *Arabidopsis*, phosphatidylcholine (PtdCho) is the major membrane lipid in organelles other than the chloroplast, but under P-limited growth conditions, a portion of this PtdCho can be replaced by the phosphate-free membrane glycolipid digalactosyldiacylglycerol (12). This tactic is taken a step further by the green alga *Chlamydomonas reinhardtii*, in which the P-free betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine (DGTS) (Fig. 1) is synthesized to the exclusion of PtdCho, regardless of P availability (13). *C. reinhardtii* and closely related species of the Volvocales algal lineage, such as *Volvox carteri*, lack PtdCho biosynthetic pathways (14, 15), and the apparent replacement of PtdCho with DGTS may reflect the intense selective pressure to optimize usage of P_i in the chronically P_i-limited freshwater environment in which these organisms live. Like PtdCho, DGTS is a quaternary amine-containing, zwitterionic lipid, and it has been demonstrated that the biophysical properties of these lipids are similar (16). However, while superficially similar in terms of net charge and steric bulk, the structure of DGTS is significantly different from that of PtdCho, in that the head group is attached to the diacylglycerol moiety via an ether linkage, which is much more chemically stable than the phosphodiester linkage of PtdCho. The ether linkage would also render DGTS impervious to phospholipases C and D, enzymes which are thought to be important for a number of fungal cell biological processes, such as sporulation and protein trafficking through the Golgi apparatus (17, 18).

The genes encoding the biochemical pathway for DGTS synthesis have been identified and characterized in the bacteria *Rhodobacter sphaeroides* and *Sinorhizobium meliloti* and the eukaryotic green alga *C. reinhardtii* (10, 14, 19, 20). The bacterial betaine lipid biosynthetic enzymes (BtaA and BtaB) (Fig. 1) are coexpressed from an operon under the control of the bacterial PHO regulon and function together in carrying out all steps of DGTS synthesis (10, 19, 20). The *C. reinhardtii* DGTS synthase protein, CrBTA1, consists of a single polypeptide containing BtaA- and BtaB-like domains, and likewise functions to carry out all steps in DGTS biosynthesis (14). Sequencing projects have revealed that the genomes of many fungal species, e.g., *Neurospora crassa* and *Kluyveromyces lactis*, encode apparent homologs of the *C. reinhardtii* BTA1 protein. We hypothesized that these uncharacterized fungal proteins would carry out the biochemical functions of the corresponding bacterial and *C. reinhardtii* proteins, as pre-
sentenced in Fig. 1. Our current report demonstrates several new findings relevant to this genomic information. (i) Fungi whose genomes encode a BTA1 homolog have the capacity to replace a significant portion of their membrane phospholipids with DGTS during P starvation. (ii) Accumulation of DGTS at the expense of phospholipids is under the control of the fungal PHO regulon, mediated by the transcriptional activator NUC-1 in Neurospora crassa, analogous to Pho4p in Saccharomyces and Candida spp. (iii) Saccharomyces cerevisiae lacks a BTA1 homolog, but expression of the BTA1 gene from K. lactis led to DGTS accumulation in this organism. (iv) The choline (Cho) auxotrophy of a pem1Δ pem2(opi3)Δ strain of S. cerevisiae was rescued by BTA1-mediated DGTS accumulation and produced a yeast strain capable of growth with no detectable PtdCho content. These findings reveal an unanticipated biochemical mechanism of fungal adaptation to P limitation and provide new evidence regarding the dispensable nature of PtdCho in fungal cell growth and membrane biogenesis.

**MATERIALS AND METHODS**

**Strains and growth conditions.** In initial phosphate starvation experiments presented in Fig. 2, N. crassa wild-type strain 74-OR 23-1A (FGSC 987) was grown in Vogel’s medium (21) in which phosphate was replaced with 20 mM morpholinenuethanesulfonic acid (MES), pH 6.0. Other experiments were conducted with N. crassa strains obtained from the Fungal Genetic Stock Center (22) as indicated for individual experiments. Phosphate was brought to the appropriate concentration with 1 M potassium phosphate, pH 6.0. Experiments involving phosphate starvation of mutant N. crassa strains were conducted with Bird medium (23), a modified Neurospora minimal medium based on Vogel’s medium that was altered to contain potassium phosphate at either 50 μM (P, starvation conditions) or 1 mM (P, replete). N. crassa mycelia were routinely grown by inoculating 50- to 200-ml liquid cultures with conidia scraped from plates or slant tubes. Liquid N. crassa cultures were incubated with shaking at 150 rpm at 30°C for 48 to 72 h. Mycelia were harvested by vacuum filtration on glass fiber filters. Wild-type strains of K. lactis (CBS2359) and S. cerevisiae (BY4742) were grown in Edinburgh minimal medium without phosphate (EMMP). EMMP is a P,-free minimal medium comparable to standard synthetic complete (SC) minimal medium, originally designed for 32P labeling of yeasts (24). Identical growth rates were observed for S. cerevisiae and K. lactis grown in either P,-replete EMMP medium or standard SC medium. K. lactis and S. cerevisiae cultures were harvested by centrifugation, and cell pellets were prepared for lipid analysis as described below.

**Expression of K. lactis BTA1 in S. cerevisiae.** The putative K. lactis BTA1 open reading frame (gene KLLA0F22198g, strain NRRL Y-1140; NCBI accession number XP_456071) was amplified from purified K. lactis genomic DNA with primers KIBTA1-Fw (5'-ATGAACCTCTACCATCCTC TCTTCAG-3') and KIBTA1-Rv (5'-ATTTATCCTCCAAAATCGAG AACCTTT-3') and cloned into pYES2.1-TOPO (Invitrogen) according to the manufacturer’s instructions. A clone with the insert in the correct orientation (pKJBTAT1) was selected by restriction mapping and subsequently sequenced, and a circularized pYES2.1 empty vector served as a control plasmid. Constructs were transformed into S. cerevisiae strain BY4741 (25) and isogenic pem1Δ pem2Δ strain CHO33 (26) by standard methods (27). Transformants were selected on SC-uracil (SC-Ura) containing 2 mM choline (Cho) chloride, and growth and lipid analysis experiments were conducted on cultures grown in SC-Ura containing either.
Betaine Lipid Biosynthesis in Fungi

RESULTS

Fungal genomes encode functional homologs of the *Chlamydomonas* BTA1 protein. We used the *CrBTA1* protein sequence to query proteomes predicted from fungal whole-genome sequences cataloged in GenBank. This approach identified BTA1 orthologs in many of these organisms, including human pathogens (*Candida* spp. and *Paracoccidioides brasiliensis*), plant pathogens (*Ustilago maydis*, *Gibberella zeae*, and *Magnaporthe oryzae*), industrially important fungi (*Pichia* spp. and *Penicillium chrysogenum*), and fungi that form mycorrhizal symbioses with plants (*Laccaria bicolor*). A multiple-sequence alignment of the previously characterized BTA1 protein of *C. reinhardtii* and the homologs from *K. lactis* and *N. crassa* is presented in Fig. S2 in the supplemental material, and it shows conservation of residues that had previously been implicated in binding AdoMet in the active site of the *BTA1* ortholog (*S. cerevisiae*). The fact that most other yeast and filamentous fungal genomes contain a *BTA1* ortholog is consistent with previous studies reporting the presence of DGTS in fungal field isolates (29). Our analysis shows that the capacity for DGTS biosynthesis is a widespread phenomenon in fungi, and that the lack of DGTS in many of the fungal field isolates reported previously (29) indicate that DGTS synthesis is repressed under growth conditions in which samples were collected.

Working from this premise, and mindful of the fact thatPi limitation induces DGTS synthesis in bacteria, we examined fungal BTA1 gene promoters for sequences associated with the Pi starvation response. As indicated in Fig. S1 in the supplemental material, we noted that *BTA1* promoters (1,000 bases immediately 5’ of the start codon) from model fungal species (*N. crassa* and *K. lactis*) contain multiple sequences that are identical or highly similar to Pi starvation response elements (5’-CAGGTG-3’) conserved among fungi (1, 30), suggesting that fungal *BTA1* genes are induced during Pi limitation by the transcription factor referred to as Pho4p in *S. cerevisiae* and NUC-1 in *N. crassa*. To test our hypothesis that Pi starvation leads to BTA1 induction and DGTS synthesis in these fungi, we grew wild-type laboratory isolates of

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2% (wt/vol) galactose or sodium lactate as a carbon source, with or without choline supplementation, as indicated in individual experiments.

**Lipid analysis.** Total lipids were prepared by ethanolic Bligh-Dyer extraction as previously described (28). As shown in Fig. 2, *N. crassa* lipids were separated by two-dimensional thin-layer chromatography (TLC) according to reference 8. In all other experiments, lipids were separated in one dimension on silica 60 TLC plates with the solvent system chloroform-methanol-acetic acid-water (100:50:16:5, by volume) or chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by volume). TLC plates in all experiments were visualized with I$_2$ vapor, and digital images were captured with a standard flatbed scanner. Lipid classes were quantified by image analysis of scanned I$_2$-stained TLC images using NIH ImageJ software.

**Microscopy.** Images were recorded on an AMG Evos fl epifluorescence microscope using a 100× oil immersion objective. Organelles were visualized with FM 4-64 (vacuole) and Nile Red (lipid droplets) with yeast strains as indicated in individual experiments. For FM 4-64 staining, cultures were grown to mid-log phase (optical density at 600 nm [OD$_{600}$] 0.4 to 0.8) in SC-Ura medium containing 2% (wt/vol) galactose as a carbon and energy source. Cells were harvested by centrifugation and suspended in fresh medium at an OD$_{600}$ of 2.0. FM 4-64 was added to a final concentration of 1.6 μM from a 1.6 mM (1 mg/ml) dimethylsulfoxide (DMSO) stock solution, and cells were incubated with shaking at 30 degrees for 30, 60, or 120 min. Nile Red for was added at a final concentration of 1 μg/ml from a 10 mg/ml stock in DMSO. Cells in late log/early stationary phase (OD$_{600}$ 1.6 to 1.9) were incubated with Nile Red for 15 min at room temperature prior to imaging. FM 4-64 images were acquired with an Evos red fluorescent protein (RFP) light cube, and Nile Red images were acquired with an Evos green fluorescent protein (GFP) light cube. Overlay images were compiled from monochromatic images using Adobe Photoshop.

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**FIG 2** Pi starvation induces DGTS accumulation in fungi. (A and B) *N. crassa* was grown in minimal medium containing the indicated amounts of Pi, and then harvested for lipid analysis. Two-dimensional TLC of Pi-replete (A) and Pi-limited (B) *N. crassa* was performed as described in the text, and lipids were visualized with I$_2$ vapor. Lipid spots are numbered as follows, in reference to authentic standards: 1, PtdIns; 2, PtdSer; 3, PtdCho; 4, PtdEtn; 5, cardiolipin; 6, DGTS. (C) *K. lactis* cultures were grown to saturation in minimal medium with the indicated initial concentration of Pi, and lipids were extracted, separated by standard TLC, and visualized as described in the text. Authentic standard compounds were purchased from Avanti Polar Lipids (Alabaster, AL, USA) or, in the case of DGTS, purified from *Chlamydomonas reinhardtii* lipid extracts.
N. crassa, K. lactis, and S. cerevisiae in defined media with various amounts of P<sub>r</sub>

As shown in Fig. 2, DGTS synthesis in N. crassa and K. lactis was repressed in P<sub>r</sub>-replete conditions and induced in P<sub>r</sub>-starved conditions. Accumulation of DGTS was verified by cochromatography with authentic DGTS from C. reinhardtii, and fast-atom bombardment mass spectrometry of a purified N. crassa DGTS gave peaks at m/z 734.6 and 736.6, consistent with DGTS-containing fatty acids totaling (carbons:double bonds) 34:2 and 34:3, typical of N. crassa diacylglycerol moieties (31). DGTS accumulation was concomitant with PtdCho depletion in N. crassa, and the threshold initial [P<sub>r</sub>] under which DGTS synthesis was induced was between 50 and 400 μM. K. lactis (Fig. 2C) also accumulated DGTS at the expense of phospholipids when the initial [P<sub>r</sub>] was below a certain threshold (between 150 μM and 1 mM). Consistent with the absence of a BTA1 homolog in its genome, S. cerevisiae did not accumulate DGTS upon P<sub>r</sub> starvation (data not shown).

**Functional Bta-1 and Nuc-1 genes are required for DGTS synthesis in N. crassa.** Studies by Metzenberg and coworkers led to the identification of the N. crassa transcription factor Nuc-1 as the principal transcriptional activator of P<sub>r</sub> starvation-induced genes, such as those encoding extracellular phosphatases and high-affinity phosphate transporters (3). To test the hypothesis that DGTS synthesis in this fungus is mediated by the Bta-1 gene as part of the NUC-1-activated PHO regulon, we analyzed N. crassa knockout strains (32, 33) bta-1Δ (FGSC 16330) and nuc-1Δ (FGSC 11448) for their ability to accumulate DGTS under conditions of P<sub>r</sub> limitation. Figure 3 provides compelling evidence that DGTS synthesis in N. crassa is under the control of the PHO regulon, as the mutant fungal strains lacking either the BTA-1 gene or its putative primary regulator, NUC-1, both showed loss of DGTS accumulation. This finding provides additional evidence that replacement of phospholipids with DGTS represents a previously unappreciated biochemical mechanism by which fungi can reduce the quantity of P<sub>r</sub> needed for membrane biogenesis, presumably reducing the total amount of P<sub>r</sub> needed for growth.

**Saccharomyces cerevisiae tolerates the complete replacement of PtdCho by DGTS.** The apparent replacement of PtdCho by DGTS in P<sub>r</sub>-starved fungi raises questions about the general function of PtdCho in membranes of these organisms. PtdCho is regarded as a largely structural component of eukaryotic membranes, and even though PtdCho and its partially N-methylated precursors are known to be dispensable in yeast under certain circumstances (34), the regulated synthesis and turnover of PtdCho is generally regarded as critical to certain essential cellular processes, such as protein secretion through the Golgi apparatus (18, 35). Given that a small amount of PtdCho remains in these P<sub>r</sub>-starved fungi, even in circumstances where P<sub>r</sub> availability is the only limit to growth, we conducted an experiment to determine whether DGTS could support all of the essential functions of PtdCho in fungal cells using S. cerevisiae, which is normally incapable of synthesizing this lipid. To test this hypothesis, we amplified the K. lactis BTA1 gene (KIBTA1) and cloned it into a yeast expression vector (pYES2.1-TOPO; Invitrogen) under the control of a galactose-inducible promoter (plasmid pKIBTA1). pKIBTA1 and an empty pYES2.1 control vector were transformed into a pem1Δ pem2Δ yeast mutant (strain CH033) (26). The pem1Δ pem2Δ strain (also referred to as cho2Δ opi3Δ, reflecting the respective standard names of these loci) lacks the methyltransferases needed for conversion of phosphatidylethanolamine (PtdEtn) to PtdCho, and this strain is dependent on uptake and metabolism of either choline (Cho) or lyso-PtdCho from the growth medium to support PtdCho synthesis (34, 36).

The pem1Δ pem2Δ strain containing either pKIBTA1 or pYES2.1 was grown to saturation in SC glucose medium lacking Ura but containing Cho (−Ura +Cho), and these cultures were diluted 1:50 into SC galactose −Ura −Cho and incubated for 6 to 8 h to deplete PtdCho. These cultures were then used to inoculate SC galactose −Ura medium with or without 2 mM Cho at an initial A<sub>600</sub> of 0.05. The Cho-containing cultures reached a final A<sub>600</sub> of ~2.2, as expected, while the vector control culture lacking Cho failed to grow. As shown in Fig. 4 and 5, the pKIBTA1 plasmid rescued growth of the pem1Δ pem2Δ strain in the absence of Cho, demonstrating that DGTS can substitute for PtdCho as the major constituent of yeast membranes and can support all of the essential functions of PtdCho in other cellular processes. To investigate this observation further and confirm the complete replacement of PtdCho by DGTS in this strain, we extracted and analyzed lipids from these cultures as described above (Fig. 4B). The Cho-supplemented, vector-bearing control strain revealed a lipid profile typical of S. cerevisiae, while strains bearing pKIBTA1 contained DGTS, showing that expression of the K. lactis BTA1 gene is sufficient to direct the total synthesis of this lipid. When grown in the absence of Cho, pem1Δ pem2Δ pKIBTA1 completely lacked PtdCho, as judged by the absence of an iodine-positive spot or detectable lipid phosphate at the expected R<sub>r</sub>. This DGTS/PtdCho

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**Figure 3** DGTS accumulation in N. crassa is dependent on the BTA-1 structural gene and P starvation transcription factor NUC-1. P-replete (A) and -limited (B) liquid cultures of strains of the indicated genotype were prepared as described in the text, and lipids were prepared and analyzed by TLC and I<sub>2</sub> vapor staining.
replacement strain can grow indefinitely in the absence of Cho (at least 50 doublings), strongly suggesting that any residual traces of PtdCho, i.e., below the sensitivity of our analytical detection methods, are not present and not involved in maintaining the growth of this strain.

**DGTS supports mitochondrial function, endocytosis, and neutral lipid synthesis in PtdCho-deficient, DGTS-accumulating S. cerevisiae.** Given the novel finding that DGTS biosynthesis could support cell growth in the absence of PtdCho synthesis, we chose to examine several processes for which membrane biogenesis or PtdCho biosynthesis is thought to be critical. Figure 5 shows representative growth curves for liquid cultures prepared as described above, using SC − Ura medium with either galactose or sodium lactate (2% [wt/vol] each) as carbon and energy sources. Consistent with the results shown in Fig. 4A, which shows the 40-h growth time point, Fig. 5A shows that the pem1Δ pem2Δ pKlBTA1 strain was able to grow in the absence of Cho supplementation at a rate nearly equivalent to that of the Cho-supplemented culture, albeit with a slight lag. This is in sharp contrast to the pem1Δ pem2Δ strain lacking KIBTA1, which fails to grow without choline supplementation. The same pattern was observed in Fig. 5B, in which galactose was replaced with lactate, which is a nonfermentable carbon source that is not expected to repress Pgal or respiratory capacity under the conditions tested.

To further characterize cellular functions that might be altered in the PtdCho-deficient, DGTS-accumulating strain, we used FM 4-64 and Nile Red as fluorescent markers of endocytosis and lipid
These results are presented in Fig. 6 and 7. Figures 6A to C show Nile Red-stained lipid droplets in PtdCho-containing, early-stationary-phase cells. Lipid droplets in these cells are typical of those previously reported for *S. cerevisiae* and appear as distinct punctate structures in the cytoplasm. In contrast, many of the PtdCho-deficient, DGTS-accumulating cells (typically >75% of a given field of cells) appear to contain lipid droplets that have coalesced into one or two larger, intensely fluorescent structures, perhaps indicating that while neutral lipid accumulation is not defective in this strain, the replacement of PtdCho with DGTS affects the morphology or assembly of lipid droplets.

Using cultures of cells with the same genotypes as those presented in Fig. 6, Fig. 7 shows the results of staining cells with FM 4-64 after a 2-h incubation with the compound, a styrene dye commonly used to assess the function of endocytic vesicle traffick-
ing to the vacuole (38). Figure 6A shows the staining pattern of wild-type cells bearing an empty expression vector, and as expected, FM 4-64 was localized to structures consistent with the limiting membrane of the vacuole. Likewise, cells which contain both PtdCho and DGTS (Fig. 6B and C) showed apparently normal staining patterns, indicating that the presence of DGTS did not interfere with retrograde trafficking of endosomes to the vacuole. Lastly, Fig. 6D presents the FM 4-64 staining pattern of the PtdCho-deficient, DGTS-accumulating pem1Δ pem2Δ pKIBTA1 strain in the absence of Cho. Again, the accumulation of the dye in vacuolar membranes is consistent with a functional endosomal trafficking pathway, at least under the conditions in which this strain was grown.

To investigate subtle alterations in the kinetics of FM 4-64 trafficking to the vacuole, we observed BY4741 pYES (wild-type) and pem1Δ pem2Δ pKIBTA1 cells (DGTS replacement strain) in the absence of choline supplementation for 30, 60, and 120 min (see Fig. S3 in the supplemental material). No alteration in fluorescence intensity of the vacuolar membrane was observed at the early time points, indicating that the rate of endosomal transport to the vacuole was not suppressed by an appreciable amount in the DGTS replacement strain. However, we do note the slightly fragmented nature of vacuoles in this strain, in which ~40 to 60% of the cells appear to have 2 to 3 smaller vacuoles, as opposed to cells of the wild-type strain, in which this phenomenon was less common (<25%). We also note what appear to be small punctate structures attached to the surface of the vacuole in ~50% of the cells of the DGTS replacement strain. These may represent vesicles that are in the process of fusing with the vacuole, and the presence of these putative fusion intermediates, along with the slightly fragmented vacuolar phenotype, indicates a subtle defect in vacuolar fusion or morphology, perhaps stemming from the replacement of PtdCho by DGTS.

**DISCUSSION**

The data provided in our current report offer novel insights of broad importance to the fields of ecophysiology and membrane biology. First, we have demonstrated that many fungi are capable of replacing the bulk of their phospholipids, especially the major membrane lipid PtdCho, with DGTS as part of a broader P starvation acquisition and conservation response to P starvation. Given that many plant species form symbiotic relationships with mycorrhizal fungi as a way of acquiring scarce P, our discovery of fungal phospholipid replacement during phosphate starvation adds a new detail to our understanding of the way these fungi regulate P homeostasis. That is, by reducing the total P necessary for fungal membrane biogenesis, DGTS biosynthesis may render more of this critical mineral nutrient available for transport to the plant, potentially enhancing plant productivity on nutrient-poor soils.

The molecular determinants of P starvation-responsive DGTS biosynthesis are also present in plant- and animal-pathogenic fungi, such as *Magnaporthe oryzae*, a fungal pathogen of rice, and *Candida* spp., which include many species that are important opportunistic pathogens of humans and other mammals. Recent work on virulence in *Candida albicans* has implicated P starvation as a critical regulator of hyphal morphogenesis in the nutrient-poor environment of the critically ill gut, inducing this organism to switch from the yeast-like, avirulent commensal form typical of the gut of healthy individuals to a virulent, invasive, hyphal form (40). These investigators characterized a number of clinical isolates of *C. albicans* cultured from the stool of critically ill patients and demonstrated that the most virulent of these isolates could be induced to assume an invasive hyphal growth phenotype upon phosphate limitation, a nutrient stress which is characteristic of the gut of fasted, critically ill patients. This finding highlights the importance of P availability to fungal cell biology in the context of disease and suggests that biochemical mechanisms, such as phospholipid replacement, are clinically relevant determinants of virulence in this organism.

*C. albicans* can grow and be propagated as epigenetically distinct white- or opaque-phase cells, and an additional recent study (41) showed that opaque-phase *C. albicans* colonies can also assume a hyphal form under conditions of P limitation, unlike white-phase cells, which (at least in the canonical laboratory isolates commonly used for physiological studies, such as strain SC5314) are not typically induced to express a hyphal phenotype with P starvation as the sole nutrient limitation. The observed induction of hyphal morphogenesis by P starvation in *C. albicans* opaque cells (but not white cells) may also be related to previous work on gene expression patterns in white versus opaque cells. Gene expression data from Tuch et al. (42) showed that genes associated with the fungal PHO regulon are induced between 5- and 50-fold in opaque-phase cells, even in the P-replete growth conditions employed in that study. The putative *C. albicans* PHO genes with high expression in opaque cells include those typically associated with enhanced P scavenging activity in starved cells, including the Pho5 phosphatase, Pho84 high-affinity phosphate transporter, and, importantly, the ORF 19.1171, which is directly adjacent to Pho84 on chromosome 1 and encodes the *C. albicans* Bta1 homolog. This differential gene expression data in white- and opaque-phase *C. albicans* suggests that even in the absence of a P starvation signal, opaque-phase cells are poised to encounter this stress. We have also observed that the response to P limitation in *C. albicans* involves the replacement of a significant portion of PtdCho and other phospholipids with DGTS (43). Naik and Riekhof (unpublished data), similar to the phenomenon we have reported in this work using *N. crassa* and *K. lactis* as model systems.

In addition to the discovery of this previously unappreciated biochemical component of the fungal adaptation to P limitation, this work led us to ask whether DGTS could completely replace PtdCho in an organism which does not have this capacity due to the lack of a native BTA1 homolog. A pem1Δ pem2Δ strain of *S. cerevisiae* provided us with a convenient platform upon which to answer this question. Expression of the BTA1 homolog from the yeast *Kluyveromyces lactis* led to DGTS formation in *S. cerevisiae* and allowed the pem1Δ pem2Δ strain to grow in the absence of Cho, with undetectable levels of PtdCho. This result demonstrates that PtdCho per se is not required for any essential cellular functions, and this rationally engineered strain will prove to be a valuable tool for further investigation of the role of PtdCho in processes such as vesicle trafficking and secretion, organelle biogenesis, and the regulation of membrane homeostasis.

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