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DGTS Production as a Phosphate Starvation Response in the Human Fungal Pathogen *Candida albicans*

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DGTS PRODUCTION AS A PHOSPHATE STARVATION RESPONSE IN THE HUMAN FUNGAL PATHOGEN *CANDIDA ALBICANS*

by

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Abstract

Betaine lipids are a class of membrane lipids with betaine head groups. Three betaine lipids are known - diacylglyceryltrimethylhomoserine (DGTS), diacylglycerylhydroxymethylalanine (DGTA), and diacylglycerylcarboxymethylcholine (DGCC). Betaine lipids are most common in algae, although DGTS, the most common betaine lipid, is also found in many bacteria and fungi. Organisms which produce betaine lipids (especially DGTS) often don't produce phosphatidylcholine (PtdCho), and DGTS structure resembles PtdCho structure without any phosphorous, leading to the hypothesis that betaine lipids may substitute for phospholipids in some organisms. This has been confirmed by discoveries that some organisms are capable of switching their membrane composition from PtdCho to DGTS in response to low environmental phosphate (P_i). Further work identifying the metabolic pathway of DGTS biosynthesis has demonstrated that DGTS biosynthesis is under control of P_i starvation response mechanisms (the PHO regulon) in bacteria and fungi. We therefore examined this response in the human fungal pathogen *Candida albicans*. Previous work in our lab showed that *C. albicans* encodes a DGTS biosynthesis (*BTA1*) ortholog (*CaBTA1*), and we here demonstrate that it is under control of the C. albicans PHO regulon and is thereby activated during P_i starvation. CRISPR/Cas9 deletion of *BTA1* resulted in a lack of DGTS

production and a significant decrease in overall growth in low-P_i conditions. Our work also suggests crosstalk between P_i starvation mechanisms, nitrogen sequestration, amino acid metabolism, and metal homeostasis. Our transcriptional data showed that *BTA1* is induced in biofilm-promoting conditions regardless of P_i concentration. However, our results revealed no significant or consistent differences between wild type and *bta1*ΔΔ mutant biofilm formation or hyphal growth. Nevertheless, because *C. albicans* experiences P_i starvation in clinically relevant settings which can induce virulence (Romanowski et al., 2012) and because *BTA1* deletion significantly impairs growth in low P_i conditions, DGTS production is likely an important tool for survival and infection in low P_i infection scenarios.

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Introduction

Betaine lipids are a class of polar glycerolipids with distinctive ether-linked betaine head groups. Betaines are a subset of zwitterions that contain a quaternary cation, although the term 'betaine' often refers to trimethylglycine, the first discovered betaine (isolated from sugar beets *Beta vulgaris*). The quaternary cation of betaines is often an ammonium cation and the anion is often a carboxyl group, so betaines are usually derived from amino acids. To date, 3 betaine membrane lipids have been discovered: diacylglyceryltrimethylhomoserine (DGTS), diacylglycerylhydroxymethyltrimethylalanine (DGTA), and diacylglycerylcarboxymethylcholine (DGCC) (Fig 1).

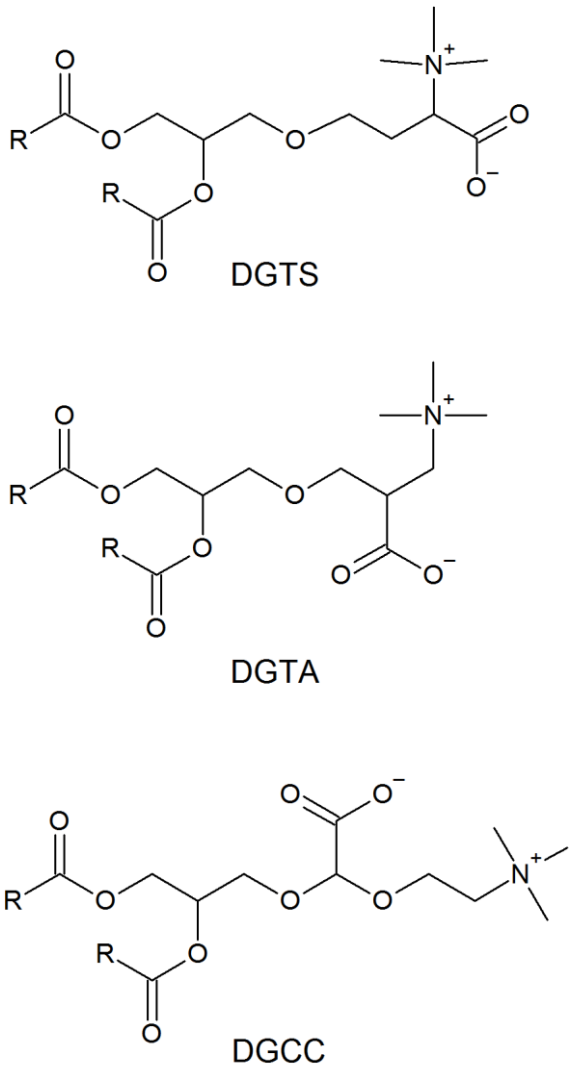

Figure 1.1 – Betaine Lipid Structures

Betaine Lipid Distribution

Betaine lipids were first discovered in algae. The first betaine lipid discovered was DGTS in 1969, when Nichols and Appleby were conducting a study of algal arachidonic acid and isolated several previously unidentified lipids from the golden alga *Ochromonas danica* (Nichols & Appleby, 1969). Several years later, one of these lipids was determined to be DGTS (Brown & Elovson, 1974); but it was not until 1990 that another of them was identified as DGTA (Vogel et al., 1990). Surveys were then conducted on many algal communities to try to determine the prevalence and distribution of these betaine lipids (Araki, Eichenberger, Sakurai, & Sato, 1991; Eichenberger, 1982; Eichenberger, Araki, & Müller, 1993; Kato, Sakai, Adachi, Ikemoto, & Sano, 1996; Sato & Furuya, 1985). The first review (Sato, 1992) of betaine lipids reported their distribution in many algae, some (lower) plants, a few fungi, and a protozoan. Many green algae (chrysophytes) and lower plants (pteridophytes and bryophytes) were reported to contain DGTS, and several brown algae (phaeophytes) contain DGTA. The third betaine lipid (DGCC) was discovered in the alga *Pavlova lutheri* (Kato et al., 1994). A second review of betaine lipids reported their distribution in even more algae, lower plants, and fungi, and noted that, like DGTA, DGCC seems to be restricted to certain algae (chromophytes). Therefore, while DGTS could be found in many algae, lower plants, fungi, and a protozoan, DGTA and DGCC were only found in chromists (DGCC only in chromophytes) (Dembitsky, 1996). A wide variety of fungi produce DGTS (Istokovics et al., 1998; Kunzler & Eichenberger, 1997; Riekhof, Naik, Bertrand, Benning, & Voelker, 2014; Senik et al., 2015), and one fungus (the pathogen *Cryptococcus neoformans*) has recently been shown to produce DGTA (Lev et al., 2019). Betaine lipids were first identified in bacteria in 1995 in *Rhodobacter sphaeroides* (Benning, Huang, & Gage, 1995) and appear to be largely limited to α-proteobacteria (Geiger, González-Silva, López-Lara, &

Sohlenkamp, 2010). Interestingly, polar lipid profiles in the North Pacific reveal that betaine lipids are found in similar abundance in both oxygenic and anoxygenic water depths, suggesting that betaine lipids are important for anoxygenic water environments (dominated by bacteria and archaea) as well as oxygenic water environments (dominated by algae and cyanobacteria) (Schubotz, Xie, Lipp, Hinrichs, & Wakeham, 2018). Aerobic anoxygenic phototrophic bacteria, made of alphaproteobacteria (known to produce betaine lipids) and gammaproteobacteria have been shown to comprise up to 24% of ocean communities (Lami et al., 2007). Only DGTS has been discovered in bacteria. To our knowledge, no betaine lipids have been detected in seed-bearing plants (angiosperms and gymnosperms) or metazoa.

Early surveys of algal betaine lipid content noted that some groups of algae produced betaine lipids to the exclusion of PC, and vice versa (Araki et al., 1991; Brown & Elovson, 1974; Dembitsky, 1996; Eichenberger, 1982; Sato, 1992; Sato & Furuya, 1985). This suggested a dichotomy between PC and DGTS which was perhaps evidence of DGTS as an ancestral membrane lipid gradually replaced by PC (Sato, 1992), and that the ability to produce betaine lipids might present a useful phylogenetic tool (Dembitsky, 1996). However, other groups of algae seemed to be less homogenous, with some members producing both DGTS and PC (Eichenberger et al., 1993; Kunzler & Eichenberger, 1997). An instructive example of both highly phylogenetic and highly variable betaine lipid distributions can be found in a survey conducted by Kato and colleagues (Kato et al., 1996). They found that of 16 haptophyte species analyzed, all of them produced DGCC (and a few produced DGTA) and only 2 species produced PC (in very small amounts); conversely, 6 rhodophytes surveyed only produced PC – a clear dichotomy between betaine lipids and PC. However, other groups such as chlorophytes and dinophytes produced both betaine lipids and (high amounts of) PC. Betaine lipids and PC seem

to occur together even more often in fungi (Kunzler & Eichenberger, 1997). So while betaine lipid distribution clearly follows phylogenetic patterns and correlates to PC absence in many scenarios, in other cases their presence in groups is highly variable and betaine lipids and PC may be found together.

Betaine Lipid Functions

Lipid Metabolism

In algae, which often produce DGTS constitutively, metabolic roles of betaine lipids have been identified. In the alga *Ochromonas danica*, extracellular radiolabeled oleate (18-C FA) was initially incorporated into DGTS, but after several hours was primarily found in other lipid classes. DGTS almost always contained 18:1 or 18:2 FA, and in the primary 18-C acceptor, phosphatidylethanolamine (PE), most radioactivity was detected in 18:2 FA. This suggests that DGTS functions as an acceptor for oleate (one of the most common FAs found in nature) where it is desaturated and then transferred to other lipid groups such as PE (Vogel & Eichenberger, 1992). A similar experiment using radiolabeled oleate in *Pavlovi lutheri* demonstrated that *P. lutheri* DGCC accomplishes similar functions as *O. Danica* DGTS. DGCC initially showed high signal (indicating oleate incorporation) followed by a drop off of signal in DGCC and an increase in signal of other lipid classes, especially MGDG (a chloroplast-specific lipid). Additionally, using radiolabeled FA biosynthesis precursors (e.g., acetate) so that only *de novo*-synthesized FAs are radiolabeled, the authors showed that DGCC also acts as an initial acceptor for *de novo* fatty acids. Together, these results suggest that DGCC acts as an acceptor for both exogenous and endogenous fatty acids, and aids in the transport of fatty acids from the cell

membrane to the chloroplast (Eichenberger & Gribi, 1997). The microalga *Phaeodactylum tricornutum*, like many algae and plants, accumulates the FA storage molecule triacylglycerol (TAG). By compiling data on fatty acid profiles of lipid classes and data on lipid degradation during nitrogen starvation, it appears that DGTS is the major FA source for TAG (Popko et al., 2016). Overall, it appears that betaine lipids can function as fatty acid transport or storage molecules in some algal species.

Temperature Adaptation

There is some evidence to suggest that, in some organisms, betaine lipids tend to be produced in higher amounts in lower temperatures. Betaine lipids showed the highest mole percentage increase of any lipid class in *P. lutheri* grown at 15°C (vs 25°C). Polyunsaturated fatty acids (PUFAs) 20:5 and 22:6 also increased at 15°C, the proportion of these PUFAs in betaine lipids increased (Tatsuzawa & Takizawa, 1995). Deletion of DGTS synthases in *Nannochloropsis oceanica* reduces growth at low temperatures (15°C) in addition to reducing growth in low Pi. In this organism, DGTS (as well as MGDG) contains a high amount of eicosapentaenoic acid (EPA) (20:5), suggesting that DGTS and MGDG may act as EPA carriers in membranes, and are thereby important for cold tolerance. Their relatively large head group may also be important for maintaining membrane fluidity (Murakami, Nobusawa, Hori, Shimojima, & Ohta, 2018). So while it seems clear that betaine lipids are important for survival in cold temperatures in this organism, it is unclear whether betaine lipids have any intrinsic cold-tolerance benefits (specific to their betaine head groups) or whether their benefit derives from their roles as carriers for PUFAs.

Low Phosphate Adaptation

The zwitterionic, phosphate (P_i)-lacking head groups of betaine lipids are crucial for their role in biological membranes. Many early studies of betaine lipid distribution noted both the frequent betaine lipid/phospholipid dichotomy in many organisms (see above), as well as the similarity between betaine lipid and phospholipid structures (particularly between DGTS and phosphatidylcholine (PC) (Fig 2)). In line with this, the phase transition temperatures (T_m) of DGTS and PC are similar but slightly (6°C) higher in DGTS (Naoki Sato & Norio Murata, 1991). Furthermore, the yeast *Saccharomyces cerevisiae*, which normally produces PC and not DGTS, can be genetically engineered to produce DGTS and no PC and is still able to accomplish basic cell functions (Riekhof et al., 2014). Together these results leave little room for doubt that DGTS and PC can and do accomplish very similar physiological roles in some organisms.

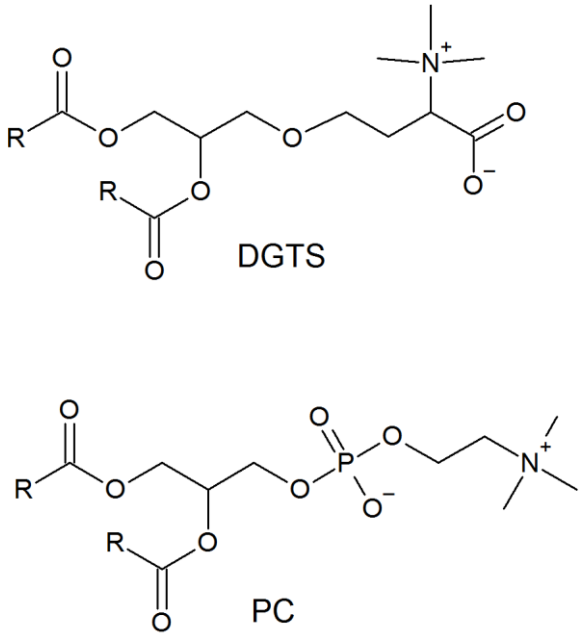

Figure 1.2 – DGTS vs PC Structure

Note that, although DGTS and PC exhibit analogous structure and function, DGTS contains no phosphorous. This is key, as *Rhodobacter sphaeroides* was shown to degrade its phospholipids and produce DGTS (along with other P_i-free lipids) in low-P_i conditions (0.1 mM vs 1 mM) (Benning et al., 1995). This discovery suggested that betaine lipids, especially DGTS, may not only function in a physiologically similar way to PC, but may also act as a P_i-starvation coping strategy. This ability has the potential to confer a significant selective advantage, as P is widely regarded as the most common limiting nutrient in most environments. Several organisms have since been shown to produce DGTS in response to P_i deprivation, including other bacteria (Geiger, Röhrs, Weissenmayer, Finan, & Thomas-Oates, 1999; Geske, Vom Dorp, Dörmann, & Hölzl, 2013), fungi (Lev et al., 2019; Riekhof et al., 2014; Senik et al., 2015) and algae (Abida et al., 2015; Ginneken, 2017; Khozin-Goldberg & Cohen, 2006; Mühlroth et al., 2017; Murakami et al., 2018; Van Mooy et al., 2009).

In 1999, a second bacterium (*Rhizobium meliloti*) was shown to produce DGTS under P_i starvation. Deletion of a high-affinity P_i transporter also induced DGTS production, even in high P_i (Geiger et al., 1999). The *E. coli* genome contains a gene called *PhoB*, which encodes a protein regulator of a suite of P_i-starvation response genes collectively known as the PHO ("Phosphate") regulon (Guan, Wanner, & Inouye, 1983; VanBogelen, Olson, Wanner, & Neidhardt, 1996). Deletion of the *PhoB* gene in *R. meliloti* results in a lack of DGTS in low-P_i conditions, demonstrating that DGTS synthesis is under control of *PhoB* and is therefore a part of the PHO regulon in *R. meliloti* (Geiger et al., 1999). Deletion of the PhoB-equivalent transcription factor (*NUC-1*) or the DGTS synthesis gene (*BTA1*) in the mold *Neurospora crassa* yielded the same results – elimination of DGTS production (Riekhof et al., 2014). By

demonstrating the location of *BTA1* (DGTS synthase) in PHO regulons, these results confirm the role of DGTS synthesis as a key component of the P_i starvation response in some organisms.

As expected from the findings detailed above, as well as from the consensus that P is the most common limiting nutrient, betaine lipids seem to be more prevalent in relatively low-P_i environments than in relatively high-P_i environments. Marine studies have observed that betaine lipids are more abundant in low-P_i seas than in high-P_i seas (Ginneken, 2017; Van Mooy et al., 2009). Additionally, in high- and low-P_i lab media, phytoplankton communities from the (relatively) P_i-rich South Pacific produced little betaine lipid, while communities from the P_i-poor Sargasso Sea did produced high amounts of betaine lipids (Van Mooy et al., 2009). The low-P_i coping benefit of betaine lipid production in indigenous algal species in the low-P_i Dutch Wadden Sea may account for the surprising lack of the highly invasive alga *Gracilaria vermiculophylla*, which has taken over other ecosystems (Ginneken, 2017). It is also interesting to note that betaine lipids were first discovered in algae, and that algae still comprise the largest group of known betaine-lipid producers (see above section on distribution). Soil communities similarly employ betaine lipids to cope with low P_i. Low-P_i soil communities had total membrane compositions with as low as 61% phospholipids (the remaining 39% almost exclusively DGTS) compared to almost 100% phospholipids in high-P_i soils, and P_i concentration was highly associated with DGTS replacement of PC (Warren, 2020). Warren (2020) estimates that at maximum DGTS production (i.e., minimum phospholipid production), the amount of P required for growth is reduced by 10% in soil communities. Van Mooy et al (2009) estimated that some of the betaine lipid-producing algae they surveyed reduced their P requirements by 10-30%. These results point to large-scale betaine lipid membrane remodeling

at population levels, and likely to selection on the basis of betaine production ability in certain environments.

Recent work has demonstrated that P_i starvation coping mechanisms in pathogens are important for virulence. Work in the Alverdy lab has shown that guts of critically ill patients experience P_i depletion severe enough to induce virulence in the opportunistic pathogens *Pseudomonas aeruginosa* (Long, Zaborina, Holbrook, Zaborin, & Alverdy, 2008) and *Candida albicans* (Romanowski et al., 2012). Several important pathogens produce DGTS: plant pathogens such as *Agrobacterium tumefaciens* (Geske et al., 2013), *Microdochium nivale* (Istokovics et al., 1998); animal pathogens such as *Epidermophyton floccosum* (Yamada & Nozawa, 1979), *Candida* species (Riekhof et al., 2014), and *Cryptococcus neoformans* (Lev et al., 2019) (see Fig 3). As a component of the PHO regulon, DGTS production by BTA1 has therefore been examined to determine its importance for virulence. In *Cryptococcus neoformans*, the opportunistic fungal pathogen that causes meningitis in mammals, deletion of the PHO regulon transcription factor Pho4 reduces virulence and increases susceptibility to a variety of stresses (Lev et al., 2017). *C. neoformans* was then shown to replace PC with DGTS and DGTA upon P_i starvation. Surprisingly, *bta1*Δ mutants didn't show an obvious growth defect on low-P_i agar plates, although their visual inspection of colony size may have overlooked small changes. Other organisms have shown decreased growth when DGTS production is eliminated (e.g., López-Lara et al., 2005; Murakami et al., 2018). However, *C. neoformans* growth is reduced in the human cell culture medium RPMI, even though RPMI has ample P_i (~ 6 mM Na_2HPO_4). Additionally, mice infected with bta1Δ survived longer than mice infected with WT, although WT CFU numbers were comparable in the brain, lungs, and blood. Clearly, BTA1 is important for virulence, however without evidence of reduced bta1Δ growth in low P_i, the mechanism by

which BTA1 aids in virulence is unclear (Lev et al., 2019). Additionally, the plant pathogen *Agrobacterium tumefaciens* has been shown to produce DGTS and other non-phosphorous lipids under P_i starvation conditions (Geske et al., 2013). Previous work had shown that P_i starvation increases biofilm attachment and total mass (Danhorn, Hentzer, Givskov, Parsek, & Fuqua, 2004). While *A. tumefaciens* virulence is reduced by lack of PC (i.e., during P_i starvation when PC is replaced by P_i-lacking lipids), the role of DGTS in particular is unknown (Aktas, Danne, Möller, & Narberhaus, 2014).

Pathogens Known to Produce DGTS		
Pathogen	**Host**	**Reference**
Cryptococcus neoformans	animal	(Lev et al., 2019)
Candida spp.	animal	(Riekhof et al., 2014)
Candida albicans	animal	(Lev et al., 2019)
Epidermophyton floccosum	animal	(Yamada & Nozawa, 1979)
Paracoccidioides brasiliensis	animal	(Riekhof et al., 2014)
Agrobacterium tumefaciens	plants	(Geske et al., 2013)
Ustilago maydis	plants	(Riekhof et al., 2014)
Gibberella zeae	plants	
Magnaporthe oryzae	plants	
Ochrobacter anthropi	animals	(Geiger et al., 2010)

Table 1.1 – Pathogens Known to Produce DGTS. This list comprises pathogens known to produce DGTS through either direct confirmation of DGTS such as by TLC analysis, or by identification of a *BTA1* homolog.

In some organisms, it seems that effects of betaine lipid synthase gene deletion will be somewhat limited due to the production of several other P_i-lacking lipids in response to P_i starvation. In fact, this phenomenon of phospholipid replacement in low P_i was first observed with *Pseudomonas diminuta* glycolipids (Minnikin, Abdolrahimzadeh, & Baddiley, 1974). *R. sphaeroides*, in addition to DGTS, also produces a glycolipid, a sulfolipid, and an ornithine lipid

in low P_i (Benning et al., 1995). Many more organisms have also been shown to alter their membrane composition with a variety of P_i-free lipids in response to P_i levels, including seed plants (Nakamura, 2013), bacteria (Geiger et al., 2010; Hölzl & Dörmann, 2007), and phytoplankton (Abida et al., 2015; Shemi et al., 2016). The plant pathogen *Agrobacterium tumefaciens*, in low P_i, produces glycolipids, ornithine lipids, and DGTS, and reduces phospholipids. Overexpression of either of two glycolipid synthesis genes results in 4-5X higher levels of glycolipids and much lower levels of DGTS. Additionally, deletion of one of the glycolipid synthesis genes (pgt) does not significantly reduce overall growth (Geske et al., 2013). Overall growth in P_i starvation of the pathogenic fungus Cryptococcus neoformans is also not significantly affected by deletion of the DGTS synthesis gene (*BTA1*) (Lev et al., 2019), perhaps owing to its complementation by other P_i-free lipids. These results suggest that under P_i starvation some organisms may produce a variety of P_i-free lipids, multiple of which may substitute for PC in membranes. If this is the case, it may be most useful to study betaine lipids in combination with other P_i-free membrane lipids (as demonstrated by Geske et al., 2013; Warren, 2020) in order to determine P_i-free lipids' significance to organisms.

Betaine Lipid Biosynthesis

The metabolic pathway of DGTS biosynthesis in algae and bacteria has been determined. Using radiolabeled methionine, it was shown in algae that DGTS was formed by addition of the 3-amino-3-carboxypropyl moiety of S-adenosylmethionine (SAM) to diacylglycerol (DAG), forming a homoserine lipid, which is then methylated (by additional SAM) three times to form DGTS (Sato, 1991; Vogel & Eichenberger, 1992) (Fig 4). It was later shown that the same mechanism is used in the bacterium *R. sphaeroides* (Hofmann & Eichenberger, 1996). In algae,

DGTA is made from DGTS by decarboxylating DGTS and introducing a new carboxyl group to make DGTA (Vogel & Eichenberger, 1992) (fig 5). The metabolic pathway of DGCC biosynthesis is not known. DGCC and DGTA are localized to the cytoplastmic membrane in *Pavlova lutheri* (Eichenberger & Gribi, 1997). In the opportunistic pathogenic fungus *Cryptococcus neoformans*, which replaces PC with DGTS and DGTA under Pi starvation, DAG fatty acid profiles of PC and DGTS are similar, suggesting that DAG can be recycled from PC in lipid-switching organisms (Lev et al., 2019).

The genes and enzymes responsible for these steps were determined in *R. sphaeroides.* 2 genes required for DGTS production were identified as 1) a 3-amino-3-carboxypropyl transferase, which carries out the 3-amino-3-carboxypropyl transfer from SAM to DAG to form a homoserine lipid; and 2) a SAM-dependent methyltransferase, which transfers the methyl groups from SAMs to the homoserine headgroup. Together, these enzymes were capable of building DGTS from diacylglycerol (DAG) and SAM (fig 4). Gene 1 was named btaA (betaine A) and gene 2 was named btaB, and together form an operon with btaA at the 5' end and btaB and the 3' end (Klug & Benning, 2001; Riekhof, Andre, & Benning, 2005). Some organisms employ slight variations of this btaA-btaB system. Both of these functions are accomplished by a single protein (CrBTA1) in *Chlamydamonas reinhardtii*, which has a btaA-like domain at the C-terminus and a btaB-like domain at the N-terminus (Riekhof, Sears, & Benning, 2005). *Nannochloropsis oceanica* also has a single-protein betaine lipid synthase, however the btaB-like domain is at the C-terminus and the btaA-like domain is at the N-terminus (reverse order of *Rs*btaA-*Rs*btaB and *Cr*BTA1), and an additional protein with only the btaB-like domain (Murakami et al., 2018).

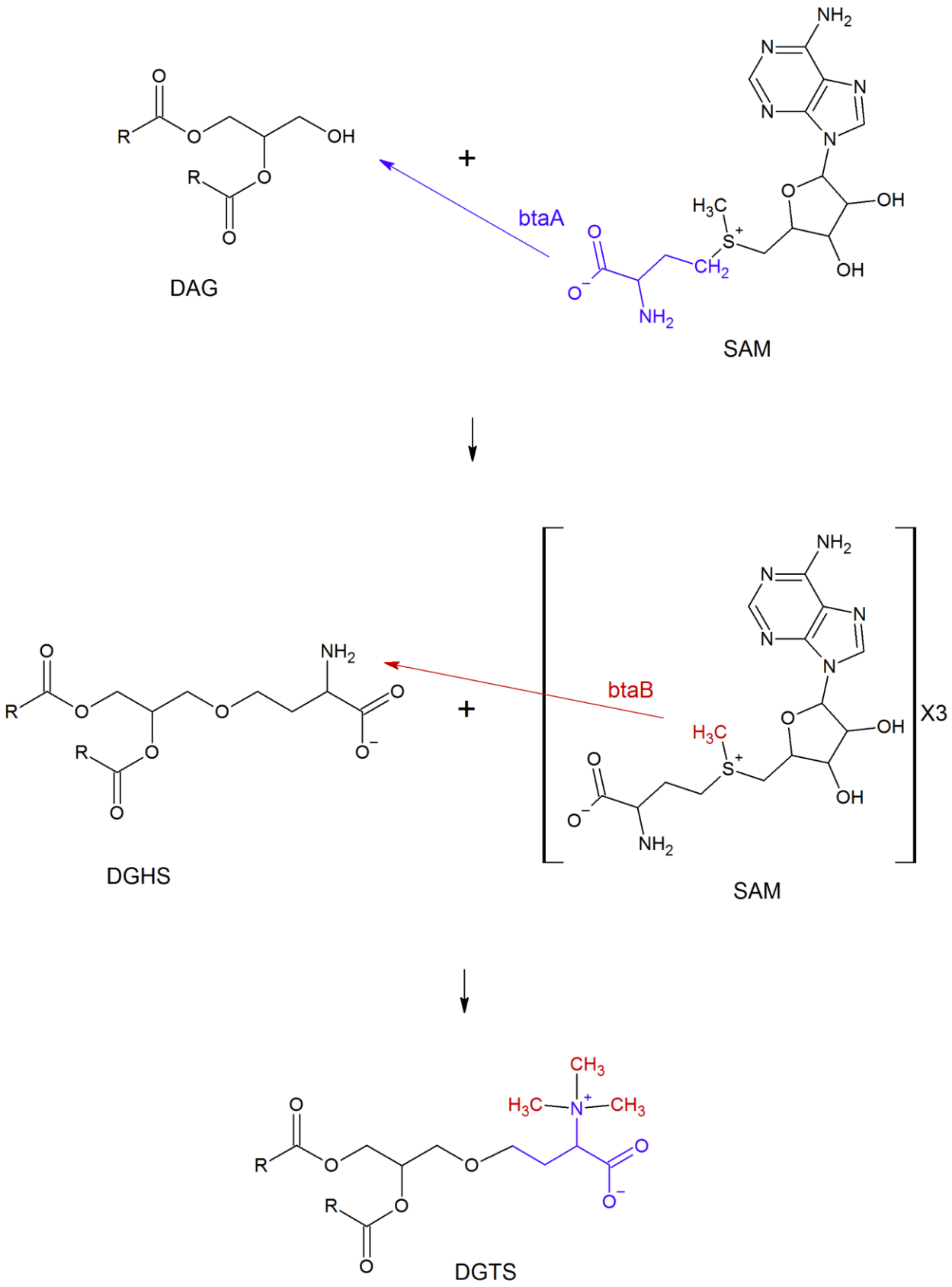

Figure 1.3 – DGTS Biosynthesis

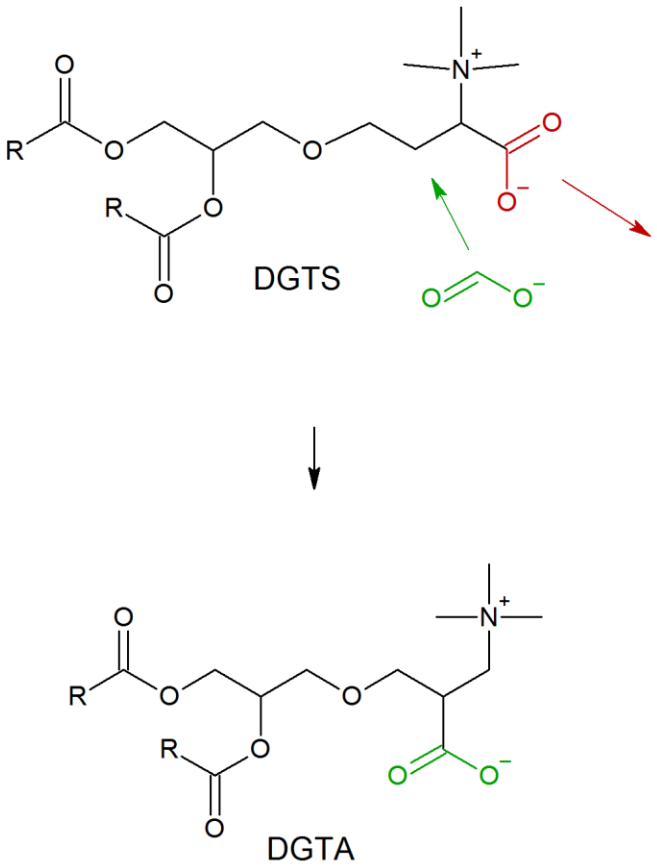

Figure 1.4 – DGTA Biosynthesis. DGTA biosynthesis is accomplished by first synthesizing DGTS followed by decarboxylation and introduction of a new carboxyl group.

The presence of these variations in arrangement of the btaA-btaB system raises intriguing questions regarding its evolution. If eukaryotes inherited the betaine lipid synthase genes from Prokaryotes, it seems that it must have been in the A-B form (as is the case in bacteria), with the two proteins subsequently fusing together to make the multi-domain BTA1 protein. And indeed, some eukaryotes (*such as C. reinhardtii* and *C. albicans*) have a single protein with this AB form. However, in other eukaryotes (such as *N. oceanica*), the order is flipped, and the protein has the BA form. Presumably, one of the domains may have been duplicated (creating either an ABA or BAB form) and then one of the excessive terminal domains might have been deleted to

yield a BA form. Many multi-domain-protein ‘architectures’ vary among organisms in their number of individual domains (perhaps indicating insertions or deletions), as well as in the presence or absence of extra domains (Björklund, Ekman, Light, Frey-Skött, & Elofsson, 2005). It appears that the order of btaA and btaB domains has little effect on function, as both A and B can function separately (Riekhof, Andre, et al., 2005), so it remains mysterious why and how such arrangements came about.

Summary and Conclusions

Betaine lipids are a class of zwitterionic glycerolipids with betaine head groups that mimic phospholipid head groups but contain no phosphorous. Thus far, three betaine lipids have been identified – DGTS, DGTA, and DGCC. The metabolic pathways of DGTS and DGTA synthesis have been established: DGTS is synthesized by the action of two protein domains (separate proteins in prokaryotes, fused together in eukaryotes), which use 4 SAM molecules to build the head group attached to a DAG molecule. Because of their phospholipid-mimicking head groups , their primary role (especially DGTS) in organisms is in their replacement of membrane phospholipids (either constitutively or inducibly) in low-P_i environments, although they seem to have roles in transporting exogenous fatty acids and perhaps in carrying unsaturated fatty acids for cold tolerance in some algae. DGTS-producing organisms show reduced growth in P_i starvation if DGTS production is inhibited, indicating that enough P_i is conserved by DGTS production to increase growth and provide a selective advantage in low P_i environments. Further, soil and aquatic microbial communities subject to chronic P_i starvation show significant betaine lipid production, while communities with relatively higher P_i content show little or no betaine lipid production, indicating large-scale selection for betaine lipid-producing organisms in P_i-

starved environments. Betaine lipids are abundant in algal species, likely because aqueous environments are often low in P_i, although DGTS also been identified in some bacteria and fungi. Many of these DGTS-producing bacteria and fungi have membranes normally composed primarily of PC but then induce DGTS production in response to low environmental P_i. Additionally, some of them are important pathogens, such as *A. tumefaciens*, *C. albicans*, and *C. neoformans*. Because P_i starvation in general is important for virulence, DGTS production is likely to play a significant role in infection, at least in low P_i infection scenarios such as those in critically ill guts. There is some evidence to suggest that deletion of *BTA1* reduces virulence for *C. neoformans*. However, it's important to note that DGTS production in response to low P_i can interact with the production of other P_i-free lipids, and these lipids studied collectively, rather than separately, may yield the most useful results.

References

Abida, H., Dolch, L. J., Meï, C., Villanova, V., Conte, M., Block, M. A., … Maréchal, E. (2015). Membrane glycerolipid remodeling triggered by nitrogen and phosphorus starvation in Phaeodactylum tricornutum. *Plant Physiology*, *167*(1), 118–136. https://doi.org/10.1104/pp.114.252395

Aktas, M., Danne, L., Möller, P., & Narberhaus, F. (2014). Membrane lipids in Agrobacterium tumefaciens: Biosynthetic pathways and importance for pathogenesis. *Frontiers in Plant Science*, *5*(MAR), 1–13. https://doi.org/10.3389/fpls.2014.00109

Araki, S., Eichenberger, W., Sakurai, T., & Sato, N. (1991). Distribution of diacylglycerylhydroxymethyltrimethyl-β-alanine (DGTA) and phosphatidylcholine in brown algae. *Plant and Cell Physiology*, *32*(5), 623–628. https://doi.org/10.1093/oxfordjournals.pcp.a078124

Benning, C., Huang, Z. H., & Gage, D. A. (1995). Accumulation of a novel glycolipid and a betaine lipid in cells of rhodobacter sphaeroides grown under phosphate limitation. *Archives of Biochemistry and Biophysics*. https://doi.org/10.1006/abbi.1995.1141

Bishop, A. C., Sun, T., Johnson, M. E., Bruno, V. M., & Patton-Vogt, J. (2011). Robust utilization of phospholipase-generated metabolites, glycerophosphodiesters, by Candida albicans: Role of the CaGit1 permease. *Eukaryotic Cell*, *10*(12), 1618–1627. https://doi.org/10.1128/EC.05160-11

Björklund, Å. K., Ekman, D., Light, S., Frey-Skött, J., & Elofsson, A. (2005). Domain rearrangements in protein evolution. *Journal of Molecular Biology*, *353*(4), 911–923. https://doi.org/10.1016/j.jmb.2005.08.067

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, *37*, 911–917.

Brown, A. E., & Elovson, J. (1974). Isolation and characterization of a novel lipid, 1(3),2-diacylglyceryl-(3)-O-4′-(N,N,N-trimethyl)homoserine, from ochromonas danica. *Biochemistry*, *13*(17), 3476–3482. https://doi.org/10.1021/bi00714a009

Carman, G. M., & Han, G.-S. (2011). Regulation of Phospholipid Synthesis in the Yeast Saccharomyces cerevisiae . *Annual Review of Biochemistry*, *80*(1), 859–883. https://doi.org/10.1146/annurev-biochem-060409-092229

Choi, H. S., Su, W. M., Han, G. S., Plote, D., Xu, Z., & Carman, G. M. (2012). Pho85p-Pho80p phosphorylation of yeast pah1p phosphatidate phosphatase regulates its activity, location, abundance, and function in lipid metabolism. *Journal of Biological Chemistry*, *287*(14), 11290–11301. https://doi.org/10.1074/jbc.M112.346023

Czabany, T., Athenstaedt, K., & Daum, G. (2007). Synthesis, storage and degradation of neutral lipids in yeast. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1771*(3), 299–309. https://doi.org/10.1016/j.bbalip.2006.07.001

Danhorn, T., Hentzer, M., Givskov, M., Parsek, M. R., & Fuqua, C. (2004). Phosphorus Limitation Enhances Biofilm Formation of the Plant Pathogen Agrobacterium tumefaciens through the PhoR-PhoB Regulatory System. *Society*, *186*(14), 4492–4501. https://doi.org/10.1128/JB.186.14.4492

Dembitsky, V. M. (1996). Betaine ether-linked glycerolipids: Chemistry and biology. *Progress in Lipid Research*. https://doi.org/10.1016/0163-7827(95)00009-7

Douglas, L. J. (2003). Candida biofilms and their role in infection. *Trends in Microbiology*, *11*(1), 30–36. https://doi.org/10.1016/S0966-842X(02)00002-1

Eichenberger, W. (1982). Distribution of diacylglyceryl-O-4′-N,N,N-trimethyl)- homoserine in different algae. *Plant Science Letters*, *24*(1), 91–95. https://doi.org/10.1016/0304-4211(82)90012-8

Eichenberger, W., Araki, S., & Müller, D. G. (1993). Betaine lipids and phospholipids in brown algae. *Phytochemistry*, *34*(5), 1323–1333. https://doi.org/10.1016/0031-9422(91)80024-U

Eichenberger, W., & Gribi, C. (1997). Lipids of Pavlova lutheri: Cellular site and metabolic role of DGCC. *Phytochemistry*, *45*(8), 1561–1567. https://doi.org/10.1016/S0031-9422(97)00201-X

Geiger, O., González-Silva, N., López-Lara, I. M., & Sohlenkamp, C. (2010). Amino acid-containing membrane lipids in bacteria. *Progress in Lipid Research*, *49*(1), 46–60. https://doi.org/10.1016/j.plipres.2009.08.002

Geiger, O., Röhrs, V., Weissenmayer, B., Finan, T. M., & Thomas-Oates, J. E. (1999). The regulator gene phoB mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-N,N,N-trimethylhomoserine in Rhizobium (Sinorhizobium) meliloti. *Molecular Microbiology*, *32*(1), 63–73. https://doi.org/10.1046/j.1365-2958.1999.01325.x

Geske, T., Vom Dorp, K., Dörmann, P., & Hölzl, G. (2013). Accumulation of glycolipids and other non-phosphorous lipids in Agrobacterium tumefaciens grown under phosphate deprivation. *Glycobiology*, *23*(1), 69–80. https://doi.org/10.1093/glycob/cws124

Ginneken, V. van. (2017). Seaweed Competition: Ulva Sp. has the Potential to Produce the Betaine Lipid Diacylglyceryl-O-4'-(N,N,N,-Trimethyl) Homoserine (DGTS) in Order to Replace Phosphatidylcholine (PC) Under Phosphate-Limiting Conditions in the P-Limited Dutch Wadden Sea and Ou. *Oceanography & Fisheries Open Access Journal*, *2*(5). https://doi.org/10.19080/ofoaj.2017.02.555596

Guan, C. D., Wanner, B., & Inouye, H. (1983). Analysis of regulation of phoB expression using a phoB-cat fusion. *Journal of Bacteriology*, *156*(2), 710–717.

Hacham, Y., Avraham, T., & Amir, R. (2002). The N-terminal region of Arabidopsis cystathionine γ-synthase plays an important regulatory role in methionine metabolism. *Plant Physiology*, *128*(2), 454–462. https://doi.org/10.1104/pp.010819

Hofmann, M., & Eichenberger, W. (1996). Biosynthesis of diacylglyceryl-N,N,N-trimethylhomoserine in Rhodobacter sphaeroides and evidence for lipid-linked N methylation. *Journal of Bacteriology*, *178*(21), 6140–6144. https://doi.org/10.1128/jb.178.21.6140-6144.1996

Hölzl, G., & Dörmann, P. (2007). Structure and function of glycoglycerolipids in plants and bacteria. *Progress in Lipid Research*. https://doi.org/10.1016/j.plipres.2007.05.001

Hong, M. Y., Chapkin, R. S., Barhoumi, R., Burghardt, R. C., Turner, N. D., Henderson, C. E., … Lupton, J. R. (2002). Fish oil increases mitochondrial phospholipid unsaturation , upregulating reactive oxygen species and apoptosis in rat colonocytes n-3 fatty acids) with the butyrate-producing fiber pectin , development . We now hypothesize that n-3 fatty acids. *Carcinogenesis*, *23*(11), 1919–1925.

Ikeh, M. A. C., Kastora, S. L., Day, A. M., Herrero-De-Dios, C. M., Tarrant, E., Waldron, K. J., … Quinn, J. (2016). Pho4 mediates phosphate acquisition in Candida albicans and is vital for stress resistance and metal homeostasis. *Molecular Biology of the Cell*, *27*(17), 2784–2801. https://doi.org/10.1091/mbc.E16-05-0266

Istokovics, A., Morita, N., Izumi, K., Hoshino, T., Yumoto, I., Sawada, M. T., … Okuyama, H. (1998). Neutral lipids, phospholipids, and a betaine lipid of the snow mold fungus Microdochium nivale. *Canadian Journal of Microbiology*, *44*(11), 1051–1059.

Karanasios, E., Han, G. S., Xu, Z., Carman, G. M., & Siniossoglou, S. (2010). A phosphorylation-regulated amphipathic helix controls the membrane translocation and function of the yeast phosphatidate phosphatase. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(41), 17539–17544. https://doi.org/10.1073/pnas.1007974107

Kato, M., Adachi, K., Hajiro-Nakanishi, K., Ishigaki, E., Sano, H., & Miyachi, S. (1994). A betaine lipid from Pavlova lutheri. *Phytochemistry*. https://doi.org/10.1016/0031-9422(94)85041-0

Kato, M., Sakai, M., Adachi, K., Ikemoto, H., & Sano, H. (1996). Distribution of betaine lipids in marine algae. *Phytochemistry*, *42*(5), 1341–1345. https://doi.org/10.1016/0031-9422(96)00115-X

Khozin-Goldberg, I., & Cohen, Z. (2006). The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte Monodus subterraneus. *Phytochemistry*, *67*, 696–701. https://doi.org/10.1016/j.phytochem.2006.01.010

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., & Salzburg, S. L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology*, *14*, R36.

Kim, H. J., Silva, J. E., Iskandarov, U., Andersson, M., Cahoon, R. E., Mockaitis, K., & Cahoon, E. B. (2015). Structurally divergent lysophosphatidic acid acyltransferases with high selectivity for saturated medium chain fatty acids from Cuphea seeds. *Plant Journal*, *84*(5), 1021–1033. https://doi.org/10.1111/tpj.13063

Klug, R. M., & Benning, C. (2001). Two enzymes of diacylglyceryl-O-4′-(N,N,N,-trimethyl)-homoserine biosynthesis are encoded by btaA and btaB in the purple bacterium Rhodobacter sphaeroides. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(10), 5910–5915. https://doi.org/10.1073/pnas.101037998

Kojic, E. M., & Darouiche, R. O. (2004). Candida Infections of Medical Devices. *Clinical Microbiology Reviews*, *17*(2), 255–267. https://doi.org/10.1128/CMR.17.2.255-267.2004

Kretschmer, M., Reiner, E., Hu, G., Tam, N., Oliveira, D. L., Caza, M., … Kronstad, J. W. (2014). Defects in phosphate acquisition and storage influence virulence of Cryptococcus neoformans. *Infection and Immunity*, *82*(7), 2697–2712. https://doi.org/10.1128/IAI.01607-14

Kunzler, K., & Eichenberger, W. (1997). Betaine lipids and zwitterionic phospholipids in plants and fungi. *Phytochemistry*, *46*(5), 883–892. https://doi.org/10.1016/S0031-9422(97)81274-5

Lami, R., Cottrell, M. T., Ras, J., Ulloa, O., Obernosterer, I., Claustre, H., … Lebaron, P. (2007). High abundances of aerobic anoxygenic photosynthetic bacteria in the South Pacific Ocean. *Applied and Environmental Microbiology*, *73*(13), 4198–4205. https://doi.org/10.1128/AEM.02652-06

Lev, S., Kaufman-Francis, K., Desmarini, D., Juillard, P. G., Li, C., Stifter, S. A., … Djordjevic, J. T. (2017). Pho4 Is Essential for Dissemination of Cryptococcus neoformans to the Host Brain by Promoting Phosphate Uptake and Growth at Alkaline pH . *MSphere*, *2*(1), 1–21. https://doi.org/10.1128/msphere.00381-16

Lev, S., Rupasinghe, T., Desmarini, D., Kaufman-Francis, K., Sorrell, T. C., Roessner, U., & Djordjevic, J. T. (2019). The PHO signaling pathway directs lipid remodeling in Cryptococcus neoformans via DGTS synthase to recycle phosphate during phosphate deficiency. *PLoS ONE*, *14*(2), 1–18. https://doi.org/10.1371/journal.pone.0212651

Long, J., Zaborina, O., Holbrook, C., Zaborin, A., & Alverdy, J. (2008). Depletion of Intestinal Phosphate following Surgical Injury Activates the Virulence of P. aeruginosa causing Lethal Gut-Derived Sepsis. *Surgery*, *144*(2), 189–197. https://doi.org/10.1038/jid.2014.371

López-Lara, I. M., Gao, J. L., Soto, M. J., Solares-Pérez, A., Weissenmayer, B., Sohlenkamp, C., … Geiger, O. (2005). Phosphorus-free membrane lipids of Sinorhizobium meliloti are not required for the symbiosis with alfalfa but contribute to increased cell yields under phosphorus-limiting conditions of growth. *Molecular Plant-Microbe Interactions*, *18*(9), 973–982. https://doi.org/10.1094/MPMI-18-0973

Magasanik, B., & Kaiser, C. A. (2002). Nitrogen regulation in Saccharomyces cerevisiae. *Gene*. https://doi.org/10.1016/S0378-1119(02)00558-9

McDermott, J. R., Rosen, B. P., & Liu, Z. (2010). Jen1p: A High Affinity Selenite Transporter in Yeast. *Molecular Biology of the Cell*, *21*, 3934–3941. https://doi.org/10.1091/mbc.E10

Minnikin, D. E., Abdolrahimzadeh, H., & Baddiley, J. (1974). Replacement of acidic phospholipids by acidic glycolipids in Pseudomonas diminuta. *Nature*, *249*(5454), 268–269. https://doi.org/10.1038/249268a0

Morgan, J. (2005). Global Trends in Candidemia: Review of Reports from 1995-2005. *Current Infectious Disease Reports*, *7*, 429–439.

Mühlroth, A., Winge, P., El Assimi, A., Jouhet, J., Maréchal, E., Hohmann-Marriott, M. F., … Bonesa, A. M. (2017). Mechanisms of phosphorus acquisition and lipid class remodeling under P limitation in a marine microalga. *Plant Physiology*, *175*(4), 1543–1559. https://doi.org/10.1104/pp.17.00621

Murakami, H., Nobusawa, T., Hori, K., Shimojima, M., & Ohta, H. (2018). Betaine Lipid Is Crucial for Adapting to Low Temperature and Phosphate Deficiency in Nannochloropsis. *Plant Physiology*, *177*(1), 181–193. https://doi.org/10.1104/pp.17.01573

Nakagawa, Y., Sakumoto, N., Kaneko, Y., & Harashima, S. (2002). Mga2p Is a Putative Sensor for Low Temperature and Oxygen to Induce OLE1 Trascription in Saccharomyces cerevisiae. *Biochemical and Biophysical Research Communications*, *291*(3), 707–713.

Nakamura, Y. (2013). Phosphate starvation and membrane lipid remodeling in seed plants. *Progress in Lipid Research*, *52*(1), 43–50. https://doi.org/10.1016/j.plipres.2012.07.002

Nakamura, Y., Koizumi, R., Shui, G., Shimojima, M., Wenk, M. R., Ito, T., & Ohtad, H. (2009). Arabidopsis lipins mediate eukaryotic pathway of lipid metabolism and cope critically with phosphate starvation. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(49), 20978–20983. https://doi.org/10.1073/pnas.0907173106

Naoki Sato, & Norio Murata. (1991). Transition of lipid phase in aqueous dispersions of diacylglyceryltrimethylhomoserine. *Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism*. https://doi.org/10.1016/0005-2760(91)90306-3

Nguyen, N., Quail, M. M. F., & Hernday, A. D. (2017). An Efficient, Rapid, and Recyclable System for CRISPR-Mediated Genome Editing in Candida albicans. *Molecular Biology and Physiology*, *2*(2), 1–10.

Nichols, B. W., & Appleby, R. S. (1969). The distribution and biosynthesis of arachidonic acid in algae. *Phytochemistry*, *8*, 1907–1915. https://doi.org/10.1016/S0031-9422(00)88075-9

Palmieri, F., Agrimi, G., Blanco, E., Castegna, A., Di Noia, M. A., Iacobazzi, V., … Walker, J. (2006). Identification of mitochondrial carriers in Saccharomyces cerevisiae by transport assay of reconstituted recombinant proteins. *Biochimica et Biophysica Acta - Bioenergetics*. https://doi.org/10.1016/j.bbabio.2006.05.023

Pflaster, E. L., Schwabe, M. J., Becker, J., Wilkinson, M. S., Parmer, A., Clemente, T. E., …

Riekhof, W. R. (2014). A high-throughput fatty acid profiling screen reveals novel variations in fatty acid biosynthesis in Chlamydomonas reinhardtii and related algae. *Eukaryotic Cell*, *13*(11), 1431–1438. https://doi.org/10.1128/EC.00128-14

Pierce, C. G., Uppuluri, P., Tummala, S., & Lopez-Ribot, J. L. (2010). A 96 well microtiter plate-based method for monitoring formation and antifungal susceptibility testing of Candida albicans biofilms. *Journal of Visualized Experiments*, (44), 1–4. https://doi.org/10.3791/2287

Popko, J., Herrfurth, C., Feussner, K., Ischebeck, T., Iven, T., Haslam, R., … Feussner, I. (2016). Metabolome analysis reveals betaine lipids as major source for triglyceride formation, and the accumulation of sedoheptulose during nitrogen-starvation of Phaeodactylum tricornutum. *PLoS ONE*, *11*(10), 1–23. https://doi.org/10.1371/journal.pone.0164673

Riekhof, W. R., Andre, C., & Benning, C. (2005). Two enzymes, BtaA and BtaB, are sufficient for betaine lipid biosynthesis in bacteria. *Archives of Biochemistry and Biophysics*, (441), 96–105. https://doi.org/10.1016/j.abb.2005.07.001

Riekhof, W. R., Naik, S., Bertrand, H., Benning, C., & Voelker, D. R. (2014). Phosphate starvation in fungi induces the replacement of phosphatidylcholine with the phosphorus-free betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine. *Eukaryotic Cell*, *13*(6), 749–757. https://doi.org/10.1128/EC.00004-14

Riekhof, W. R., Sears, B. B., & Benning, C. (2005). Annotation of Genes Involved in Glycerolipid Biosynthesis in. *Eukaryotic Cell*, *4*(2), 242–252. https://doi.org/10.1128/EC.4.2.242

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2009). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140. https://doi.org/10.1093/bioinformatics/btp616

Romanowski, K., Zaborin, A., Valuckaite, V., Rolfes, R. J., Babrowski, T., Bethel, C., … Alverdy, J. C. (2012). Candida albicans isolates from the gut of critically ill patients respond to phosphate limitation by expressing filaments and a lethal phenotype. *PLoS ONE*, *7*(1). https://doi.org/10.1371/journal.pone.0030119

Rosenfeld, L., Reddi, A. R., Leung, E., Aranda, K., Jensen, L. T., & Culotta, V. C. (2010). The effect of phosphate accumulation on metal ion homeostasis in Saccharomyces cerevisiae. *Journal of Biological Inorganic Chemistry*, *15*(7), 1051–1062. https://doi.org/10.1007/s00775-010-0664-8

Sato, N. (1991). Lipids in Cryptomonas CR-1. II. Biosynthesis of Betaine Lipids and Galactolipids. *Plant and Cell Physiology*, *32*(6), 845–851. https://doi.org/10.1093/oxfordjournals.pcp.a078152

Sato, N. (1992). Betaine Lipids. *The Botanical Magazine Tokyo*, *105*, 185–197.

Sato, N., & Furuya, M. (1985). Distribution of diacylglyceryltrimethylhomoserine and phosphatidylcholine in non-vascular green plants. *Plant Science*, *38*(2), 81–85. https://doi.org/10.1016/0168-9452(85)90134-7

Schubotz, F., Xie, S., Lipp, J. S., Hinrichs, K. U., & Wakeham, S. G. (2018). Intact polar lipids in the water column of the eastern tropical North Pacific: Abundance and structural variety of non-phosphorus lipids. *Biogeosciences*, *15*(21), 6481–6501. https://doi.org/10.5194/bg-15-6481-2018

Senik, S. V., Maloshenok, L. G., Kotlova, E. R., Shavarda, A. L., Moiseenko, K. V., Bruskin, S. A., … Psurtseva, N. V. (2015). Diacylglyceryltrimethylhomoserine content and gene expression changes triggered by phosphate deprivation in the mycelium of the

basidiomycete Flammulina velutipes. *Phytochemistry*, *117*(1), 34–42. https://doi.org/10.1016/j.phytochem.2015.05.021

Shemi, A., Schatz, D., Fredricks, H. F., Van Mooy, B. A. S., Porat, Z., & Vardi, A. (2016). Phosphorus starvation induces membrane remodeling and recycling in Emiliania huxleyi. *New Phytologist*, *211*(3), 886–898. https://doi.org/10.1111/nph.13940

Tatsuzawa, H., & Takizawa, E. (1995). Changes in lipid and fatty acid composition of Pavlova lutheri. *Phytochemistry*, *40*(2), 397–400. https://doi.org/10.1016/0031-9422(95)00327-4

Torriani-Gorini, A., Yagil, E., & Silver, S. (1994). *Phosphate in Microorganisms: Cellular and Molecular Biology*. Washington, DC: ASM Press.

Van Mooy, B. A. S., Fredricks, H. F., Pedler, B. E., Dyhrman, S. T., Karl, D. M., Koblížek, M., … Webb, E. A. (2009). Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature*, *458*(7234), 69–72. https://doi.org/10.1038/nature07659

VanBogelen, R. A., Olson, E. R., Wanner, B. L., & Neidhardt, F. C. (1996). Global analysis of proteins synthesized during phosphorus restriction in Escherichia coli. *Journal of Bacteriology*, *178*(15), 4344–4366. https://doi.org/10.1128/jb.178.15.4344-4366.1996

Vardi, N., Levy, S., Gurvich, Y., Polacheck, T., Carmi, M., Jaitin, D., … Barkai, N. (2014). Sequential feedback induction stabilizes the phosphate starvation response in budding yeast. *Cell Reports*, *9*(3), 1122–1134. https://doi.org/10.1016/j.celrep.2014.10.002

Vogel, G., & Eichenberger, W. (1992). Betaine Lipids in Lower Plants. Biosynthesis of DGTS and DGTA in Ochromonas danica (Chrysophyceae) and the Possible Role of DGTS in Lipid Metabolism. *Plant and Cell Physiology*, *33*(4), 427–436. https://doi.org/10.1093/oxfordjournals.pcp.a078271

Vogel, G., Woznica, M., Gfeller, H., Müller, C., Stämpfli, A. A., Jenny, T. A., & Eichenberger, W. (1990). 1(3),2-Diacylglyceryl-3(1)-O-2′-(hydroxymethyl)(N,N,N,-trimethyl)-β-alanine (DGTA): A novel betaine lipid from Ochoromonas danica (Chrysophyceae). *Chemistry and Physics of Lipids*, *52*(2), 99–109. https://doi.org/10.1016/0009-3084(90)90154-J

Warren, C. R. (2020). Soil microbial populations substitute phospholipids with betaine lipids in response to low P availability. *Soil Biology and Biochemistry*, *140*(November 2019), 107655. https://doi.org/10.1016/j.soilbio.2019.107655

Yamada, T., & Nozawa, Y. (1979). An unusual lipid in the human pathogenic fungus Epidermophyton floccosum. *Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism*. https://doi.org/10.1016/0005-2760(79)90239-X

CHAPTER 2: PHOSPHATE STARVATION INDUCES REPLACEMENT OF PHOSPHOLIPIDS WITH THE BETAINE LIPID DIACYLGLYCEROLTRIMETHYLHOMOSERINE IN THE HUMAN FUNGAL PATHOGEN *CANDIDA ALBICANS*

Introduction

Microorganisms rely on their external environment for nutrients. Microbes can experience rapid shifts from nutrient excess to deficiency, and this environmental variation necessitates the presence of nutrient starvation responses as well as response regulatory mechanisms for optimal growth and survival (Vardi et al., 2014). Phosphorus, in the form of inorganic phosphate (PO_4^{2-}; P_i), is an essential macronutrient for all organisms, and is necessary for the biosynthesis of lipids, nucleic acids, cofactors, and phosphorylated metabolites (Torriani-Gorini, Yagil, & Silver, 1994); consequently, microbes possess several strategies to cope with P_i starvation. P_i starvation in the fungi *Neurospora crassa*, *Kluyveromyces lactis* (Riekhof et al., 2014), Cryptococcus neoformans, and Candida albicans (Lev et al., 2019) leads to the replacement of the ubiquitous membrane phospholipid phosphatidylcholine (PtdCho) with the phosphorous-free betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine (DGTS). In ascomycetes, this process is under the control of the NUC-1/Pho4p transcription factor which regulates a suite of genes associated with P_i scavenging and uptake called the PHO regulon (Riekhof et al., 2014). Replacement of PtdCho with DGTS is thus an important Pi starvation coping strategy in some organisms.

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C. albicans is a common member of the human gut microbiome and an important opportunistic human pathogen, capable of forming systemic infections with ~ 40% lethality rate (Morgan, 2005), especially in intensive care and immunocompromised patients. Harsh conditions in guts of critically ill patients reduce populations of normal gut flora and select for *C. albicans,* and are sufficiently low in P_i to induce virulence in certain clinical isolates, which can be attenuated with P_i supplementation (Romanowski et al., 2012). Thus, in many cases the ability of *C. albicans* to respond to and cope with low P_i environments is crucial to its ability to persist in and infect critically ill patients, and so these responses are important areas for ongoing research.

We have previously identified a *BTA1* homolog in *C. albicans* (*Ca*BTA1) (Riekhof et al., 2014). We now demonstrate that the biochemical pathways responsible for the turnover of phospholipids and the biosynthesis of DGTS in *Candida albicans*, including the betaine lipid synthase (C1_11490C; *Ca*BTA1), are coordinately regulated as a core component of its PHO regulon, and that DGTS production confers a survival advantage in low-P_i conditions. Additionally, our work suggests important crosstalk between P_i starvation mechanisms, nitrogen sequestration, amino acid metabolism, and metal homeostasis. DGTS production is therefore an important component of its P_i starvation response and may prove to be a useful drug target in antifungal chemotherapies.

Results

Identification of DGTS under P_i starvation

To confirm the expression of *Ca*BTA1 under phosphate limitation, we performed lipid analysis by thin layer chromatography (TLC) followed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of isolated lipid species. Identification and characterization of DGTS in the wild type SN152 and *bta1*ΔΔ mutant (Fungal Genetic Stock Center) was done by ESI-MS/MS. Under P_i starvation, a decrease of PC and a corresponding quantitative increase of DGTS in the wild type was observed. The absence of DGTS in the *bta1*ΔΔ mutant was confirmed by TLC (Fig 2.1A). Individual bands of DGTS and PC were isolated and further characterized by direct infusion electrospray ionization-MS/MS with a neutral loss of 236.1 and precursor ion scan of 184.1 to confirm PC and DGTS molecular species respectively (Fig. 2.1B).

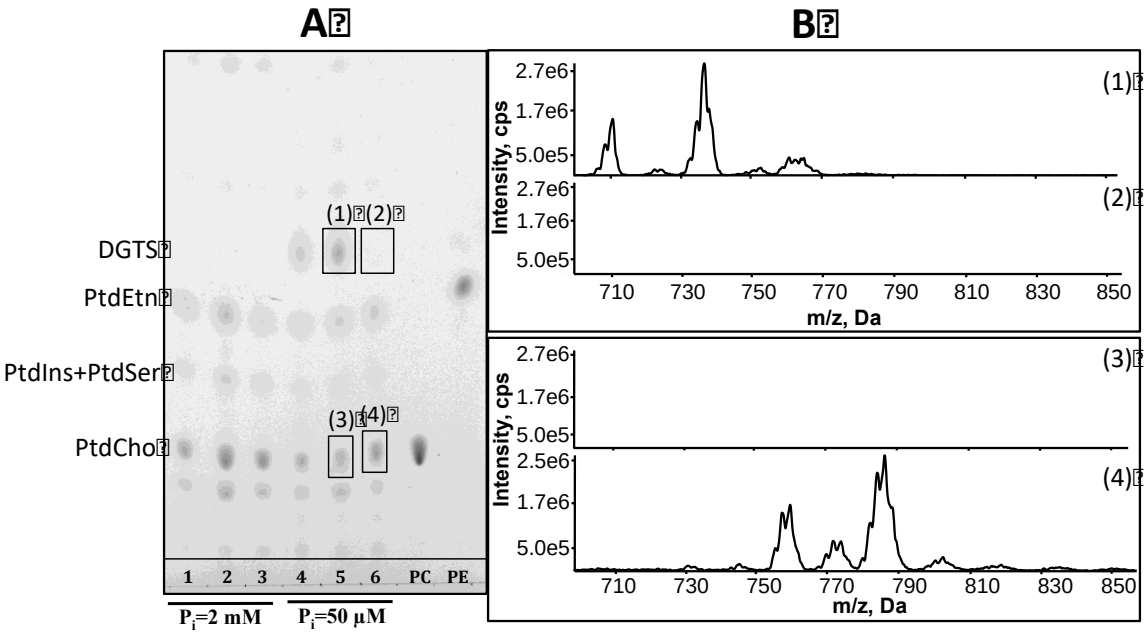

Figure 2.1 - Switch from PtdCho to DGTS in low-P_i conditions. Identification of DGTS by (A) thin-layer chromatography (TLC), with lanes of lipid extracts of samples grown in high P_i (1-3) and low P_i (4-6) conditions (2 mM and 50 µM, respectively): 1,4 - SC5314; 2,5 - SN152; 3,6 – SN152-derived bta1ΔΔ; PC - phosphatidylcholine (PtdCho); PE – phosphatidylethanolamine (PtdEtn); and (B) Electrospray ionization mass spectrometry (ESI-MS/MS). (1, 3) Chromatogram showing the presence of DGTS (1) and absence of PtdCho (3) in WT in low P_i (50 µM) conditions, and (2, 4) chromatogram showing the presence of PtdCho (2) and absence of DGTS (4) in WT in high P_i (2 mM) conditions.

DGTS vs PtdCho Fatty Acid Profiles

ESI-/MS/MS scans from DGTS identification were quantified, and molecular weights of individual molecular species were used to calculate the fatty acid composition of PC and DGTS (Fig 2.2). Gas chromatography of fatty acid methyl esters was used to assess the overall cellular fatty acid profile (Pflaster et al., 2014) (Fig. 2.3). A significant difference in the composition of molecular species of DGTS and PC was detected under P_i starvation. The major molecular species of DGTS were 32:1 (total carbon atoms:number of double bonds), 34:1, and 34:2, along with some odd chain fatty acids (Fig. 2.2). The most abundant molecular species of PC present were 34:2, 36:3, 36:4, 36:5 with some odd chain fatty acids (35:2, 35:3, 37:2 and 37:3). In summary, the DGTS fatty acid profile primarily consisted of 32- and 34-carbon fatty acids, whereas PC consists primarily of 34- and 36-carbon unsaturated fatty acids (Fig. 2.2).

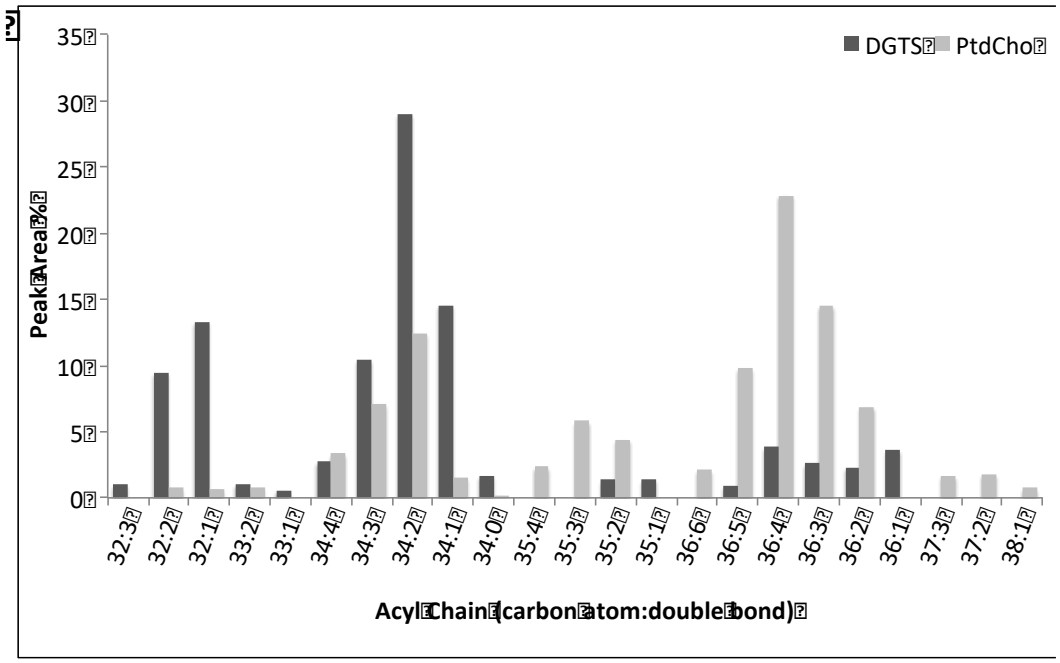

Figure 2.2 - PtdCho vs DGTS Fatty Acids in low-P_i conditions. Most abundant molecular species of WT DGTS (in low P_i) and PtdCho (in high P_i) analyzed by ESI-MS/MS.

Several studies have reported the importance of the balance between saturated and unsaturated fatty acids for maintaining membrane fluidity, which is in turn important for cell integrity and function. Presumably, therefore, the replacement of PC with DGTS in P_i starvation – and the corresponding replacement of the PC-specific fatty acid profile with that of DGTS – might change this balance. In order to assess this, we measured the unsaturation of fatty acid classes in WT and *bta1*ΔΔ whole-cell lipid extracts under P_i starvation (Fig. 2.3A). We calculated the unsaturation index by taking the sum of the fatty acid class mole percentage multiplied by the number of double bonds in that species (Hong et al., 2002). The unsaturation indices of *C. albicans* WT and *bta1*ΔΔ fatty acid profiles show a significant decrease in unsaturation under P_i starvation due to a decrease in 18:2 and an increase in 18:1. This result correlates well with our gene expression data, which indicated increases in expression of OLE1 (stearoyl-CoA desaturase), FAD2, and FAD3 in WT in P_i starvation. Fig. 2.3B shows the ratio of 16 and 18-carbon acyl chains, showing the presence of more 18-carbon fatty acids under P_i starvation. The unsaturation indices of PC fatty acids were significantly higher than those of DGTS, indicating that DGTS had fewer double bonds per acyl chain.

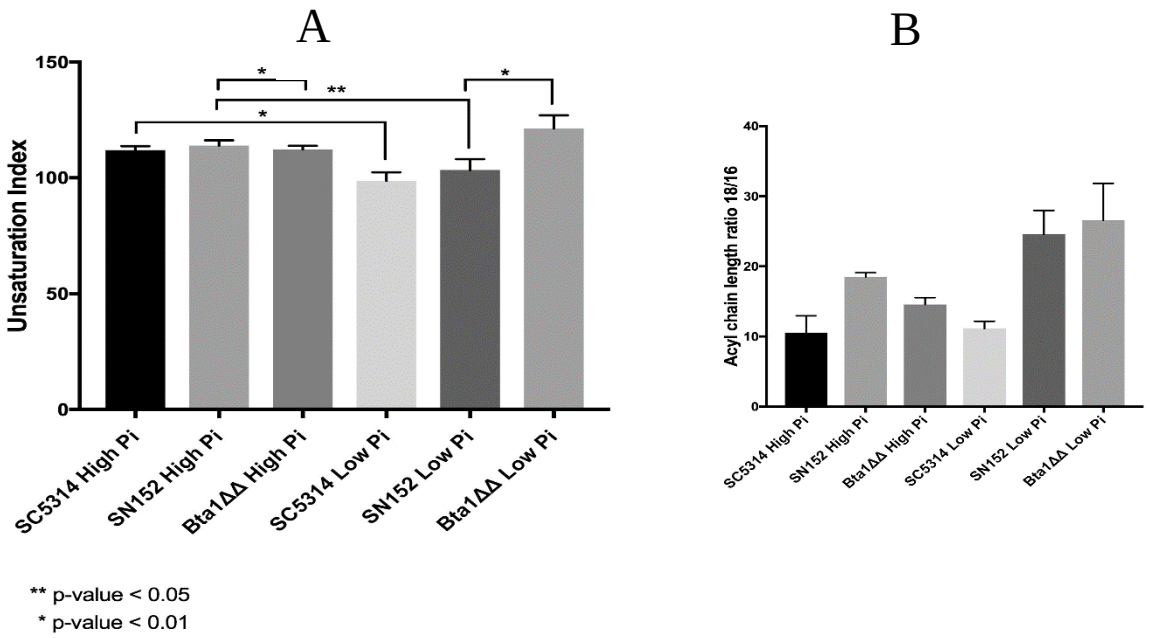

Figure 2.3 – Fatty Acid Unsaturation of PtdCho vs DGTS. Combined PtdCho and DGTS fatty acid compositions in two different wild type strains (SC5314 and SN152) and an SN152-derived *bta1*ΔΔ in high and low-P_i conditions (2 mM and 50 µM, respectively). (A) Unsaturation Index (fatty acid class mole% multiplied by the number of double bonds in that species) of each fatty acid class. (B) Ratio of 18- to 16-carbon acyl chains.

RNA–Sequencing Analysis and validation by qRT-PCR

We performed RNA-sequencing-based transcriptional profiling to determine the P_i starvation response roles of *BTA1* and DGTS in lipid homeostasis, biofilm formation, hyphal formation, iron and copper homeostasis, and pathogenicity. WT SN152 and a SN152-derived *bta1*ΔΔ mutant strain was used for differential gene expression studies. Comparable transcriptional regulation was done under phosphate deplete and replete conditions. We observed that *BTA1* and *PHO84* were highly induced in wild type under Pi limited condition, which supports our hypothesis that the BTA1 is expressed under phosphate limited conditions and is a tightly regulated component of the PHO regulon in *C. albicans*, as has been previously reported in *N. crassa* and *K. lactis* (Riekhof et al., 2014).

Fig. 2.4 shows the numbers of up- and down-regulated genes in WT vs *bta1*ΔΔ in P_i starvation. 69 genes were downregulated only in WT and 30 genes were downregulated only in *bta1*ΔΔ; 78 genes were downregulated in both WT and *bt*a1ΔΔ. In the same conditions, 57 genes were upregulated only in WT and 40 genes were downregulated only in *bta1*ΔΔ; 173 genes were upregulated in both WT and *bta1*ΔΔ.

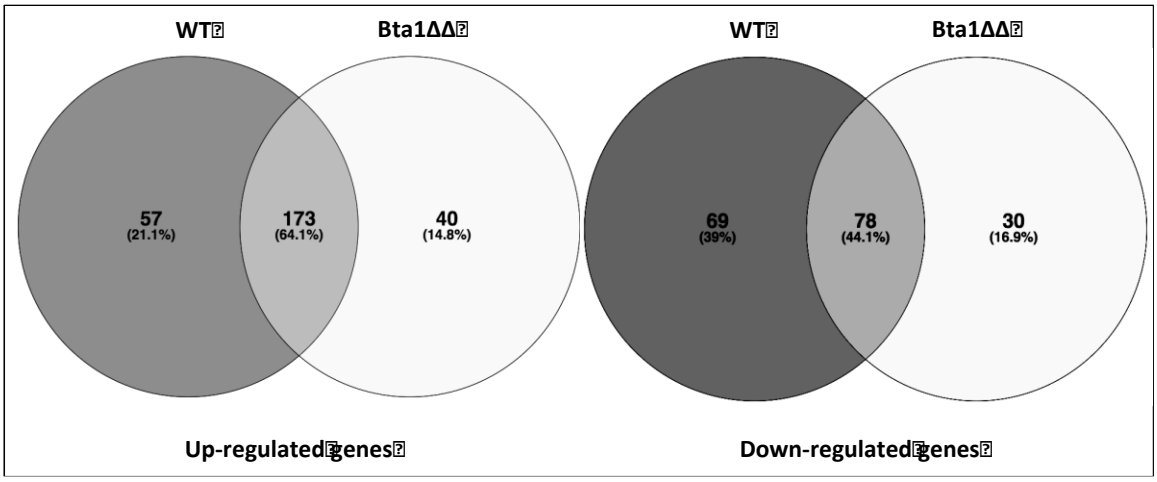

Figure 2.4 - Venn diagram of differentially expressed genes in WT vs *bta1*ΔΔ in P_i starvation. Differentially expressed genes were selected on the basis of the negative binomial distribution of the count reads using EdgeR, FDR adjusted p-value< 0.05.

An examination of genes involved in P_i regulation and storage revealed that high-affinity P_i transporters PHO84 and PHO89 as well as acid phosphatases PHO100, PHO112, and PHO113 were all significantly upregulated; the low-affinity phosphate transporter PHO87 was significantly downregulated. In addition, we observed induction of *GIT1*, a gene which encodes permease for utilizing exogenous glycerophosphoinositol and glycerophosphocholine as P_i sources (Bishop, Sun, Johnson, Bruno, & Patton-Vogt, 2011).

P_i limitation led to the differential regulation (at least a 2-fold change in expression) of 377 genes in WT and 321 genes in *bta1*ΔΔ. In WT, 230 were upregulated and 147 were downregulated. To analyze the differentially expressed genes at the functional level, we performed Gene Ontology (GO) and KEGG pathway enrichment analyses using DAVID

(Database for Annotation, Visualization and Integrated Discovery; https://david.ncifcrf.gov) to determine the enriched biological processes (BPs) and pathways using p-value <= 0.05.

GO analysis (Fig 2.5, 2.6) revealed that several of the genes differentially regulated by P_i starvation were involved in important biological process and molecular functions. The significantly upregulated pathways were involved in cellular response to drugs, glycine catabolism, ribosome biogenesis, and rRNA maturation and processing. A major role of nutrient signaling is the management of ribosome biogenesis and the translational apparatus in response to nutrient starvation. The biological processes significantly downregulated were involved in cell adhesion, cellular response to starvation, filamentous growth, pathogenesis, iron and copper transport. The downregulated genes involved in filamentous growth or hyphal morphology were ECE1, ALS1, ECM22, HWP1, QDR1 and SAP2.

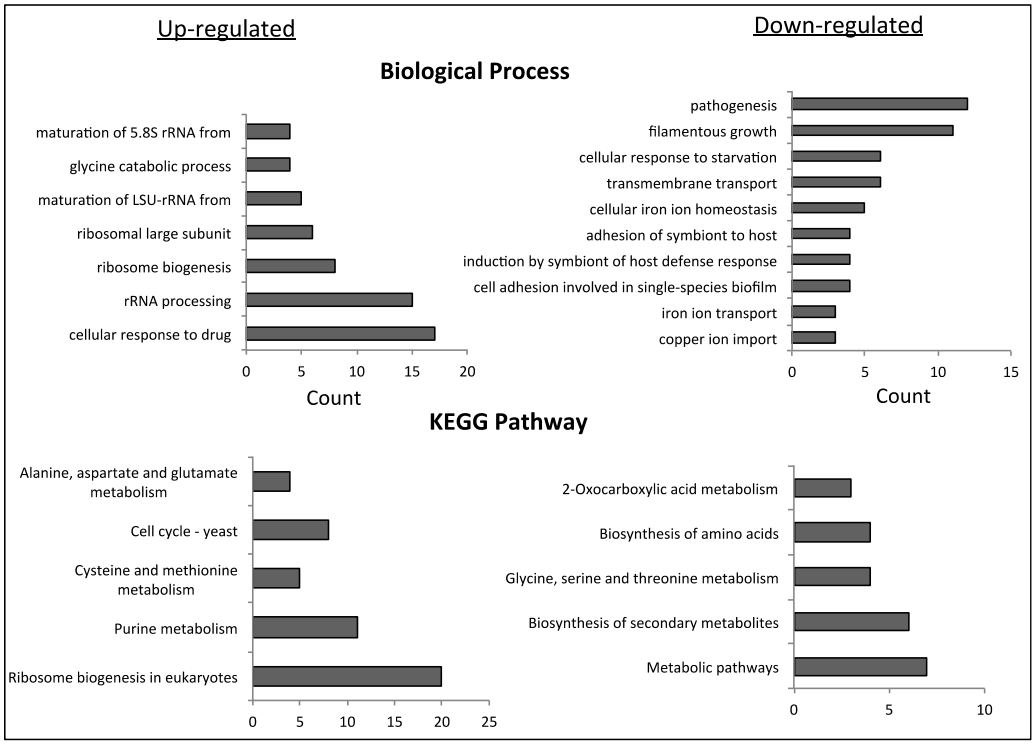

Figure 2.5 – Differentially Expressed Gene Analysis (EdgeR). Histograms show significantly enriched gene term distribution and KEGG analyses with differentially expressed genes in wild type in high (2 mM) vs low (50 µM) P_i to obtain the enriched biological processes (BPs) and pathways using p-value <= 0.05. Counts indicate the number of differentially expressed genes (DEGs) per category. GO term enrichment and KEGG analyses were performed with DAVID bioinformatics tool. DEG analysis was done by EdgeR software package.

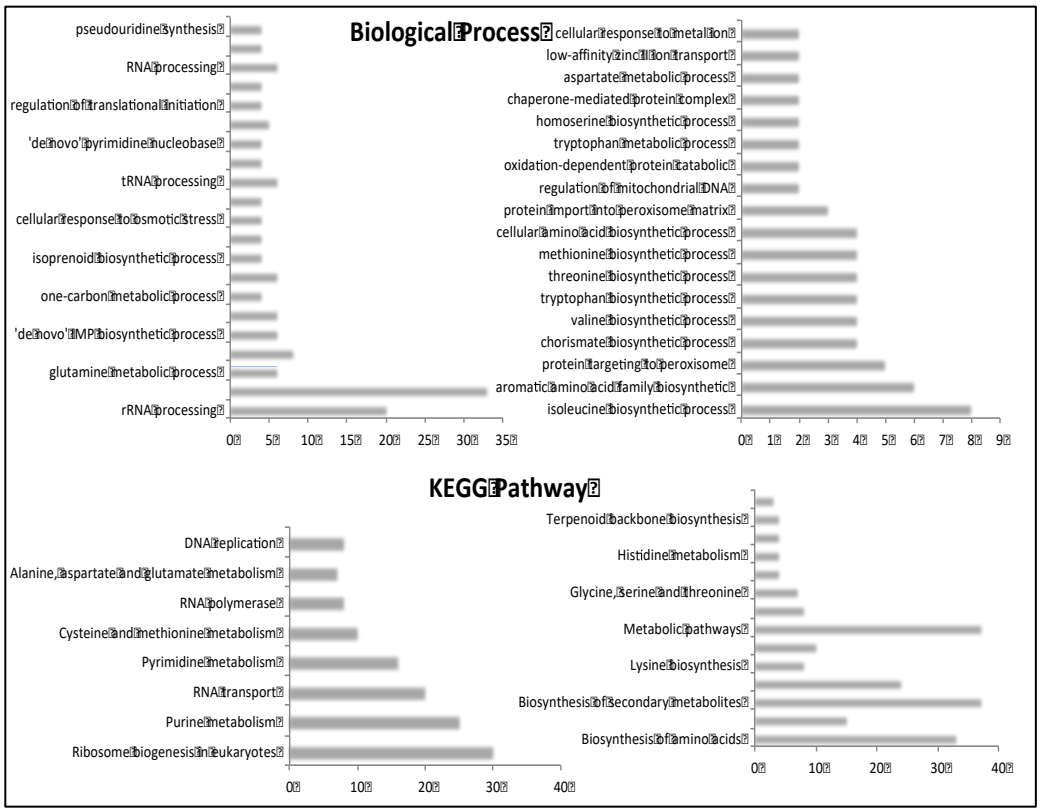

Figure 2.6 - Differentially Expressed Gene Analysis (TopHat and Cufflinks). Histograms show significantly enriched gene term distribution and KEGG analyses with differentially expressed genes in wild type in high (2 mM) vs low (50 µM) P_i to obtain the enriched biological processes (BPs) and pathways using p-value <= 0.05. Counts indicate the number of differentially expressed genes (DEGs) per category. GO term enrichment and KEGG analyses were performed with DAVID bioinformatics tool. DEG analysis was done by TopHat and Cufflinks open-source software tools.

We then verified that the expression of BTA1 is under the control of the PHO regulon by performing quantitative real-time PCR (qPCR) analysis as shown in figure 2.7. We observed a significant increase in BTA1 and PHO84 expression under P_i starvation. We also checked the changes in expression levels of some of the genes in our proposed pathway - *PLD1*, *DPP1* (now shown), and *SAM*2 - in WT under low- and high-P_i conditions and no significant fold changes were observed.

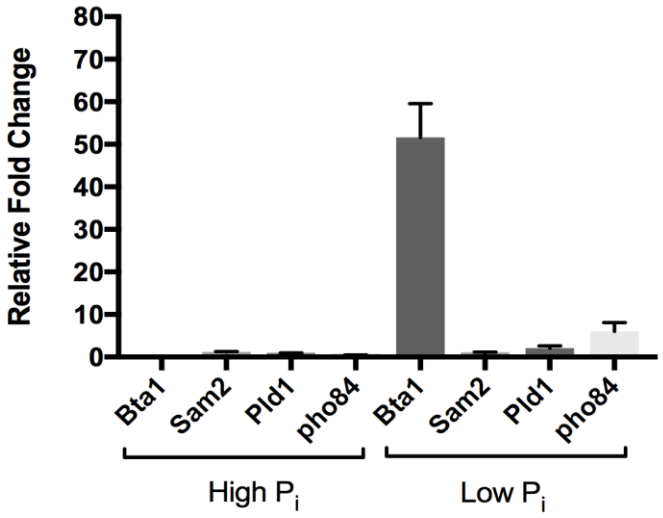

Figure 2.7 – Upregulation of *BTA1* and *PHO84*. Gene expression of *BTA1, SAM2, DPP1, PLD1* and *PHO84* in wild type in high- and low-P_i (2 mM and 50 µM, respectively) synthetic complete (SC) media by quantitative real time PCR (qRT-PCR). Data represent relative fold changes normalized to *Ca*ACT1 as an internal control. The P-Value is 0.003127. The result is significant at $p < 0.05$.

Pathways associated with phospholipid degradation and DGTS synthesis are induced by P_i starvation

Given the genomic context of the *BTA1* gene coupled with RNAseq data for P_i-starved cultures, we postulate a regulatory network of DGTS synthesis. Fig 2.8A shows a 35kb region of C. albicans chromosome 1, which contains a cluster of genes which were then shown by RNAseq analysis to be upregulated under P_i starvation, including *SAM2, PLB1, GDE1, GPT2, SLC1, PAH1,* and *PHO84* in addition to *BTA1*.We therefore predicted these genes to all be coordinately regulated by the PHO regulon for the synthesis of DGTS in response to P_i starvation. We propose a biochemical pathway (Fig 2.8B) for the synthesis of DGTS using fatty acids and glycerol-3-phosphate released from PC and phosphatidylethanolamine (PE) degradation.

We propose (Fig 2.8B) that PtdCho is deacylated by the PLB1 enzyme, the predominant phospholipase B isoform of *C. albicans* (Bishop et al., 2011), which is responsible for the breakdown of lipids into fatty acids and water-soluble glycerophosphocholine (GroPCho). The second step is catalyzed by the glycerophosphodiesterase, GDE1, which hydrolyzes GroPCho to choline and glycerol-3-phosphate (Gro-3-P). Gro-3-P acyltransferases (*Ca*GPT2 or SCT1) acylate Gro-3-P at the *sn-1* position yielding lysophosphatidic acid (lyso-PtdOH), which is then acylated at the *sn-2* position to yield phosphatidic acid (PtdOH). This reaction is catalyzed by a 1-acylglycerol-3-phosphate acyltransferase (AGPAT; alternatively called a lysophospholipid acyltransferase, LPT). The genes known to encode an AGPAT/LPT are SLC1 and *Sc*ALE1/*Ca*LPT1 (Carman & Han, 2011; Czabany, Athenstaedt, & Daum, 2007). Dephosphorylation of PtdOH is catalyzed by a phosphatidate phosphatase (*Ca*PAH1) yielding

diacylglycerol (DAG). Thus, DAG is synthesized from PtdCho by a cluster of genes likely regulated by the PHO regulon. In validation of this model, it has been demonstrated that eukaryotic Pah1 is responsible for membrane lipid remodeling under P_i starvation (Nakamura et al., 2009). Further, in P_i starvation the Pho85p-Pho80p complex phosphorylates Pah1p, thereby regulating Pah1p activity (Choi et al., 2012). This regulation of Pah1p by Pho85p-Pho80p involves important cellular trafficking. Briefly, Pah1p is phosphorylated by Pho85-Pho80 in the cytosol, resulting in its recruitment into the nuclear/ER membrane. Here it is dephosphorylated by the Nem1p-Spo7p protein phosphatase complex, allowing Pah1p to attach to the cytoplasmic membrane and dephosphorylate PtdOH into DAG (Karanasios, Han, Xu, Carman, & Siniossoglou, 2010).

The metabolic pathway of DGTS biosynthesis by BTA1 from DAG has already been demonstrated in bacteria and the fungi *K. lactis* and *N. crassa* (Riekhof et al., 2014). Likewise, *Ca*BTA1 is activated during P_i starvation and is responsible for DTGS synthesis from DAG and S-adenosylmethionine (SAM). SAM2 (also induced in P_i starvation) encodes the only S-adenosylmethionine synthetase in C. albicans, synthesizing SAM from methionine and ATP. 1 SAM molecule acts as the donor of the four-carbon homoserine carbon skeleton to the DAG molecule, and 3 more SAM molecules act as methyl donors for N-trimethylation of the diacylglycerylhomoserine intermediate, yielding DGTS. In summary, PtdCho is degraded to yield DAG, which is then used to make DGTS. This network is further validated by thin layer chromatography (TLC) of lipid extracts, which suggests the replacement of PtdCho with DGTS.

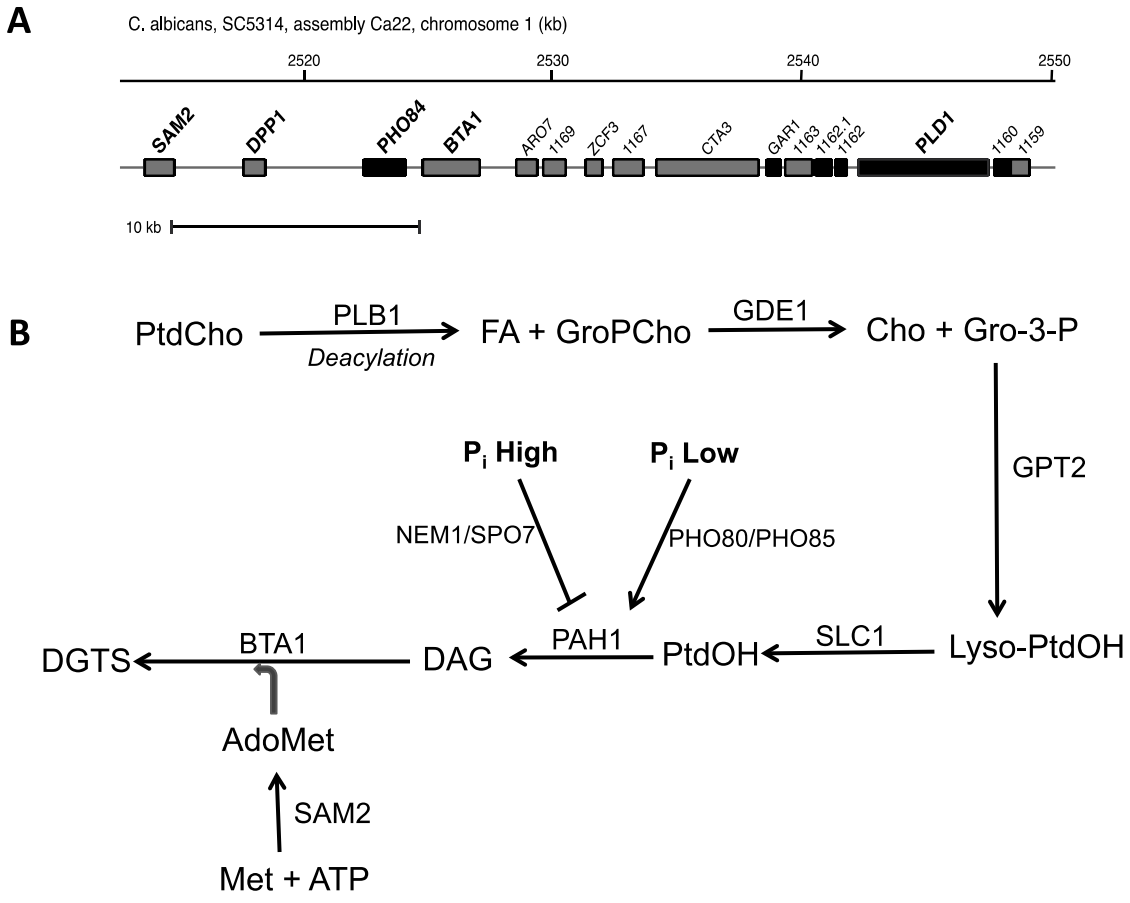

Figure 2.8 - Proposed regulatory pathway of DGTS synthesis in P_i starvation. (A) a 35-kb region of *C. albicans* chromosome 1 consisting of co-regulated genes responsible for DGTS synthesis. (B) Proposed pathway of DGTS biosynthesis in *C. albicans* under P_i starvation. Abbreviations: PtdCho, phosphatidylcholine; GroPCho, glycerophosphocholine; FA, fatty acid; G-3-P, glycerol- 3-phosphate; Lyso-PA, Lyso-phosphatidic acid; PtdOH, phosphatidic acid; DAG, diacylglycerol; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; AdoMet, S-adenosylmethionine

Amino Acid Profiling

Pathway analysis using the KEGG pathway database showed that biosynthesis of free amino acids (glycine, serine, and threonine) and biosynthesis of secondary metabolites were downregulated, whereas alanine, aspartate, glutamate, cysteine, methionine, and purine metabolic pathways were significantly upregulated in response to P_i starvation. These results were validated by measuring amino acid levels in wild type under P_i starvation using ultra-performance liquid chromatography (UPLC).

Our amino acid profiling data (Fig 2.9) showed a significant decrease in the total free amino acid levels in WT (SN152) under P_i starvation. We observed a significant change in the relative concentrations of free amino acids. Some amino acid concentrations decreased, e.g. alanine, aspartic acid, lysine, ornithine, and serine, while glutamine levels increased.

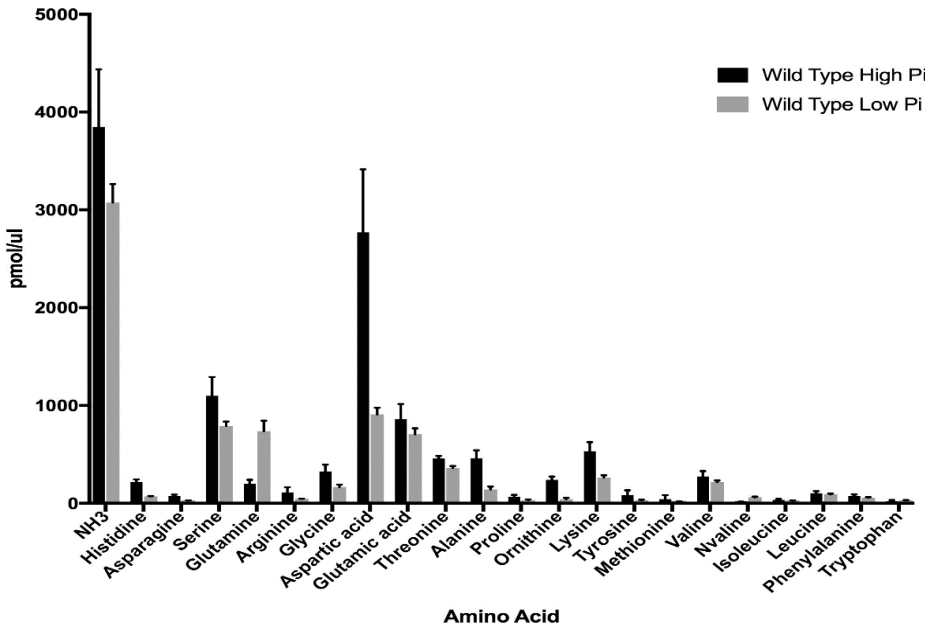

Fig. 2.9 - Amino Acid composition of WT in high (2 mM) and low (50 µM) P_i

In support of the above data, our Gene Ontology and KEGG pathway analysis revealed amino acid and ammonium permeases (GAP1 and MEP2; responsible for amino acid and ammonium transport through the plasma membrane) were downregulated in WT under P_i starvation. It has been previously observed that in the presence of the preferred nitrogen source (e.g., glutamine, glutamate, or ammonium), Gap1 is repressed transcriptionally and post-transcriptionally (Magasanik & Kaiser, 2002). GST1, which encodes a glutathione S-transferase involved in oxidative stress responses and cellular detoxification, is downregulated. SAP2, a secreted aspartyl protease considered to play a fundamental role in *Candida* virulence by providing amino acids to the pathogen by degrading host proteins, was also significantly repressed. AAT22 encodes an aspartate aminotransferase, which plays a role in the metabolism of nitrogen and amino acids by catalyzing the reversible transfer of the amino group from L-aspartate to 2-oxoglutarate to form oxaloacetate and L-glutamate (Palmieri et al., 2006), is significantly upregulated. JEN1, which is a lactate transporter and also facilitates selenite accumulation inside cells (McDermott, Rosen, & Liu, 2010), is upregulated.

Cellular response to metal ions

Metal ions such as iron, copper, and zinc are essential nutrients, playing crucial roles in fundamental cellular processes and in regulating pathogenicity in *C. albicans*. It has been demonstrated that intracellular P_i binds metal cations and that regulating internal P_i concentration is important for metal homeostasis (Rosenfeld et al., 2010). We therefore examined our RNA-seq data to investigate P_i-metal interactions in WT under P_i limitation.

Our RNA-Seq data showed that genes involved in iron, copper, and zinc transport were significantly downregulated in wild type under P_i starvation. Genes belonging to members of the

CFL (CFL2, CFL4, FRP1 and FRP2) ferric reductase, the FET (FET34) multicopper ferroxidase, the FTR (FTR2) plasma membrane iron permease family, the iron/zinc-iron transporter (FTH1, RBT5 and ZRT1), as well as copper transporters (AMO2) were all. One of the SFU1 (Suppressor of Ferric Uptake) target genes *RBT5*, which also functionally coordinates with CaTUP1 was downregulated, whereas CCC1, a manganese transporter, was upregulated.

bta1ΔΔ mutants do not show consistent changes in colony morphology or hyphal formation

In order to evaluate the role of BTA1 in low-P_i growth, colony morphology, hyphal formation, and biofilm formation, multiple *bta1*ΔΔ mutants were created. Two *bta1*ΔΔ strains were constructed using a Candida albicans-specific, CRISPR/Cas9-mediated gene deletion method (Nguyen, Quail, & Hernday, 2017) in an AHY940 WT background. *BTA1* deletion was confirmed by colony PCR (cPCR) with primer sets internal (Fig 2.10A) to and external (Fig 2.10B) to the *BTA1* locus, as well as TLC lipid analysis (Fig 2.11).

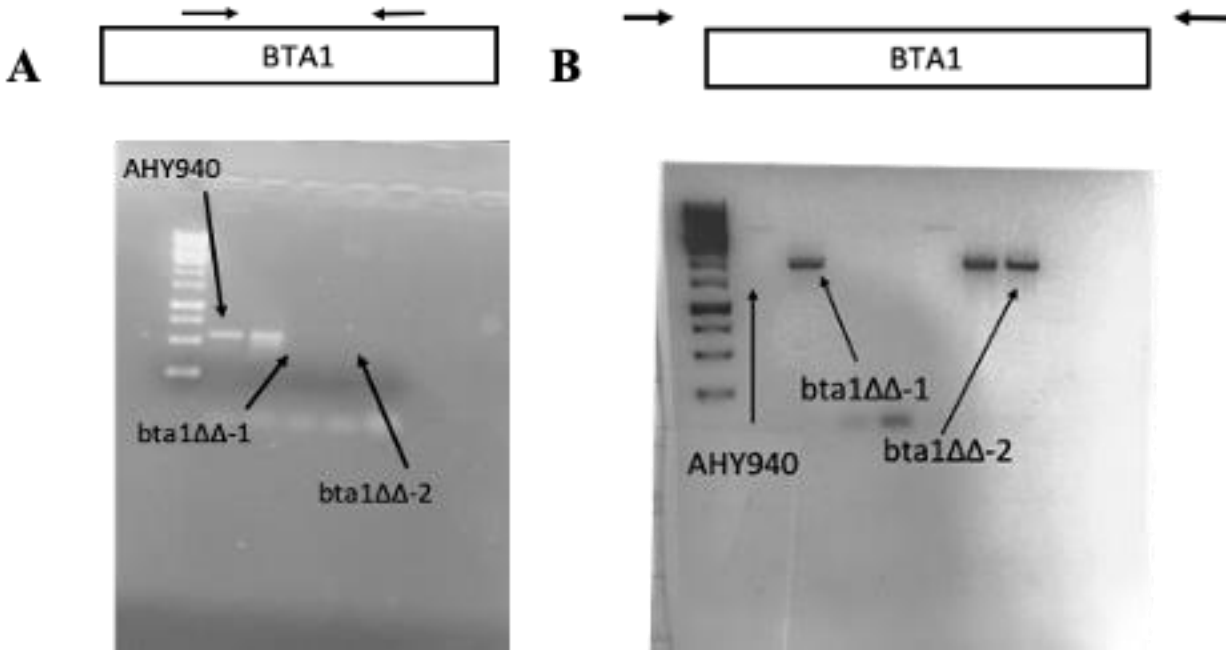

Figure 2.10 – cPCR of CRISPR/Cas9 *bta1*ΔΔ mutants. Primer sets internal (A) and external (B) to *BTA1* were used. Primers external to BTA1 create fragments so large they amplify poorly and show only faint bands; thus, only *bta1*ΔΔ bands are clearly visible.

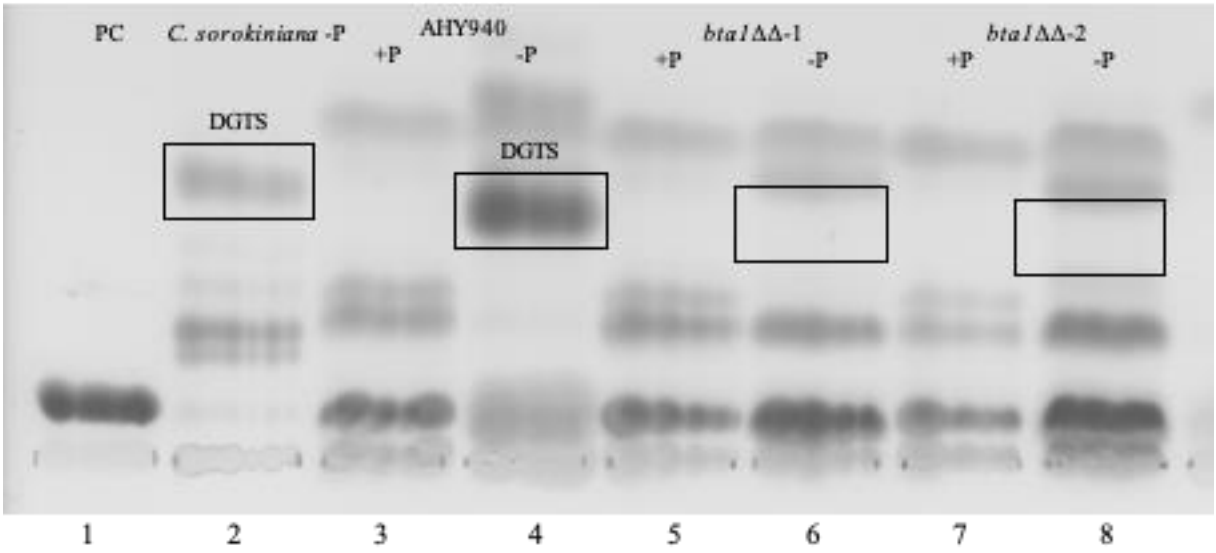

Figure 2.11 – DGTS Production Absent in CRISPR *bta1*ΔΔ Mutants. Identification of DGTS using thin-layer chromatography (TLC). Lane 1 – PtdCho standard; Lane 2 – *C. sorokiniana* in low-P_i media (DGTS standard); Lanes 3-4 – WT CRISPR background strain AHY940 in high and low P_i, respectively; lanes 5-6 - *bta1*ΔΔ-1 in high and low P_i, respectively; lanes 7-8 - *bta1*ΔΔ-2 in high and low P_i, respectively.

Colony morphologies of WT and *bta1*ΔΔ mutants were evaluated on spider (a standard hyphae-inducing growth medium) plates. Though *bta1*ΔΔ mutants exhibited colony morphologies different from WT in these conditions, mutants exhibited morphologies distinct from each other as well (Fig 2.12A). Next, WT and *bta1*ΔΔ mutants were grown on high- and low-P_i PNMC and RPMI 1640 (also hyphae-inducing) plates (Fig 2.12B, C), yielding the same inconsistent differences in colony morphology. Further, *bta1*ΔΔ mutants exhibited no noticeable differences in hyphal formation or length compared to WT in liquid spider media (Fig 2.13).

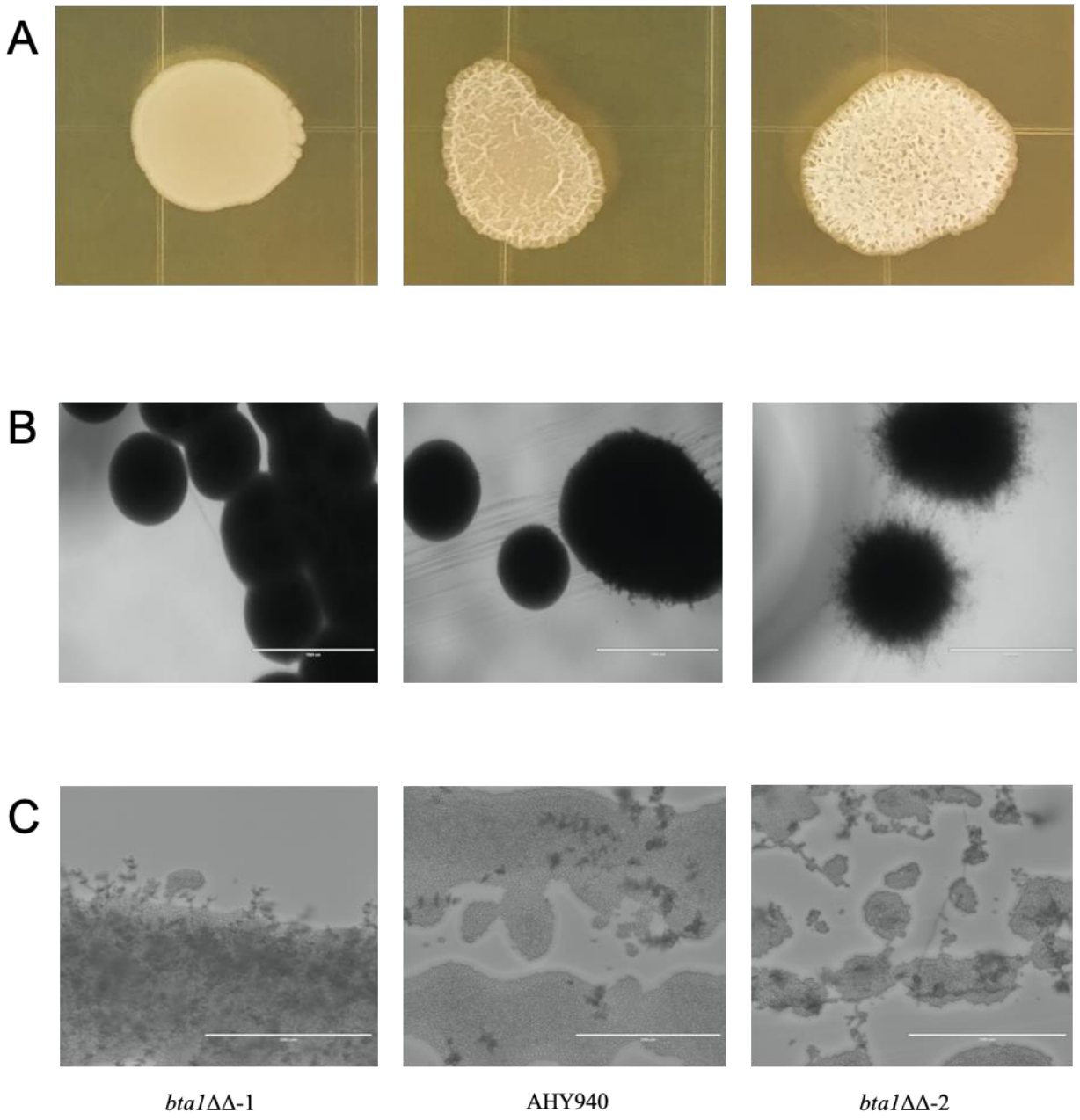

Figure 2.12 – WT (center) vs *bta1*ΔΔ Colony Morphologies. Colonies were grown at 37 °C for 24 – 48 hours on (A) spider media, (B) low-P_i PNMC media, and (C) 50 µM P_i RPMI media solid plates.

Figure 2.13 – WT vs *bta1*ΔΔ Hyphal Morphology. Cultures were grown aerobically in liquid spider media at 37 °C and shaken at 150 rpm for 24 hours.

bta1ΔΔ mutants do not show consistent changes in biofilm formation

Our GO analysis showed that *BTA1* is upregulated in certain biofilm-inducing growth conditions regardless of P_i concentration, leading to the hypothesis that bta1ΔΔ mutants would show decreased biofilm formation. Biofilms were then grown in the human cell medium RPMI 1640 prepared with 2 mM or 50 µM P_i. Imaging with confocal laser scanning microscopy (CLSM) revealed no consistent and significant differences in biofilm formation (Fig 2.14) in either high or low P_i. Strains were further evaluated for their biofilm formation ability in low- and high-P_i RPMI 1640 with a *C. albicans* XTT/menadione assay (Pierce, Uppuluri, Tummala, & Lopez-Ribot, 2010), which measures the number of metabolically active cells by their ability to reduce XTT. Results show no significant difference between WT (AHY940) and *bta1*ΔΔ (AHY940-derived) strains (see Fig 2.15).

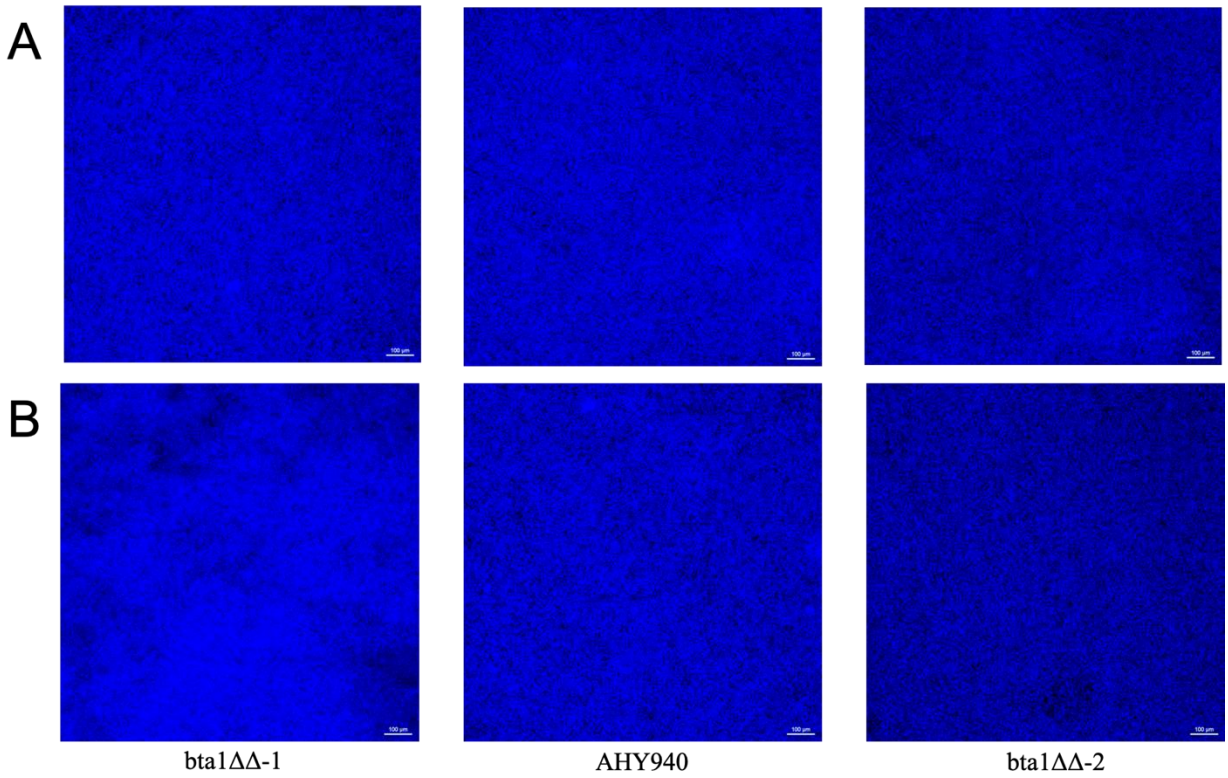

Figure 2.14 – Confocal Laser Scanning Microscopy (CLSM) images of WT vs *bta1*ΔΔ. Grown in high (A) and low (B) phosphate RPMI 1640. Biofilms were stained with calcofluor white.

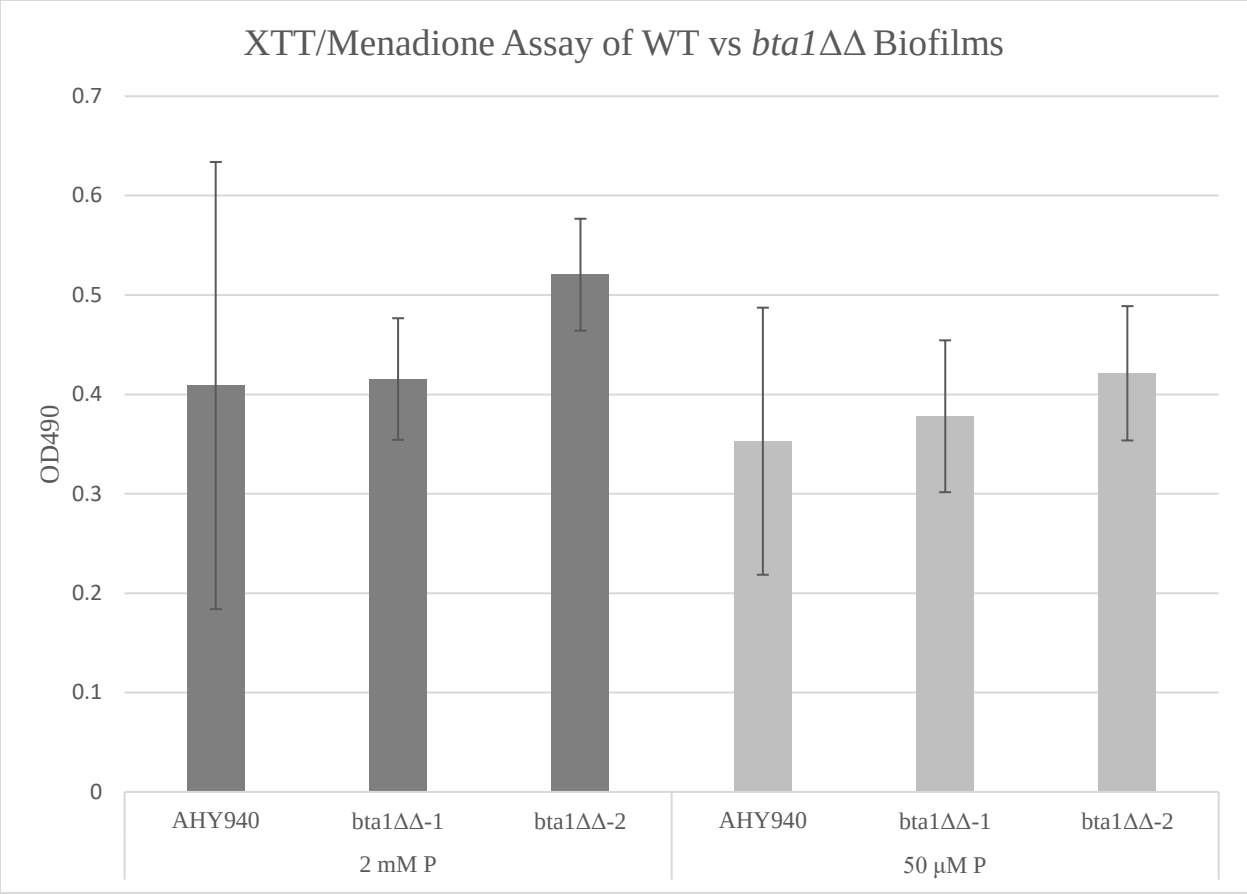

Figure 2.15 – XTT/Menadione Assay of WT vs *bta1*ΔΔ Biofilms. Data points represent averages of 3 wells of each of 3 biological replicates for each strain. Error bars represent standard deviations for each strain.

bta1ΔΔ mutants show reduced growth in P_i starvation

*bta1*ΔΔ mutants were evaluated for their ability to grow in synthetic complete (SC) media under P_i starvation. WT and *bta1*ΔΔ strains showed equivalent growth in high-P_i (2 mM) SC (Fig 2.16A), but *bta1*ΔΔ mutants showed a decreased maximum cell density in low-P_i (50 µM) SC with *bta1*ΔΔ mutants reaching a max OD_{600} reading of approximately 0.9 – 1.0, and WT reaching a max OD_{600} reading of approximately 1.3 (Fig 2.16B). In comparing *bta1*ΔΔ and

*pho4*ΔΔ mutant growth to WT in low P_i (30 µM), the *pho4*ΔΔ mutant showed almost no growth in low P_i, while *bta1*ΔΔ mutants showed moderately reduced growth (Fig 2.17).

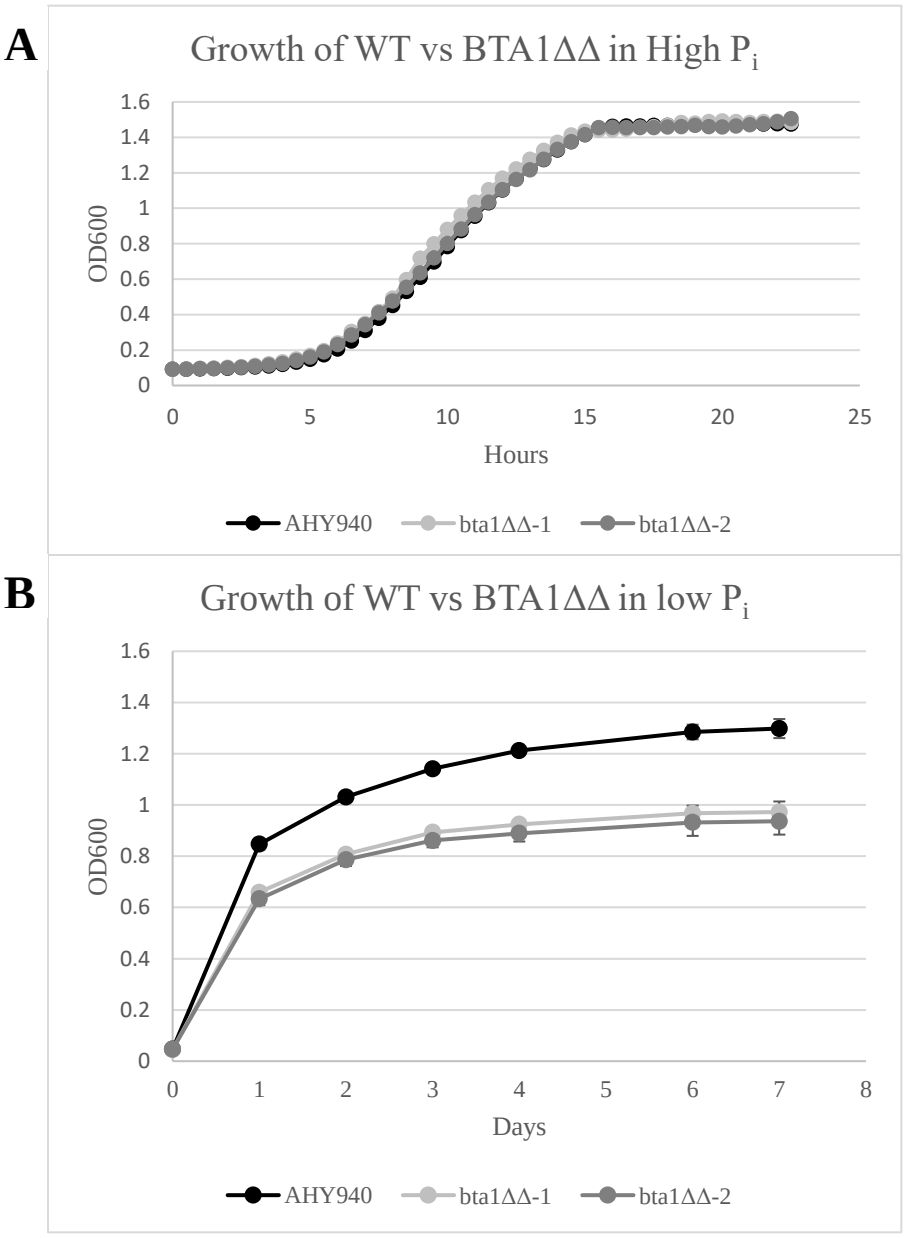

Figure 2.16 – Growth of WT (AHY940) and *bta1*ΔΔ mutants in (A) high- and (B) low-P_i SC media. Data points in low P_i graph represent averages of 3 wells each of 3 biological replicates for each strain. Error bars in low P_i graph represent standard deviations for each strain. Data points in high P_i graphs are single replicates, but are representative of multiple consistent experiments

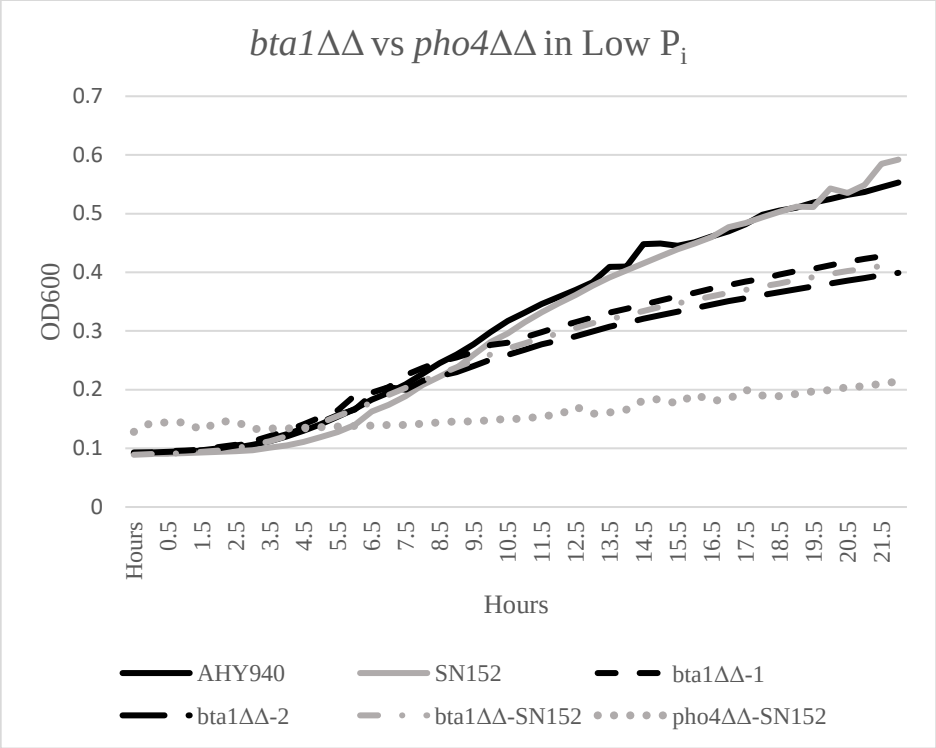

Figure 2.17 – *BTA1* vs *PHO4* Effects on Low Pi Growth. WT mutant backgrounds (AHY940, SN152), *bta1*ΔΔ mutants (AHY940- and SN152-derived), and an SN152-derived *pho4*ΔΔ strain in high- and low-P_i SC media. Data points are single replicates.

Discussion

Our data suggest some new insights into survival strategies of *C. albicans* under P_i starvation. Here we demonstrate that in *Candida albicans* the *BTA1* gene is solely responsible for the synthesis of the betaine lipid DGTS in response to P_i starvation, and that the expression of *BTA1* is under control of the fungal PHO regulon. We also propose a regulatory pathway of betaine lipid synthesis by which PtdCho is degraded and replaced with DGTS.

Our data indicate that molecular species of DGTS and PtdCho differ in their fatty acid compositions. DGTS fatty acids were more saturated (contained fewer double bonds) and had shorter acyl chains. The relatively higher saturation and shorter acyl chain length of DGTS likely lower cell membrane fluidity, meaning the cell is less permeable to various ions and therefore requires less energy expenditure to maintain homeostasis. It has been reported in many organisms that manipulating membrane fluidity via fatty acid saturation/unsaturation is an important response to cold stress (Murakami et al., 2018; Nakagawa, Sakumoto, Kaneko, & Harashima, 2002). Lowering maintenance costs by decreasing membrane fluidity in these ways (i.e., by decreasing desaturase and elongase acitivity) may be one of the key general stress response mechanisms of cells.

Under P_i starvation the desaturases *OLE2*, *FAD2* and *FAD3* are highly upregulated. This is surprising given that, as noted above, DGTS (present in low P_i starvation) is more saturated than PtdCho (present in high P_i). This may indicate that, compared to PtdCho, DGTS is a poor substrate for desaturases, thereby resulting in more saturation in low P_i despite desaturase upregulation.

Our transcriptome data shows evidence of crosstalk between transcriptional regulation of phosphate and nitrogen stress responses. When P_i is limited, the cell lowers its metabolic activity

to conserve energy. Our amino acid analysis showed a decrease in the total free amino acid pool in wild type under P_i starvation, consistent with our transcriptome data which demonstrated the downregulation of amino acid and ammonium permeases, which are important for amino acid transport and metabolism. The aspartate aminotransferase (AAT22), on the other hand, was upregulated. Aspartate aminotransferase catalyzes the transfer of the amino group from aspartate to 2-oxoglutarate to form oxaloacetate and glutamate (Palmieri et al., 2006) and thereby contributes to the sequestering of glutamate for nitrogen storage. This storage of nitrogen in glutamate (which lowers the amount of readily available nitrogen in the cytoplasm) is likely important in low P_i conditions to keep the proper balance between cytoplasmic nitrogen and P_i pools; as both nitrogen and P_i are required for nucleic acid synthesis, imbalance in the ratio of the two nutrients might inhibit nucleic acid synthesis.

The effects of perturbations in P_i homeostasis have previously been studied in *S. cerevisiae*, the pathogenic fungus *C. neoformans,* and *C. albicans* (Ikeh et al., 2016; Kretschmer et al., 2014; Rosenfeld et al., 2010). These studies demonstrate that cytoplasmic P_i binds metal ions and is important for maintaining metal homeostasis. Further, in *pho*4Δ mutants (which are unable to control cytoplasmic polyphosphate synthesis) genes involved in iron, zinc and copper acquisition were downregulated but intracellular iron levels were increased, resulting in impaired resistance to metal ions. A lack of cytoplasmic P_i appears to decrease metal-binding capacity, to which cells respond by decreasing metal transport into the cytoplasm. In line with these findings, our transcriptome data revealed that genes involved in iron, copper, and zinc transport were significantly downregulated in response to Pi starvation.

DGTS production has been shown to reduce the total P requirement for soil communities (Warren, 2020), and algae show reduced growth in low P_i conditions if the betaine lipid synthase

is knocked out (López-Lara et al., 2005; Murakami et al., 2018). Likewise, our data show that *bta1*ΔΔ growth in low P_i is significantly reduced (OD_{600} ~ 0.9-1.0) compared to WT (OD_{600} ~ 1.3), corresponding to, roughly, a 30-40% decrease in total cell number. In *C. albicans*, *PHO4* encodes a transcription factor which activates a whole suite of P_i starvation response genes (Ikeh et al., 2016), and *pho4*ΔΔ mutant growth is almost completely eliminated in low P_i conditions. Given that *BTA1* is only one of many genes induced by *PHO4* to obtain and conserve P_i in starvation conditions, *BTA1* effects on low P_i growth are surprisingly high. DGTS production thus represents an extremely important piece of the total P_i starvation response.

Recent work has demonstrated that P_i starvation response mechanisms in pathogens, including *C. albicans* (Romanowski et al., 2012), are important for virulence, and several important pathogens produce DGTS. The pathogenic fungus *Cryptococcus neoformans* produces DGTS, and a bta1Δ mutant showed reduced lethality in mice and reduced CFU after growth in RPMI 1640 liquid media compared to WT (Lev et al., 2019). Further, our GO analysis shows that some biofilm genes are induced in low P_i conditions. This led us to investigate whether *BTA1* deletion affected *C. albicans* virulence.

Because hyphal formation is crucial to *C. albicans* virulence, we first investigated whether *bta1*ΔΔ mutants showed decreased hyphal formation. Our results show that on both high and low P_i hyphae-inducing media, *bta1*ΔΔ strains do exhibit different colony morphologies from WT; however, colony morphologies of each mutant also differ from each other, and thus suggest that these morphologies are being controlled by other genetic differences between the two mutants (Fig 2.8), perhaps as a result of CRISPR background mutations. bta*1ΔΔ*-2 shows more aerial growth, while *bta1*ΔΔ-1 colonies look similar to those grown on non-hyphae inducing media such as YPD. AHY940 colonies look like an intermediate between the two

mutants, alternatively suggesting that AHY940 may comprise populations with background genetics similar to both *bta1*ΔΔ-1 and *bta1*ΔΔ-2, representatives of which each happened to be selected in CRISPR gene editing. Hyphal formation appears to be unaffected. Biofilm formation is crucial for *C. albicans* virulence (Douglas, 2003; Kojic & Darouiche, 2004), so we investigated whether *BTA1* deletion might affect biofilm formation. However, microscopy analysis and XTT assays of WT and *bta1*ΔΔ biofilms showed no consistent or significant difference in biofilm formation ability. Importantly, however, domesticated *C. albicans* lab strains do not always show virulence comparable to clinical isolates in response to P_i starvation, and virulent responses to P_i starvation may vary significantly among all strains (Romanowski et al., 2012). Regardless, *BTA1* is crucial for growth in low P_i conditions, and therefore may prove to be important for pathogenesis in more realistic infection models involving P_i-depleted guts.

In summary, our current study demonstrates that: i. P_i starvation induces the degradation of phosphatidylcholine and its replacement with the P_i–free lipid DGTS; ii. Transcriptomic changes under P_i starvation suggest an increased flux of carbon from serine and glycine into the C1-pathway and increased flux into methionine, S-adenosylmethionine, DGTS, and polyamines; iii. Amino acid levels and transcriptomic data suggest that nitrogen is sequestered into glutamate in response to low P_i, perhaps to maintain stoichiometry between N and P as well as lower overall cell metabolism; iv. Expression of genes important for metal transport is reduced in low P_i conditions, likely because P_i is an important counterion for binding and stabilizing intracellular metal ions; v. Biofilm genes are induced in low P_i conditions, but no consistent or significant defects in hyphal or biofilm formation in *bta1*ΔΔ strains were detected; iv. Deletion of the *BTA1* gene results in a significant reduction of total cell growth. Taken together, these

results demonstrate the importance of DGTS production to growth in low P_i and provide new insights regarding biochemical adaptations to P_i starvation.

Methods

Strains and Growth Conditions

Candida albicans wild types (SC5314, SN152, and AHY940) and SN152-derived *bta1*ΔΔ and *pho4*ΔΔ mutant strains were obtained from the Fungal Genetic Stock Center. For pre-inoculum and routine maintenance, cells were grown aerobically overnight at 30°C in/on YPD media containing yeast extract (10 g/L), peptone (20 g/L), and dextrose (20 g/L). For phosphate starvation experiments, cells were grown aerobically at 30°C in synthetic complete (SC) media (0.67% w/v yeast nitrogen base without inositol, 2% w/v glucose, complete amino acid mixture) containing high (2 mM) or low (50 μM) phosphate concentrations and buffered with 2.4 g/L HEPES. For biofilm experiments, RPMI 1640 media were made according to the USBiological R8999-04A RPMI 1640 Medium (w/o Amino Acids, Sodium Phosphate) formulation (pH 7.4) and supplemented with high (2 mM) or low (50 μM) phosphate concentrations.

Multiple *bta1*ΔΔ mutants were constructed for low-P_i growth and hyphal and biofilm assays using a *C. albicans*-specific CRISPR/Cas9 method (Nguyen et al., 2017). Briefly, an "A" fragment consisting of a promoter, Cas9 gene, and portions of *C. albicans LEU2* and *NAT* (nourseothricin resistance) genes is joined via overlap extension stitching PCR to a "B" fragment consisting of a gRNA gene and the remaining *LEU2* and *NAT* gene portions. The resulting "C" fragment, along with a donor DNA "repair" fragment consisting of upstream and downstream portions of the target gene, is integrated directly into the single leu2 locus of WT strain AHY940, allowing for selection with nourseothricin for successful transformation. Transformed colonies are then selected for loss of CRISPR/Cas9 machinery (and thereby restoration of the single

LEU2 allele) in SC – leucine and verified via cPCR and TLC. Primers and DNA oligonucleotides were ordered from Integrated DNA Technologies (idtdna.com), and plasmids were ordered from Addgene (addgene.org). The *BTA1* CRISPR target sequence was designed according to the Nguyen et al protocol recommendation using the "Design and Analyze Guides" tool on Benchling (www.benchling.com) with the following settings: Design Type, single guide; Guide Length, 20bp; Genome, CA22 (Candida albicans SC5314 (Diploid)); PAM, NGG.

Name	**Sequence**	
AHO1096	GACGGCACGGCCACGCGTTTAAACCGCC	
AHO1097	CCCGCCAGGCGCTGGGGTTTAAACACCG	
AHO1098	CAAATTAAAAATAGTTTACGCAAG	
AHO1237	AGGTGATGCTGAAGCTATTGAAG	
AHO1238	TGTATTTTGTTTTAAAATTTTAGTGACTGTTTC	
BTA1gRNA	CGTAAACTATTTTTAATTTGAAGGCATTAGAAGACATGTGGTTTTAGAGCTAGAAATAGC	
cPCR (Internal)	Fw	TGACAACCTTAGAATCCACAC
	Rv	TACAAGGTTCATACCGTACAG
cPCR (External)	Fw	ACACAATCAACAATTTCCCGAG
	Rv	CGTTTGACTTTCTTGATTCCAG

Table 2.1– CRISPR/Cas9 Primers Used in This Study

Name	Description
pADH110	NAT 2 of 2, pSNR52
pADH119	gRNA conserved, C. albicans LEU2 2 of 2
pADH137	C. albicans LEU2 1 of 2, pENO1, Cas9, NAT 1of 2
Sequence	
BTA1dDNAtop	AAAGTTTACTACTACAATTTTTTTTTTGGTG AAACCAATTTGCTTCAGAATAGTTAGAATAT AAGTTTTGTTATATATAAATTGTTAGTTGTAG TCTTG
BTA1dDNAbtm	CAAGACTACAACTAACAATTTATATATAACA AAACTTATATTCTAACTATTCTGAAGCAAATT GGTTTCACCAAAAAAAAAAATTGTAGTAGTA AACTTT

Table 2.2 – CRISPR/Cas9 Plasmids and DNA Oligonucleotides Used in This Study

Lipid Extraction and Analysis

Total lipid was extracted using Bligh-Dyer method (Bligh & Dyer, 1959) using chloroform/methanol (2:1, v/v). Briefly, cultures were grown to log phase, harvested, and lysed using acid-washed glass beads (Sigma). Low-P_i cultures were grown to 50 mL volumes to achieve adequate cell mass, while high-P_i cultures were grown to 4 mL. Individual phospholipids were separated by thin layer chromatography (TLC) on silica 60 plates (Sigma-Aldrich) using a chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5 v/v) solvent system. Bands were visualized by brief exposure of the plate to iodine vapor. For identification of individual lipids by ESI-MS/MS, bands were separated by TLC, the silica was scraped into a glass tube, and the purified compound extracted in chloroform/methanol (2:1), and then dried under N2 gas.

Electrospray ionization-tandem mass spectroscopy (ESI-MS/MS)

Extracted lipids were then analyzed for phospholipid identification and confirmation using a direct infusion ESI-MS/MS approach. Briefly, the extracted lipids were resuspended in 500 μL of chloroform:methanol (2:1) and spiked with 15:0-PC as an internal standard. Samples were then diluted 5000X in water/isopropyl alcohol/methanol (55:35:10 v/v/v) containing ammonium formate (25 mM) and 0.4% formic acid, and directly infused into the mass spectrometer at a rate of 10 μL/min. Instrument settings were as follows: source temperature - 300°C; ESI needle voltage - 5.5 kV; declustering potential - 90; entrance potential - 10; curtain gas, 10; gas 1 - 50 arbitrary units; gas 2 - 40 arbitrary units; nitrogen gas was used as a collision gas (H. J. Kim et al., 2015).

Lipids were analyzed with a triple quadrupole/linear ion trap mass spectrometer (Sciex QTRAP 4000) in positive ion mode for precursor scans of 184.1 and 236.1 for PtdCho and DGTS respectively. PtdCho was identified with a precursor ion scan of m/z 184.1 and DGTS was detected in positive ion mode as neutral loss of 236.1. The scan was taken over the mass range of 200-1000 m/z with a cycle time of 2 seconds. DGTS showed two peaks at m/z 736.8 and 710.8 with major molecular species of 32:2 and 34:2 (total carbon atoms:total double bonds). In the chromatogram, PtdCho showed four predominant peaks at m/z 784.6, 782.6, 758.7 and 757.7 with major molecular species of 36:5, 36:3, 32:3 and 32:1 respectively.

RNA isolation and gene expression by qRT-PCR

Cultures were grown to A_{600} 0.8-1.0 on SC-minimal media (described previously) with high (2 mM) and low (50 μM) Phosphate concentrations. Total RNA was extracted using OMEGA E.Z.N.A Yeast RNA Kit according to manufacturer's instructions. RNA purification was done using GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific). cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). qRT-PCR was done on Eppendorf Realplex^2 Mastercycler using SYBR GreenER qPCR Supermix (Invitrogen).

Primer3 software was used to design forward and reverse qRT-PCR primers for *Ca*BTA1, *Ca*SAM2, *Ca*DPP1, *Ca*PHO4 and *Ca*PLD1 (Table I). Gene expression was analyzed using $\Delta\Delta C_T$ and normalized to *Ca*ACT1 used as endogenous control. Primers were checked for specificity by melting curve analysis and visualizing in gel electrophoresis. Controls were used lacking the template and reverse transcriptase. Primers for the particular genes did not show any amplification when their respective deletion mutants were used. Wild type expression in low phosphate was normalized to their respective internal controls (HKG) and then used to compare with expression in high phosphate. All experiments were performed in triplicate.

No.	Primer Name	Sequence
1.	q*Ca*BTA1-Fw	5'-CCCAACTTTCAATGCTGCTA-3'
	q*Ca*BTA1-Rv	5'-ATGTCCCTGTTAACCAACCC-3'
2.	q*Ca*SAM2-Fw	5'-CCTTCTCAGGCAAGGATTTC-3'
	q*Ca*SAM2- Rv	5'-GCATAGGAGAACTGCACCAA-3'
3.	q*Ca*DPP1-Fw	5'-TTGGTGGGAATTGAAGTTTG-3'
	q*Ca*DPP1- Rv	5'-TCCAACCAACCATAATGACAA-3'
4.	q*Ca*PHO4-Fw	5'-ATGTCGACGAACACGATGAT-3'
	q*Ca*PHO4- Rv	5'-GTGGAGCAATTTCTTGAGCA-3'
5.	q*Ca*PLD1-Fw	5'-CTCAGTGGTTTGTTGACGCT-3'
	q*Ca*PLD1- Rv	5'-AAGCCACCAATCGTGTATCA-3'
6.	q*Ca*ACT1- Fw	5'-CATGGTTGGTATGGGTCAAA-3'
	q*Ca*ACT1- Rv	5'-TATCGTCCCAGTTGGAAACA-3'

Table 2.3 – qRT-PCR Primers used in this study

RNA-Sequencing Analysis

Briefly, wild type SN152 and SN152-derived *bta1*ΔΔ mutant strains were grown to log phase in low- and high-P_i conditions. Cells were then collected and washed with UltraPureTM DNase/RNase-free distilled water. Total RNA was extracted using OMEGA E.Z.N.A Yeast RNA Kit according to manufacturer's instructions. RNA purification was done using GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific). RNA samples were sent to GENEWIZ for Next Generation Sequencing (www.genewiz.com). Sequencing was performed on the Illumina HiSeq2500 platform in a 1x50bp single-read configuration in Rapid Run mode,

with a total of at least 120 million reads per lane or ~15M reads per sample. Reads were aligned to the Candida genome (version 22) with Tophat2 (D. Kim et al., 2013) and used to calculate differentially expressed genes with the EdgeR software package (Robinson, McCarthy, & Smyth, 2009). Venn diagrams were constructed using Venny (version 2.1.0) online freeware and statistical comparisons were performed with GraphPad Prism (version 6).

Amino Acid Profiling by Ultra-Performance Liquid Chromatography (UPLC)

Wild type SN152 was grown aerobically at 30°C to A_{600} 0.8-1.0 in synthetic complete media containing high (2 mM) or low (50 μM) phosphate concentrations and frozen in liquid nitrogen. The samples for free amino acid (FAA) analysis were processed as described by Hacham et al. (Hacham, Avraham, & Amir, 2002). Briefly, frozen samples (10 mL each) were mechanically lysed by glass beads (acid-washed) in the presence of 600 μL of water:chloroform:methanol (3:5:12, v/v). Samples were centrifuged and supernatants were collected. A second extraction with another 600 μL of water:chloroform:methanol (3:5:12, v/v) was performed on each of the remaining residues, followed by centrifugation and collection of supernatant, and supernatants were pooled. Chloroform (300 μL) and water (450 μL) were added, and the resulting mixture was centrifuged again. The upper water:methanol layer was collected and dried. The samples were resuspended in HCl 20 mM and further used for total free amino acid quantification on an Agilent 1290 Infinity II UPLC.

The extracted amino acids were derivatized using AccQ-Tag reagents (Waters, Milford, MA, USA) according to the manufacturer's protocol. Briefly, 70 μL of AccQ-Tag Ultra borate buffer was added to 10 μL of the biological extract to ensure optimum pH. 20 μL of AccQ-Tag reagent previously dissolved in 1.0 mL of AccQ-Tag Ultra reagent diluent were added to each

sample and the tubes were incubated at 55 °C for 10 min. The same steps were followed for the external standard calibration curve. Liquid chromatographic separation was performed on the AccQ-Tag Ultra column (Waters, 2.1 mm I.D. × 100 mm, 1.7 μm) at 43°C with a 1290 Infinity II UPLC system (Agilent, Santa Clara, CA, USA). The UPLC is equipped with the Flexible quaternary pump running at a flow rate of 0.7 mL/min. Mobile phases A-D are: A = 100% Waters AccQ-Tag Eluent A; B = 10% Waters AccQ-Tag Eluent B; C = 100% Milli-Q water; D = 100% Waters AccQ-Tag Eluent B. The gradient was as follows : T = 0, 9.9% A, 90.1% C; T = 0.29 min, 9.9% A, 90.1% C; T = 4.84 min, 9.1% A, 70% B, 20.9% C, 0% D; T = 6.45 min, 8% A, 15.6% B, 58.9% C, 17.5% D; T = 6.65 min, 8% A, 15.6% B, 58.9% C, 17.5% D; T = 7.04 min, 7.8% A, 0% B, 71.9% C, 20.3% D; T = 7.64 min, 13.7% A, 0% B, 36.3% C, 50% D ; T = 8.89 min, 13.7% A, 0% B, 36.3% C, 50% D ; T = 8.98 min, 9.9% A, 0% B, 90.1% C, 0% D . The signal was detected using the PDA detector at 260 nm with a sampling rate of 40 Hz. 1 μL of sample and standards were injected for analysis. The concentrations of amino acids were calculated using the external standard calibration curve. The concentrations in pmol/μL for each amino acid were detected in each sample and are calculated using a series of standard dilutions run before the samples.

Growth Curves

For AHY940 vs AHY940-derived *bta1*ΔΔ strain growth curve (figure 2.12B), strains were grown overnight in YPD at 30°C, washed twice in ddH_20, and incubated for 7 hours in 0-P_i SC media. Strains were again washed twice in ddH_20 and counted using a Countess II FL Automated Cell Counter (Thermofisher). 10^5 cells were added to wells in tissue-culture treated 24-well plates, 1 mL of low-P_i (50 μM) or high-P_i (2 mM) SC media were added to each well, and OD_{600} readings were taken daily with a Synergy HI Hybrid Reader (BioTek). Three wells of each of three replicates for each strain were used.

For other growth curves (figure 2.12A, 2.13), strains were grown overnight in YPD, diluted in ddH_2O, counted, and standardized to WT cell count (~10^5). Cells were added to 2X serial dilutions of 2 mM P_i SC into 0 mM P_i SC in 96 well plates and grown in the plate reader set for 30C every 30 minutes.

Colony Morphology

Colony morphologies of WT and *bta1*ΔΔ mutants were evaluated on YPD and standard hyphae-inducing (spider, lee's) solid plates and liquid media. Strains were grown overnight in YPD at 30°C, washed twice and diluted in 1 mL PBS, 10 μL cell suspension was added to plates, and plates were grown at 37°C for 48 – 72 hours.

Biofilm Microscopy

10^5 cells of each strain were added to 2 mL of 2 mM or 50 μM P_i RPMI 1640 media in a 24-well plate and incubated for 2 hours at 37C and 5% CO_2 to allow for cell adherence. RPMI media were then aspirated and replaced with 2 mL of the same fresh media and incubated for 48

hours at 37C and 5% CO_2. Media were again aspirated and replaced with 1 mL calcofluor white solution and incubated at 37C for 30 minutes. Samples were then imaged with Nikon A1-NiE laser scanning confocal microscope (Nikon Instruments, Melville, NY). The samples were excited at a wavelength of 405 nm and emission at wavelengths between 425 nm and 475 nm were collected. Images were acquired by using 10x objective lens with the help of NIS elements software and by Z stacking (Z series) of 1µm slices. Settings for laser power, detector gain, and offset for individual set of samples were unchanged during acquisition. 3 random areas were imaged for each sample.

Biofilm XTT Assay

An XTT/menadione assay was carried out based on (Pierce et al., 2010) to evaluate biofilm formation. Strains were pre-starved in 50 µM P_i SC media at 30C for 48 hours, washed in 0.9% sterile saline, and counted using a Countess II FL Automated Cell Counter (Thermofisher). Cells were diluted to 10^7 cells/mL in 2 mM or 50 µM RPMI 1640 medium, 100-µL aliquots were transferred to wells of a tissue culture-treated 96 well plate, and samples were incubated at 37C, 5% CO_2 for 48 hours. RPMI 1640 media were then aspirated from each well and 100 µL of XTT/menadione solution (0.5 g/L XTT in 0.9% sterile saline, 1 mM menadione) was added to each well. Plates were incubated for 2 hours at 37C, 80 µL of XTT/menadione solution was aspirated from each well and transferred to a new plate, and OD_{490} readings were taken. Three wells of each of three replicates for each strain were used.

References

Abida, H., Dolch, L. J., Meï, C., Villanova, V., Conte, M., Block, M. A., … Maréchal, E. (2015). Membrane glycerolipid remodeling triggered by nitrogen and phosphorus starvation in Phaeodactylum tricornutum. *Plant Physiology*, *167*(1), 118–136. https://doi.org/10.1104/pp.114.252395

Aktas, M., Danne, L., Möller, P., & Narberhaus, F. (2014). Membrane lipids in Agrobacterium tumefaciens: Biosynthetic pathways and importance for pathogenesis. *Frontiers in Plant Science*, *5*(MAR), 1–13. https://doi.org/10.3389/fpls.2014.00109

Araki, S., Eichenberger, W., Sakurai, T., & Sato, N. (1991). Distribution of diacylglycerylhydroxymethyltrimethyl-β-alanine (DGTA) and phosphatidylcholine in brown algae. *Plant and Cell Physiology*, *32*(5), 623–628. https://doi.org/10.1093/oxfordjournals.pcp.a078124

Benning, C., Huang, Z. H., & Gage, D. A. (1995). Accumulation of a novel glycolipid and a betaine lipid in cells of rhodobacter sphaeroides grown under phosphate limitation. *Archives of Biochemistry and Biophysics*. https://doi.org/10.1006/abbi.1995.1141

Bishop, A. C., Sun, T., Johnson, M. E., Bruno, V. M., & Patton-Vogt, J. (2011). Robust utilization of phospholipase-generated metabolites, glycerophosphodiesters, by Candida albicans: Role of the CaGit1 permease. *Eukaryotic Cell*, *10*(12), 1618–1627. https://doi.org/10.1128/EC.05160-11

Björklund, Å. K., Ekman, D., Light, S., Frey-Skött, J., & Elofsson, A. (2005). Domain rearrangements in protein evolution. *Journal of Molecular Biology*, *353*(4), 911–923. https://doi.org/10.1016/j.jmb.2005.08.067

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, *37*, 911–917.

Brown, A. E., & Elovson, J. (1974). Isolation and characterization of a novel lipid, 1(3),2-diacylglyceryl-(3)-O-4′-(N,N,N-trimethyl)homoserine, from ochromonas danica. *Biochemistry*, *13*(17), 3476–3482. https://doi.org/10.1021/bi00714a009

Carman, G. M., & Han, G.-S. (2011). Regulation of Phospholipid Synthesis in the Yeast Saccharomyces cerevisiae . *Annual Review of Biochemistry*, *80*(1), 859–883. https://doi.org/10.1146/annurev-biochem-060409-092229

Choi, H. S., Su, W. M., Han, G. S., Plote, D., Xu, Z., & Carman, G. M. (2012). Pho85p-Pho80p phosphorylation of yeast pah1p phosphatidate phosphatase regulates its activity, location, abundance, and function in lipid metabolism. *Journal of Biological Chemistry*, *287*(14), 11290–11301. https://doi.org/10.1074/jbc.M112.346023

Czabany, T., Athenstaedt, K., & Daum, G. (2007). Synthesis, storage and degradation of neutral lipids in yeast. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1771*(3), 299–309. https://doi.org/10.1016/j.bbalip.2006.07.001

Danhorn, T., Hentzer, M., Givskov, M., Parsek, M. R., & Fuqua, C. (2004). Phosphorus Limitation Enhances Biofilm Formation of the Plant Pathogen Agrobacterium tumefaciens through the PhoR-PhoB Regulatory System. *Society*, *186*(14), 4492–4501. https://doi.org/10.1128/JB.186.14.4492

Dembitsky, V. M. (1996). Betaine ether-linked glycerolipids: Chemistry and biology. *Progress in Lipid Research*. https://doi.org/10.1016/0163-7827(95)00009-7

Douglas, L. J. (2003). Candida biofilms and their role in infection. *Trends in Microbiology*, *11*(1), 30–36. https://doi.org/10.1016/S0966-842X(02)00002-1

Eichenberger, W. (1982). Distribution of diacylglyceryl-O-4′-N,N,N-trimethyl)- homoserine in different algae. *Plant Science Letters*, *24*(1), 91–95. https://doi.org/10.1016/0304-4211(82)90012-8

Eichenberger, W., Araki, S., & Müller, D. G. (1993). Betaine lipids and phospholipids in brown algae. *Phytochemistry*, *34*(5), 1323–1333. https://doi.org/10.1016/0031-9422(91)80024-U

Eichenberger, W., & Gribi, C. (1997). Lipids of Pavlova lutheri: Cellular site and metabolic role of DGCC. *Phytochemistry*, *45*(8), 1561–1567. https://doi.org/10.1016/S0031-9422(97)00201-X

Geiger, O., González-Silva, N., López-Lara, I. M., & Sohlenkamp, C. (2010). Amino acid-containing membrane lipids in bacteria. *Progress in Lipid Research*, *49*(1), 46–60. https://doi.org/10.1016/j.plipres.2009.08.002

Geiger, O., Röhrs, V., Weissenmayer, B., Finan, T. M., & Thomas-Oates, J. E. (1999). The regulator gene phoB mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-N,N,N-trimethylhomoserine in Rhizobium (Sinorhizobium) meliloti. *Molecular Microbiology*, *32*(1), 63–73. https://doi.org/10.1046/j.1365-2958.1999.01325.x

Geske, T., Vom Dorp, K., Dörmann, P., & Hölzl, G. (2013). Accumulation of glycolipids and other non-phosphorous lipids in Agrobacterium tumefaciens grown under phosphate deprivation. *Glycobiology*, *23*(1), 69–80. https://doi.org/10.1093/glycob/cws124

Ginneken, V. van. (2017). Seaweed Competition: Ulva Sp. has the Potential to Produce the Betaine Lipid Diacylglyceryl-O-4'-(N,N,N,-Trimethyl) Homoserine (DGTS) in Order to Replace Phosphatidylcholine (PC) Under Phosphate-Limiting Conditions in the P-Limited Dutch Wadden Sea and Ou. *Oceanography & Fisheries Open Access Journal*, *2*(5). https://doi.org/10.19080/ofoaj.2017.02.555596

Guan, C. D., Wanner, B., & Inouye, H. (1983). Analysis of regulation of phoB expression using a phoB-cat fusion. *Journal of Bacteriology*, *156*(2), 710–717.

Hacham, Y., Avraham, T., & Amir, R. (2002). The N-terminal region of Arabidopsis cystathionine γ-synthase plays an important regulatory role in methionine metabolism. *Plant Physiology*, *128*(2), 454–462. https://doi.org/10.1104/pp.010819

Hofmann, M., & Eichenberger, W. (1996). Biosynthesis of diacylglyceryl-N,N,N-trimethylhomoserine in Rhodobacter sphaeroides and evidence for lipid-linked N methylation. *Journal of Bacteriology*, *178*(21), 6140–6144. https://doi.org/10.1128/jb.178.21.6140-6144.1996

Hölzl, G., & Dörmann, P. (2007). Structure and function of glycoglycerolipids in plants and bacteria. *Progress in Lipid Research*. https://doi.org/10.1016/j.plipres.2007.05.001

Hong, M. Y., Chapkin, R. S., Barhoumi, R., Burghardt, R. C., Turner, N. D., Henderson, C. E., … Lupton, J. R. (2002). Fish oil increases mitochondrial phospholipid unsaturation , upregulating reactive oxygen species and apoptosis in rat colonocytes n-3 fatty acids) with the butyrate-producing fiber pectin , development . We now hypothesize that n-3 fatty acids. *Carcinogenesis*, *23*(11), 1919–1925.

Ikeh, M. A. C., Kastora, S. L., Day, A. M., Herrero-De-Dios, C. M., Tarrant, E., Waldron, K. J., … Quinn, J. (2016). Pho4 mediates phosphate acquisition in Candida albicans and is vital for stress resistance and metal homeostasis. *Molecular Biology of the Cell*, *27*(17), 2784–2801. https://doi.org/10.1091/mbc.E16-05-0266

Istokovics, A., Morita, N., Izumi, K., Hoshino, T., Yumoto, I., Sawada, M. T., … Okuyama, H. (1998). Neutral lipids, phospholipids, and a betaine lipid of the snow mold fungus Microdochium nivale. *Canadian Journal of Microbiology*, *44*(11), 1051–1059.

Karanasios, E., Han, G. S., Xu, Z., Carman, G. M., & Siniossoglou, S. (2010). A phosphorylation-regulated amphipathic helix controls the membrane translocation and function of the yeast phosphatidate phosphatase. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(41), 17539–17544. https://doi.org/10.1073/pnas.1007974107

Kato, M., Adachi, K., Hajiro-Nakanishi, K., Ishigaki, E., Sano, H., & Miyachi, S. (1994). A betaine lipid from Pavlova lutheri. *Phytochemistry*. https://doi.org/10.1016/0031-9422(94)85041-0

Kato, M., Sakai, M., Adachi, K., Ikemoto, H., & Sano, H. (1996). Distribution of betaine lipids in marine algae. *Phytochemistry*, *42*(5), 1341–1345. https://doi.org/10.1016/0031-9422(96)00115-X

Khozin-Goldberg, I., & Cohen, Z. (2006). The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte Monodus subterraneus. *Phytochemistry*, *67*, 696–701. https://doi.org/10.1016/j.phytochem.2006.01.010

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., & Salzburg, S. L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology*, *14*, R36.

Kim, H. J., Silva, J. E., Iskandarov, U., Andersson, M., Cahoon, R. E., Mockaitis, K., & Cahoon, E. B. (2015). Structurally divergent lysophosphatidic acid acyltransferases with high selectivity for saturated medium chain fatty acids from Cuphea seeds. *Plant Journal*, *84*(5), 1021–1033. https://doi.org/10.1111/tpj.13063

Klug, R. M., & Benning, C. (2001). Two enzymes of diacylglyceryl-O-4′-(N,N,N,-trimethyl)-homoserine biosynthesis are encoded by btaA and btaB in the purple bacterium Rhodobacter sphaeroides. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(10), 5910–5915. https://doi.org/10.1073/pnas.101037998

Kojic, E. M., & Darouiche, R. O. (2004). Candida Infections of Medical Devices. *Clinical Microbiology Reviews*, *17*(2), 255–267. https://doi.org/10.1128/CMR.17.2.255-267.2004

Kretschmer, M., Reiner, E., Hu, G., Tam, N., Oliveira, D. L., Caza, M., … Kronstad, J. W. (2014). Defects in phosphate acquisition and storage influence virulence of Cryptococcus neoformans. *Infection and Immunity*, *82*(7), 2697–2712. https://doi.org/10.1128/IAI.01607-14

Kunzler, K., & Eichenberger, W. (1997). Betaine lipids and zwitterionic phospholipids in plants and fungi. *Phytochemistry*, *46*(5), 883–892. https://doi.org/10.1016/S0031-9422(97)81274-5

Lami, R., Cottrell, M. T., Ras, J., Ulloa, O., Obernosterer, I., Claustre, H., … Lebaron, P. (2007). High abundances of aerobic anoxygenic photosynthetic bacteria in the South Pacific Ocean. *Applied and Environmental Microbiology*, *73*(13), 4198–4205. https://doi.org/10.1128/AEM.02652-06

Lev, S., Kaufman-Francis, K., Desmarini, D., Juillard, P. G., Li, C., Stifter, S. A., … Djordjevic, J. T. (2017). Pho4 Is Essential for Dissemination of Cryptococcus neoformans to the Host Brain by Promoting Phosphate Uptake and Growth at Alkaline pH . *MSphere*, *2*(1), 1–21. https://doi.org/10.1128/msphere.00381-16

Lev, S., Rupasinghe, T., Desmarini, D., Kaufman-Francis, K., Sorrell, T. C., Roessner, U., & Djordjevic, J. T. (2019). The PHO signaling pathway directs lipid remodeling in Cryptococcus neoformans via DGTS synthase to recycle phosphate during phosphate deficiency. *PLoS ONE*, *14*(2), 1–18. https://doi.org/10.1371/journal.pone.0212651

Long, J., Zaborina, O., Holbrook, C., Zaborin, A., & Alverdy, J. (2008). Depletion of Intestinal Phosphate following Surgical Injury Activates the Virulence of P. aeruginosa causing Lethal Gut-Derived Sepsis. *Surgery*, *144*(2), 189–197. https://doi.org/10.1038/jid.2014.371

López-Lara, I. M., Gao, J. L., Soto, M. J., Solares-Pérez, A., Weissenmayer, B., Sohlenkamp, C., … Geiger, O. (2005). Phosphorus-free membrane lipids of Sinorhizobium meliloti are not required for the symbiosis with alfalfa but contribute to increased cell yields under phosphorus-limiting conditions of growth. *Molecular Plant-Microbe Interactions*, *18*(9), 973–982. https://doi.org/10.1094/MPMI-18-0973

Magasanik, B., & Kaiser, C. A. (2002). Nitrogen regulation in Saccharomyces cerevisiae. *Gene*. https://doi.org/10.1016/S0378-1119(02)00558-9

McDermott, J. R., Rosen, B. P., & Liu, Z. (2010). Jen1p: A High Affinity Selenite Transporter in Yeast. *Molecular Biology of the Cell*, *21*, 3934–3941. https://doi.org/10.1091/mbc.E10

Minnikin, D. E., Abdolrahimzadeh, H., & Baddiley, J. (1974). Replacement of acidic phospholipids by acidic glycolipids in Pseudomonas diminuta. *Nature*, *249*(5454), 268–269. https://doi.org/10.1038/249268a0

Morgan, J. (2005). Global Trends in Candidemia: Review of Reports from 1995-2005. *Current Infectious Disease Reports*, *7*, 429–439.

Mühlroth, A., Winge, P., El Assimi, A., Jouhet, J., Maréchal, E., Hohmann-Marriott, M. F., … Bonesa, A. M. (2017). Mechanisms of phosphorus acquisition and lipid class remodeling under P limitation in a marine microalga. *Plant Physiology*, *175*(4), 1543–1559. https://doi.org/10.1104/pp.17.00621

Murakami, H., Nobusawa, T., Hori, K., Shimojima, M., & Ohta, H. (2018). Betaine Lipid Is Crucial for Adapting to Low Temperature and Phosphate Deficiency in Nannochloropsis. *Plant Physiology*, *177*(1), 181–193. https://doi.org/10.1104/pp.17.01573

Nakagawa, Y., Sakumoto, N., Kaneko, Y., & Harashima, S. (2002). Mga2p Is a Putative Sensor for Low Temperature and Oxygen to Induce OLE1 Trascription in Saccharomyces cerevisiae. *Biochemical and Biophysical Research Communications*, *291*(3), 707–713.

Nakamura, Y. (2013). Phosphate starvation and membrane lipid remodeling in seed plants. *Progress in Lipid Research*, *52*(1), 43–50. https://doi.org/10.1016/j.plipres.2012.07.002

Nakamura, Y., Koizumi, R., Shui, G., Shimojima, M., Wenk, M. R., Ito, T., & Ohtad, H. (2009). Arabidopsis lipins mediate eukaryotic pathway of lipid metabolism and cope critically with phosphate starvation. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(49), 20978–20983. https://doi.org/10.1073/pnas.0907173106

Naoki Sato, & Norio Murata. (1991). Transition of lipid phase in aqueous dispersions of diacylglyceryltrimethylhomoserine. *Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism*. https://doi.org/10.1016/0005-2760(91)90306-3

Nguyen, N., Quail, M. M. F., & Hernday, A. D. (2017). An Efficient, Rapid, and Recyclable System for CRISPR-Mediated Genome Editing in Candida albicans. *Molecular Biology and Physiology*, *2*(2), 1–10.

Nichols, B. W., & Appleby, R. S. (1969). The distribution and biosynthesis of arachidonic acid in algae. *Phytochemistry*, *8*, 1907–1915. https://doi.org/10.1016/S0031-9422(00)88075-9

Palmieri, F., Agrimi, G., Blanco, E., Castegna, A., Di Noia, M. A., Iacobazzi, V., … Walker, J. (2006). Identification of mitochondrial carriers in Saccharomyces cerevisiae by transport assay of reconstituted recombinant proteins. *Biochimica et Biophysica Acta - Bioenergetics*. https://doi.org/10.1016/j.bbabio.2006.05.023

Pflaster, E. L., Schwabe, M. J., Becker, J., Wilkinson, M. S., Parmer, A., Clemente, T. E., …

Riekhof, W. R. (2014). A high-throughput fatty acid profiling screen reveals novel variations in fatty acid biosynthesis in Chlamydomonas reinhardtii and related algae. *Eukaryotic Cell*, *13*(11), 1431–1438. https://doi.org/10.1128/EC.00128-14

Pierce, C. G., Uppuluri, P., Tummala, S., & Lopez-Ribot, J. L. (2010). A 96 well microtiter plate-based method for monitoring formation and antifungal susceptibility testing of Candida albicans biofilms. *Journal of Visualized Experiments*, (44), 1–4. https://doi.org/10.3791/2287

Popko, J., Herrfurth, C., Feussner, K., Ischebeck, T., Iven, T., Haslam, R., … Feussner, I. (2016). Metabolome analysis reveals betaine lipids as major source for triglyceride formation, and the accumulation of sedoheptulose during nitrogen-starvation of Phaeodactylum tricornutum. *PLoS ONE*, *11*(10), 1–23. https://doi.org/10.1371/journal.pone.0164673

Riekhof, W. R., Andre, C., & Benning, C. (2005). Two enzymes, BtaA and BtaB, are sufficient for betaine lipid biosynthesis in bacteria. *Archives of Biochemistry and Biophysics*, (441), 96–105. https://doi.org/10.1016/j.abb.2005.07.001

Riekhof, W. R., Naik, S., Bertrand, H., Benning, C., & Voelker, D. R. (2014). Phosphate starvation in fungi induces the replacement of phosphatidylcholine with the phosphorus-free betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine. *Eukaryotic Cell*, *13*(6), 749–757. https://doi.org/10.1128/EC.00004-14

Riekhof, W. R., Sears, B. B., & Benning, C. (2005). Annotation of Genes Involved in Glycerolipid Biosynthesis in. *Eukaryotic Cell*, *4*(2), 242–252. https://doi.org/10.1128/EC.4.2.242

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2009). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140. https://doi.org/10.1093/bioinformatics/btp616

Romanowski, K., Zaborin, A., Valuckaite, V., Rolfes, R. J., Babrowski, T., Bethel, C., … Alverdy, J. C. (2012). Candida albicans isolates from the gut of critically ill patients respond to phosphate limitation by expressing filaments and a lethal phenotype. *PLoS ONE*, *7*(1). https://doi.org/10.1371/journal.pone.0030119

Rosenfeld, L., Reddi, A. R., Leung, E., Aranda, K., Jensen, L. T., & Culotta, V. C. (2010). The effect of phosphate accumulation on metal ion homeostasis in Saccharomyces cerevisiae. *Journal of Biological Inorganic Chemistry*, *15*(7), 1051–1062. https://doi.org/10.1007/s00775-010-0664-8

Sato, N. (1991). Lipids in Cryptomonas CR-1. II. Biosynthesis of Betaine Lipids and Galactolipids. *Plant and Cell Physiology*, *32*(6), 845–851. https://doi.org/10.1093/oxfordjournals.pcp.a078152

Sato, N. (1992). Betaine Lipids. *The Botanical Magazine Tokyo*, *105*, 185–197.

Sato, N., & Furuya, M. (1985). Distribution of diacylglyceryltrimethylhomoserine and phosphatidylcholine in non-vascular green plants. *Plant Science*, *38*(2), 81–85. https://doi.org/10.1016/0168-9452(85)90134-7

Schubotz, F., Xie, S., Lipp, J. S., Hinrichs, K. U., & Wakeham, S. G. (2018). Intact polar lipids in the water column of the eastern tropical North Pacific: Abundance and structural variety of non-phosphorus lipids. *Biogeosciences*, *15*(21), 6481–6501. https://doi.org/10.5194/bg-15-6481-2018

Senik, S. V., Maloshenok, L. G., Kotlova, E. R., Shavarda, A. L., Moiseenko, K. V., Bruskin, S. A., … Psurtseva, N. V. (2015). Diacylglyceryltrimethylhomoserine content and gene expression changes triggered by phosphate deprivation in the mycelium of the

basidiomycete Flammulina velutipes. *Phytochemistry*, *117*(1), 34–42. https://doi.org/10.1016/j.phytochem.2015.05.021

Shemi, A., Schatz, D., Fredricks, H. F., Van Mooy, B. A. S., Porat, Z., & Vardi, A. (2016). Phosphorus starvation induces membrane remodeling and recycling in Emiliania huxleyi. *New Phytologist*, *211*(3), 886–898. https://doi.org/10.1111/nph.13940

Tatsuzawa, H., & Takizawa, E. (1995). Changes in lipid and fatty acid composition of Pavlova lutheri. *Phytochemistry*, *40*(2), 397–400. https://doi.org/10.1016/0031-9422(95)00327-4

Torriani-Gorini, A., Yagil, E., & Silver, S. (1994). *Phosphate in Microorganisms: Cellular and Molecular Biology*. Washington, DC: ASM Press.

Van Mooy, B. A. S., Fredricks, H. F., Pedler, B. E., Dyhrman, S. T., Karl, D. M., Koblížek, M., … Webb, E. A. (2009). Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature*, *458*(7234), 69–72. https://doi.org/10.1038/nature07659

VanBogelen, R. A., Olson, E. R., Wanner, B. L., & Neidhardt, F. C. (1996). Global analysis of proteins synthesized during phosphorus restriction in Escherichia coli. *Journal of Bacteriology*, *178*(15), 4344–4366. https://doi.org/10.1128/jb.178.15.4344-4366.1996

Vardi, N., Levy, S., Gurvich, Y., Polacheck, T., Carmi, M., Jaitin, D., … Barkai, N. (2014). Sequential feedback induction stabilizes the phosphate starvation response in budding yeast. *Cell Reports*, *9*(3), 1122–1134. https://doi.org/10.1016/j.celrep.2014.10.002

Vogel, G., & Eichenberger, W. (1992). Betaine Lipids in Lower Plants. Biosynthesis of DGTS and DGTA in Ochromonas danica (Chrysophyceae) and the Possible Role of DGTS in Lipid Metabolism. *Plant and Cell Physiology*, *33*(4), 427–436. https://doi.org/10.1093/oxfordjournals.pcp.a078271

Vogel, G., Woznica, M., Gfeller, H., Müller, C., Stämpfli, A. A., Jenny, T. A., & Eichenberger, W. (1990). 1(3),2-Diacylglyceryl-3(1)-O-2′-(hydroxymethyl)(N,N,N,-trimethyl)-β-alanine (DGTA): A novel betaine lipid from Ochoromonas danica (Chrysophyceae). *Chemistry and Physics of Lipids*, *52*(2), 99–109. https://doi.org/10.1016/0009-3084(90)90154-J

Warren, C. R. (2020). Soil microbial populations substitute phospholipids with betaine lipids in response to low P availability. *Soil Biology and Biochemistry*, *140*(November 2019), 107655. https://doi.org/10.1016/j.soilbio.2019.107655

Yamada, T., & Nozawa, Y. (1979). An unusual lipid in the human pathogenic fungus Epidermophyton floccosum. *Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism*. https://doi.org/10.1016/0005-2760(79)90239-X