



Central Role of the Trehalose Biosynthesis Pathway in the Pathogenesis of Human Fungal Infections: Opportunities and Challenges for Therapeutic Development

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SUMMARY	1
INTRODUCTION	2
TREHALOSE PATHWAY	2
What Is Trehalose?	2
History of Trehalose	3
Biochemical Properties of Trehalose	4
Trehalose Biosynthesis in Fungi	4
Specific Functions of Trehalose in Fungi	7
TPS1 (TREHALOSE-6-PHOSPHATE SYNTHASE)	11
TPS2 (TREHALOSE-6-PHOSPHATE PHOSPHATASE)	14
REGULATORY SUBUNITS	16
TPS3/TSL1	16
NONCANONICAL TREHALOSE BIOSYNTHESIS IN FUNGI	17
TREHALASES	18
TREHALOSE TRANSPORTERS	19
REGULATION OF THE TREHALOSE PATHWAY	19
FUTURE RESEARCH DIRECTIONS AND CLINICAL POTENTIAL	21
ACKNOWLEDGMENTS	24
REFERENCES	25
AUTHOR BIOS	30

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SUMMARY Invasive fungal infections cause significant morbidity and mortality in part due to a limited antifungal drug arsenal. One therapeutic challenge faced by clinicians is the significant host toxicity associated with antifungal drugs. Another challenge is the fungistatic mechanism of action of some drugs. Consequently, the identification of fungus-specific drug targets essential for fitness *in vivo* remains a significant goal of medical mycology research. The trehalose biosynthetic pathway is found in a wide variety of organisms, including human-pathogenic fungi, but not in humans. Genes encoding proteins involved in trehalose biosynthesis are mechanistically linked to the metabolism, cell wall homeostasis, stress responses, and virulence of *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. While there are a number of pathways for trehalose production across the tree of life, the TPS/TPP (trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase) pathway is the canonical pathway found in human-pathogenic fungi. Importantly, data suggest that proteins involved in trehalose biosynthesis play other critical roles in fungal metabolism and *in vivo* fitness that remain to be fully elucidated. By further defining the biology and functions of trehalose and its biosynthetic pathway components in pathogenic fungi, an opportunity exists to leverage this pathway as a potent antifungal drug target. The goal of this review is to cover the known roles of this important molecule

and its associated biosynthesis-encoding genes in the human-pathogenic fungi studied to date and to employ these data to critically assess the opportunities and challenges facing development of this pathway as a therapeutic target.

KEYWORDS fungal pathogenesis, fungal virulence, trehalose, antifungal agents, antifungal therapy, carbon metabolism, cell wall

INTRODUCTION

The incidence of invasive fungal infections (IFIs) has increased significantly over the last 4 decades, largely due to increased use of aggressive chemotherapies for malignancies, potent immunosuppressive regimens for organ transplantation, and the HIV/AIDS pandemic (1–4). *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis* are the most common genera that cause so-called opportunistic invasive fungal infections, but the incidence of many other fungal diseases is also increasing across the globe (1–9).

There are many clinical challenges in promoting positive patient outcomes for these human fungal infections that are too often lethal. For instance, a major challenge is that fungi are eukaryotes that share cellular structures and metabolic pathways with humans. Therefore, current antifungal drug options are limited and are fraught with serious side effects in humans. Currently, there are four main treatment classes of antifungal drugs for invasive disease. These classes target the fungal membrane (azoles and polyenes), cell wall (echinocandins), and RNA/DNA synthesis (flucytosine) (5). Although newer antifungal drugs (voriconazole, posaconazole, and isavuconazole) have been developed from previous antifungal drug structures, these are still limited by the route of administration, the spectrum of activity, reduced fungicidal properties, drug-drug interactions, toxicity, and bioavailability (6). For instance, drug-drug interactions with agents metabolized by the P450 cytochrome system may limit the use of triazoles, such as voriconazole (5, 7). Recently, multiple case reports of antifungal drug resistance, especially against the azoles and now the echinocandins, have been reported (5, 6, 8–11). Further, a multidrug-resistant species of *Candida*, *Candida auris*, is now emerging in clinics across the world (12). Thus, there is a critical need for novel antifungal drugs that are fungicidal but have reduced off-target side effects for the patients who desperately need them.

One strategy for discovering new and potent antifungal drugs is to target unique fungal metabolic pathways important for fungal fitness and virulence *in vivo*. Trehalose biosynthesis is one of the pathways that broadly exists in fungi but not in humans (13, 14). Recent studies on the molecular genetics of this pathway have revealed that components of the trehalose biosynthesis pathway are essential for *Candida*, *Cryptococcus*, and *Aspergillus* species to cause invasive diseases in vertebrates (13–21). However, much remains to be learned about the mechanisms through which trehalose biosynthesis affects fungus-host interactions. This knowledge is essential not only for determining the therapeutic efficacy of targeting this pathway for each respective disease but also for defining the best target for each fungal pathogen. Consequently, the goal of this review is to analyze and present the data on the role of the trehalose pathway in human fungal pathogenesis in order to assess the opportunities and challenges facing therapeutic development of this pathway.

TREHALOSE PATHWAY

What Is Trehalose?

Trehalose is a nonreducing sugar containing two glucose subunits with an α,α -1,1-glycosidic linkage (Fig. 1A). It is found in plants, fungi, lichens, algae, a wide variety of bacteria, insects, and invertebrates but not in mammals (outside their microbiomes) (13, 14). Interestingly, trehalases (trehalose-degrading enzymes) are found in the kidney and the brush border of the small intestine in mammals, including humans (14). In fungi, trehalose is present in spores, fruiting bodies, and vegetative cells such as hyphae. Trehalose is rapidly depleted after germination and used in central fungal metabolism and in response to specific environmental stresses (13).

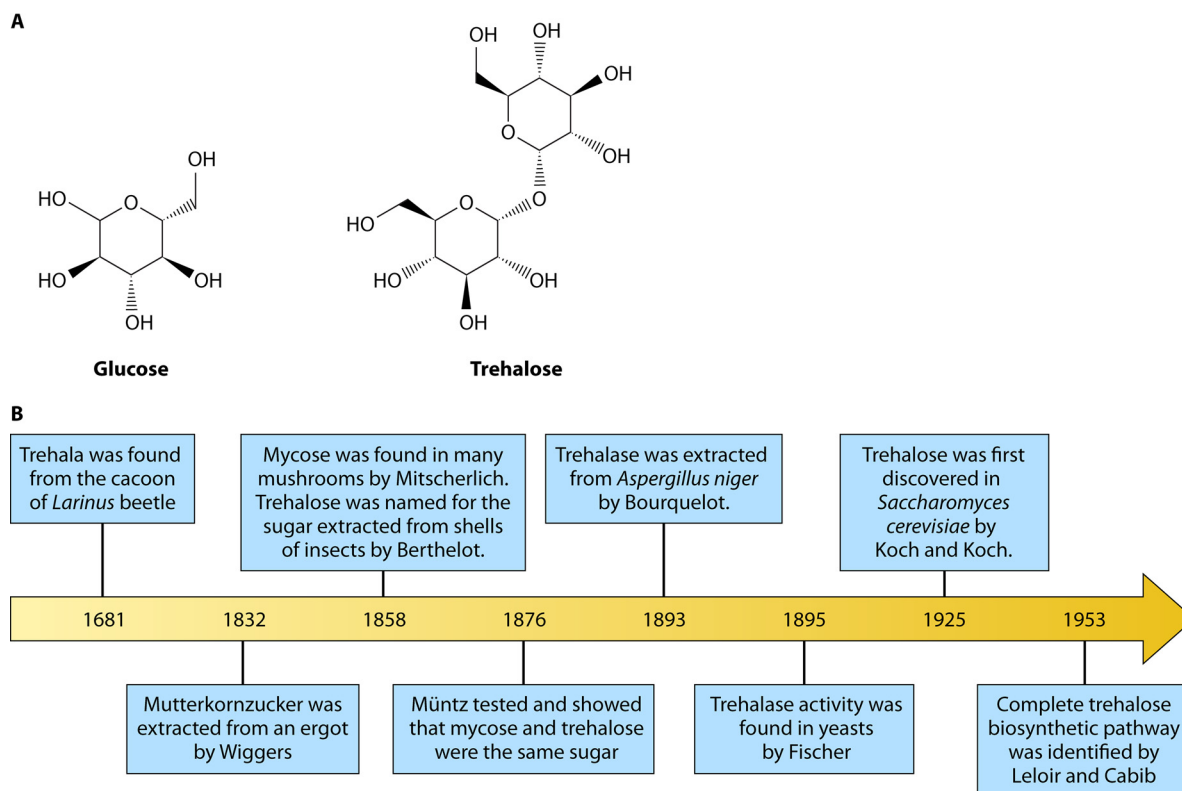


FIG 1 (A) Chemical structures of glucose and trehalose. Trehalose consists of two glucose molecules with an α,α -1,1-glycosidic linkage. (B) Timeline of trehalose and trehalase-related enzyme discovery.

History of Trehalose

The *Pharmacopoea Persica*, written in 1681 by Friar Ange of Toulouse, contains the first record of trehalose, recorded as *trehala* in the cocoons of *Larinus* beetles (22–24). Around a century later, in 1832, H. A. Wiggers discovered that undisturbed solutions of ergot formed crystals of an odorless nonreducing sugar that he named “mutterkornzucker” (an ergot sugar) (24, 25). This new sugar was resistant to hydrolysis and polarized light to a greater extent than sucrose (24). In 1858, Mitscherlich found the same sugar in mushrooms and called it *mycose*, while Berthelot also found this sugar in cocoon-like shells of various insects from the Middle East (24). Berthelot extracted this sugar from the shell and named it *trehalique glucose* or *trehalose* (24). He also found similarity between *trehalose* and *mycose* (24). In 1876, Müntz tried to find trehalose in brewer’s yeast, i.e., *Saccharomyces cerevisiae*. However, he could not extract trehalose by using a water and alcohol separation approach and erroneously concluded that this sugar did not exist in this important yeast. Yet he also tested and concluded that *mycose* and trehalose were the same sugar (26). Thereafter, Bourquelot extracted trehalase from the filamentous fungus *Aspergillus niger* and Fischer discovered trehalase activity in yeasts, in 1893 and 1895, respectively (26, 27). By 1925, Koch and Koch had observed an unknown sugar on the sides of a flask from an alcohol extract of Fleischmann’s yeast, *S. cerevisiae*, that had been undisturbed for several months (26). The complete trehalose biosynthetic pathway in *Saccharomyces* was identified and carefully described in 1953 and 1958 by Leloir and Cabib (28, 29). Lemieux and Bauer successfully synthesized trehalose in 1954 (24, 30). The amount of trehalose in a given sample can be detected by monitoring glucose levels after treatment with trehalase, a highly specific enzyme, which cleaves trehalose into two D-glucose units (14, 31). Given the seminal role that *S. cerevisiae* has played in the understanding of trehalose biosynthesis and function in fungi, it is used for comparative purposes throughout this review. The timeline for trehalose discovery is summarized in Fig. 1B.

Biochemical Properties of Trehalose

Trehalose's formal name is α -D-glucopyranosyl α -D-glucopyranoside ($C_{12}H_{22}O_{11} \cdot 2H_2O$ [trehalose dihydrate] or $C_{12}H_{22}O_{11}$ [anhydrous trehalose]) (Fig. 1A). The functions of trehalose proposed by Colaço and Roser in 1995 are myriad and include water replacement, glass transformation, and chemical stability (32). Water replacement by molecules forms hydrogen bonds and protects the surface structure. Trehalose does not form direct internal hydrogen bonds but instead forms four hydrogen bonds with two water molecules. This structure allows special molecular flexibility around the disaccharide bond and may allow trehalose to interact with phospholipid or polar groups of certain macromolecules (Fig. 2A) (32). However, surprisingly, there is still no direct evidence of specific interactions between trehalose and proteins (32). For pathogenic fungi, interactions between trehalose and other molecules are unreported and an area for further investigation.

With regard to glass transformation, sugars may solidify in a glass state that helps biomolecules to stabilize and protect small hydrophobic volatile esters from evaporation in cold and desiccating environments. The glass state of trehalose is different from other sugars because it does not retain water molecules and thus does not form crystals as other sugars do (Fig. 2B). Due to this unique property, the trehalose glass state is stable at high temperatures and under desiccation conditions (33, 34). What role this property of trehalose has, if any, on fungal biology is unclear.

As a nonreducing sugar with a low free energy of activation of the glycosidic linkage, trehalose is more resistant to hydrolysis than other disaccharides. Under mildly acidic conditions, other disaccharides go through a Maillard (browning) reaction that forms many compounds, e.g., furans, imidazoles, and *N*-nitroso derivatives, which may negatively affect dried food nutrition. However, trehalose is relatively stable in this context and does not hydrolyze (32). In fact, O'Brien observed in 1996 that under suboptimal conditions, trehalose undergoes $\sim 2,000$ -fold fewer Maillard reactions than those observed for sucrose (35). Consequently, although there is still some controversy about the mechanisms of trehalose's protective properties, it is well known and widely used in food preservative processes and in mammalian cell and plant preservations (35). For example, it is used as a cryogenic preservative for spermatozoa and ovarian tissue and also in the formulation of commercial products such as Herceptin and Avastin. Its unique properties that preserve protein structures allow its use in everyday products (36). Further experiments to unravel the biological properties of trehalose are necessary to fully reveal its biological mechanisms and its potential for preservation of fragile cells and molecules. This is particularly true for the role of trehalose itself in the pathogenesis mechanisms of human-pathogenic fungi.

Trehalose Biosynthesis in Fungi

Several trehalose biosynthesis pathways have been identified throughout the tree of life, and five of these pathways are currently well described (37, 38). The first pathway consists of two main enzymes: trehalose-6-phosphate synthase (TPS or Tps1p) and trehalose-6-phosphate phosphatase (TPP or Tps2p) (Fig. 3A) (28, 29). TPS converts UDP-glucose and glucose-6-phosphate (G6P) into UDP and trehalose-6-phosphate (T6P). T6P is converted by the TPP into trehalose and free inorganic phosphate (P_i). This canonical pathway is found in a wide variety of organisms, such as eubacteria, archaea, fungi, plants, and insects (13). It is the most well-studied trehalose biosynthesis pathway in human-pathogenic fungi. The second pathway found in some fungi was reported in 1971 (39) and was characterized for the mushrooms *Agaricus bisporus* (40), *Grifola frondosa* (41), and *Schizophyllum commune* (42). It is also found in some bacteria (43–46) and protists (47). This pathway is found in the model filamentous fungus *Neurospora crassa*, as evidenced by the identification and characterization of the *ccg-9* gene (48), which encodes a trehalose phosphorylase enzyme (TreP). TreP converts glucose-1-phosphate (G1P) and glucose into trehalose (Fig. 3B). This pathway may be reversible, but to date, that property has been shown only *in vitro* (40, 49). This noncanonical pathway of trehalose biosynthesis in fungi is likely present in the

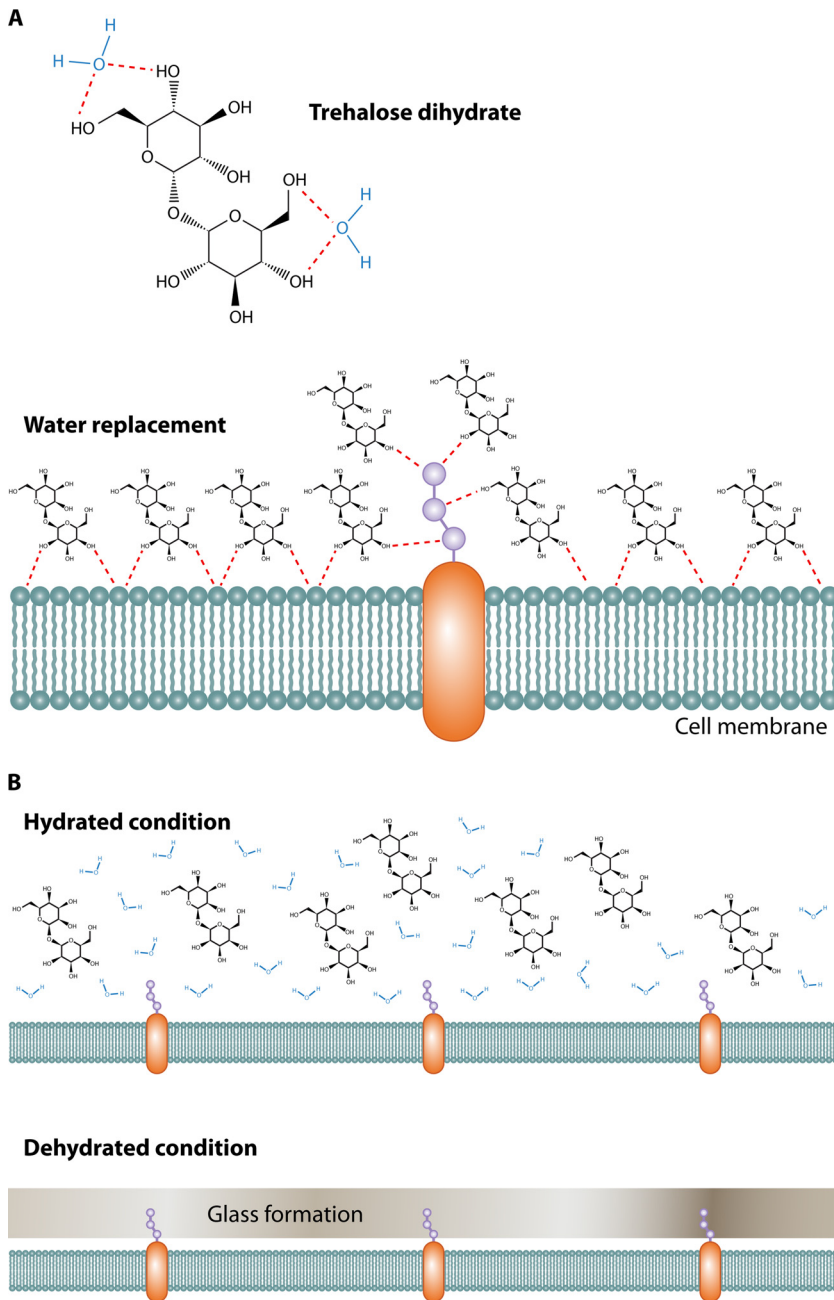


FIG 2 (A) Trehalose forms hydrogen bonds with two water molecules and functions as a replacement for water by interacting with the phospholipids or other macromolecules on the cell membrane to protect their structures under stress conditions. (B) Trehalose forms a glass state without any water retention or crystallization, thereby protecting the cell membrane under dehydrated conditions.

human fungal pathogen *Aspergillus fumigatus*, as suggested by the presence of 2 TreP-encoding genes, but so far has not been characterized fully for *A. fumigatus* or other pathogenic fungi (21). Thus, it remains unclear whether trehalose can be produced through TreP in human-pathogenic fungi, and the role of the TreP genes in the pathogenesis of fungi is unknown. In the context of assessing the value of targeting trehalose biosynthesis for therapeutic development, it seems warranted to define all possible trehalose biosynthesis pathways in human-pathogenic fungi. This is a significant challenge, as a noncanonical pathway may be functional only under specific environmental or cellular conditions. Regardless, if an alternative pathway can com-

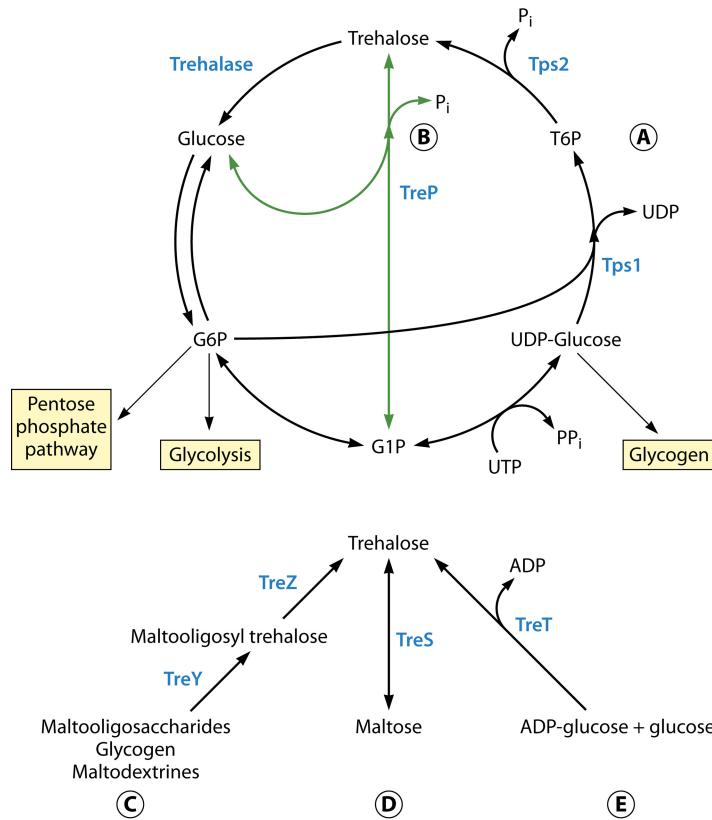


FIG 3 Trehalose biosynthesis pathways in fungi. (A) The Tps1/Tps2 (TPS/TPP) pathway consists of two main enzymes: trehalose-6-phosphate synthase (Tps1) and trehalose-6-phosphate phosphatase (Tps2). (B) The alternative trehalose phosphorylase pathway (TreP) consists of a trehalose phosphorylase enzyme that reversibly converts glucose and glucose-1-phosphate (G1P) into trehalose and inorganic phosphate (P_i). (C) The TreY/TreZ pathway consists of a maltooligosyl trehalose synthase enzyme (TreY) and a maltooligosyl trehalose trehalohydrolase (TreZ). TreY converts maltooligosaccharides, glycogen, and starch (maltodextrins) into maltooligosyl trehalose, and TreZ releases trehalose from maltooligosyl trehalose. (D) Trehalose synthase (TreS) changes maltose directly into trehalose. (E) ADP-glucose and glucose are reversibly converted into trehalose by trehalose glycosyl-transferring synthase (TreT).

plement inhibition of the primary pathway, this must be taken into consideration in targeting these pathways for therapeutic development.

A third pathway is found in *Arthrobacter* spp. and *Sulfolobus* spp. (50–52) and is related to the maltooligosyl trehalose synthase enzyme TreYp. TreYp changes the maltooligosaccharides glycogen and starch (maltodextrins) into maltooligosyl trehalose. Trehalose is released from maltooligosyl trehalose by the enzyme maltooligosyl trehalose trehalohydrolase (TreZp) (Fig. 3C). There are some bacteria, e.g., *Pimelobacter* sp. (53, 54), that can change maltose directly into trehalose by using a trehalose synthase (TreSp). The TreSp pathway is the fourth trehalose biosynthesis pathway (Fig. 3D). The fifth pathway was discovered recently in *Thermococcus litoralis* and other extremophilic archaea, e.g., *Pyrococcus* spp. (55, 56). This pathway utilizes a trehalose glycosyl-transferring synthase (TreTp) to reversibly generate trehalose by using ADP-glucose and glucose (Fig. 3E) (55, 56). However, the last three pathways have not been described in fungi. Importantly, the existence of multiple pathways of trehalose biosynthesis highlights the central role of trehalose production in a variety of organisms capable of causing human disease. This broad importance of trehalose in the physiology of many microbes may have therapeutic implications that need to be considered at a deeper level (discussed later in this review).

Trehalose is degraded into two glucose molecules by trehalase enzymes (57). Fungal trehalases are divided into two groups, namely, nonregulatory and regulatory trehalases (57). Nonregulatory trehalases, found in the ascomycetes and basidiomycetes,

function under acidic conditions (pH 3.5 to 5.5) and have high heat stability (57). They do not possess rapid activity changes during periods of fast trehalose mobilization and are not activated by phosphorylation (57). Regulatory trehalases, found in *Phycomyces blakesleeanus*, *Mucor rouxii*, *Pichia pastoris*, *Piptocephalis* spp., and *S. cerevisiae*, function under neutral conditions (pH 6.0 to 7.5) and have low heat stability (57). Unlike nonregulatory trehalases, they are rapidly activated during trehalose mobilization (57). A summary of the known genes encoding proteins important for trehalose metabolism in fungi is presented in Table 1.

Specific Functions of Trehalose in Fungi

Trehalose has critical functions in fungal biology. There is a hypothesis that trehalose serves as an alternative carbon source for some fungi, though how this relates to pathogenesis is unclear (57). One possibility that has not been tested experimentally is that trehalose produced by the microbiota of the human body provides a carbon source for fungi under certain pathological or homeostatic conditions. Trehalose is critically important for filamentous fungal conidium survival and germination, likely due to its function as a carbon source (57). An energy reserve, i.e., glycogen, usually accumulates when nutrients are rich (58). In contrast, trehalose accumulates in yeast after glucose depletion, at the beginning of the stationary phase. In the stationary phase, yeast cells use glycogen initially, but trehalose is utilized under extreme starvation conditions (58, 59). From these data, it can be concluded that trehalose is less likely to be a main reserve carbohydrate in *S. cerevisiae* but rather serves as an alternative carbon source under severe stress conditions. In human-pathogenic fungi, the role of trehalose as a reserve carbohydrate or an alternative carbon source is still unclear, and this area still remains to be explored in depth.

Trehalose has a critical role as a general stress protectant in fungi, especially in response to dehydration and thermal stress. For example, trehalose is proposed to form hydrogen bonds with proteins and to interact with the polar head groups of phospholipids to maintain membrane structure under dehydration conditions. Trehalose also inhibits both fusion and lipid-phase transitions under anhydrobiotic conditions (60, 61). Under these conditions, trehalose may form hydrogen bonds with proteins, but as discussed above, specific molecular interactions remain elusive and it is unclear whether these conditions and mechanisms influence fungus-host interactions *in vivo*. However, growth at human body temperature may be considered an extreme stress for many fungi. In *S. cerevisiae* at suboptimal environmental temperatures, the total amount of trehalose (e.g., after 1 h of exposure to 40°C) increases dramatically (62, 63). Under the same conditions, the trehalose phosphate synthase (TPS) and neutral trehalase enzymes have increased activity to both produce and degrade trehalose during heat stress and the recovery phase, respectively (62, 63). The trehalose phosphate synthase complex accumulates under heat stress conditions, and a *tps1* null mutant of *S. cerevisiae* has a growth defect in glucose-containing medium at high temperatures (64).

Consequently, it is perhaps not surprising that trehalose is also important for thermal stress survival of human-pathogenic fungi, including *Candida albicans* (15), *Cryptococcus neoformans* (19), and the rarely pathogenic fungus *Aspergillus nidulans* (65–67). A trehalose-deficient mutant ($\Delta tpsA/B$) of *A. fumigatus* is sensitive to heat shock, as suggested by a significant decrease in viability at 50°C (68). However, at normal human body temperature, loss of TpsA and TpsB in *A. fumigatus* does not affect viability. Thus, mechanisms other than trehalose biosynthesis *per se* appear to support the ability of *A. fumigatus* to thrive at human body temperature. Conversely, trehalose biosynthesis-encoding genes are important for cold shock in *Escherichia coli* (69). Trehalose also accumulates in *S. cerevisiae* after 12 h of exposure to 10°C (late cold response), but a *tps1/tps2* double null mutant still survives at 10°C (70). This result suggests the existence of an unknown pathway for yeast cold adaptation. Nonetheless, it has been hypothesized that trehalose may still prevent the aggregation of denatured proteins under cold conditions and thus keep the cell membrane intact (13).

TABLE 1 Genes involved in the trehalose pathway in fungi¹²

Fungal species	Gene name	Gene product	Function/properties of gene product	Phenotype of mutant	Reference(s)
Yeasts					
<i>Saccharomyces cerevisiae</i>	<i>TPS1</i>	Trehalose-6-phosphate synthase	Converting UDP-glucose and G6P into T6P	Inability to grow on glucose and other fermentable sugars	64
	<i>TPS2</i>	Trehalose-6-phosphate phosphatase	Dephosphorylating T6P into trehalose and inorganic phosphate	Inability to grow at temp above 34°C and accumulation of T6P	115, 116
	<i>TSL1</i>	Regulatory subunit	Controlling TPS complex activity	Decreased trehalose synthase activity	96, 123, 124, 126
	<i>TPS3</i>	Regulatory subunit	Controlling TPS2 activity	No effect on trehalose synthase activity	124–126
	<i>NTH1</i>	Neutral trehalase	Degrading trehalose into glucose at pH 7.0, controlling intracellular trehalose degradation	Susceptible to heat shock	137, 138
<i>Schizosaccharomyces pombe</i>	<i>NTH2</i>	Neutral trehalase	No significant trehalase activity	Susceptible to heat shock	137, 138
	<i>ATH1</i>	Acid trehalase	Degrading trehalose into glucose at pH 5.0, controlling trehalose utilization	Inability to grow on trehalose as a carbon source	142
	<i>TPS1</i>	Trehalose-6-phosphate synthase	Converting UDP-glucose and G6P into T6P	No growth defect on glucose-containing medium	208
<i>Candida albicans</i>	<i>TPP1</i>	Main trehalose-6-phosphate phosphatase	Dephosphorylating T6P into trehalose and inorganic phosphate	Inability to grow at temp above 37°C and accumulation of T6P	209
	<i>TPS1</i>	Trehalose-6-phosphate synthase	Converting UDP-glucose and G6P into T6P	Inability to grow on glucose at 42°C, hyphal transformation defect at 37°C, increased sensitivity to oxidative stress, and attenuation of virulence in systemic mouse model	15, 16
	<i>TPS2</i>	Trehalose-6-phosphate phosphatase	Dephosphorylating T6P into trehalose and inorganic phosphate	Decreased growth at 42°C and attenuation of virulence and dissemination in systemic mouse model	17, 18
	<i>TPS3</i>	Putative regulatory subunit	Binding site for Cap1p at the promoter related to oxidative stress tolerance; involved with Efg1p, Tsa1p, and biofilm formation	ND	127–130
<i>Cryptococcus neoformans</i>	<i>MTC1</i>	Cytosolic trehalase	Degrading trehalose into glucose, inhibited by ATP, increased activity in stationary phase	No virulence defects in systemic mouse model	57, 145, 146
	<i>ATC1</i>	Cell wall-linked trehalase	Degrading trehalose into glucose, increased activity in resting cells or in trehalose- or glycerol-containing media	Significant virulence defect in systemic mouse model	57, 145, 146
	<i>TPS1</i>	Trehalose-6-phosphate synthase	Converting UDP-glucose and G6P into T6P	Prominent growth defect on a glucose-containing medium at 37°C, increased susceptibility to oxidative stress, osmotic, stress, and antifungal drugs, decreased virulence in animal models	19
<i>Cryptococcus gattii</i>	<i>TPS2</i>	Trehalose-6-phosphate phosphatase	Dephosphorylating T6P into trehalose and inorganic phosphate	Accumulation of T6P and increased sensitivity to high temp	19
	<i>NTH1</i>	Trehalase	Degrading trehalose into glucose	No apparent phenotypes related to virulence	19
	<i>TPS1</i>	Trehalose-6-phosphate synthase	Converting UDP-glucose and G6P into T6P	Prominent growth defect on glucose-containing medium at 37°C, defects in cell wall integrity and melanin and capsule synthesis, decreased virulence in animal models	20
<i>Cryptococcus gattii</i>	<i>TPS2</i>	Trehalose-6-phosphate phosphatase	Dephosphorylating T6P into trehalose and inorganic phosphate	Accumulation of T6P and increased sensitivity to high temp, decreased virulence in animal models	20
	<i>NTH1</i>	Trehalase	Degrading trehalose into glucose	No apparent phenotypes related to virulence	20

(Continued on following page)

TABLE 1 (Continued)

Fungal species	Gene name	Gene product	Function/properties of gene product	Phenotype of mutant	Reference(s)
Molds <i>Aspergillus nidulans</i>	<i>tpsA</i>	Trehalose-6-phosphate synthase	Converting UDP-glucose and G6P into T6P	Lack of trehalose, reduced viability under constant exposure to sublethal heat or oxidative stress	65–67
	<i>orlA</i>	Trehalose-6-phosphate phosphatase	Dephosphorylating T6P into trehalose and inorganic phosphate	Defects in chitin production and lysis at 42°C, reduced activity of GFAT at 28°C	75–77
	<i>treA</i>	Acid trehalase	Degrading trehalose into glucose	Essential for growing on trehalose but not related to intracellular mobilization of the trehalose pool	67
<i>Aspergillus niger</i>	<i>tpsA</i>	Trehalose-6-phosphate synthase	Most important for trehalose production during conidiation, induced by different derepressing carbon sources	Reduced trehalose content	109, 110
	<i>tpsB</i>	Trehalose-6-phosphate synthase	Induced by heat shock	No apparent phenotypes	109, 110
	<i>tpsC</i>	Trehalose-6-phosphate synthase	Expressed at low level at 72 h	No apparent phenotypes	110
	<i>tpsA</i>	Trehalose-6-phosphate phosphatase	Dephosphorylating T6P into trehalose and inorganic phosphate	Reduction of T6P and trehalose and abnormal growth, conidiation, and accumulation of T6P	110
	<i>tpsB</i>	Putative regulatory subunit	Possible alternative T6P phosphatase	Increased sensitivity to oxidative, salt, and acid stresses	110
	<i>tpsC</i>	Putative regulatory subunit	Possible alternative T6P phosphatase	No apparent phenotypes	110
<i>Aspergillus fumigatus</i>	<i>tpsA</i>	Trehalose-6-phosphate synthase	Converting UDP-glucose and G6P into T6P	No apparent phenotypes	68
	<i>tpsB</i>	Trehalose-6-phosphate synthase	Converting UDP-glucose and G6P into T6P	No apparent phenotypes, lack of trehalose with loss of both <i>tpsA</i> and <i>tpsB</i>	68
	<i>tpsC</i>	Putative trehalose-6-phosphate synthase	Low expression at 37°C and under other stress conditions	No apparent phenotypes	68
	<i>tpsD</i>	Putative trehalose-6-phosphate synthase	Low expression at 37°C and under other stress conditions	No apparent phenotypes	68
	<i>orlA</i>	Trehalose-6-phosphate phosphatase	Dephosphorylating T6P into trehalose and inorganic phosphate	Accumulation of T6P, defects in cell wall integrity, no lysis of germlings under temp stress, and significant virulence defect in invasive aspergillosis mouse model	21
	<i>TPS1</i>	Trehalose-6-phosphate synthase	Converting UDP-glucose and G6P into T6P, regulating G6P metabolism, pentose phosphate pathway, and carbon and nitrogen source utilization	Inability to grow in glucose-containing medium, inability to cause invasive disease in rice	204, 207, 210–215
<i>Magnaporthe oryzae</i>	<i>TPS2</i>	Trehalose-6-phosphate phosphatase	Dephosphorylating T6P into trehalose and inorganic phosphate	ND	213
	<i>TPS3</i>	Regulatory subunit	Regulating trehalose synthesis	ND	213
	<i>NTH1</i>	Neutral trehalase	Degrading trehalose into glucose	Decreased T6P and trehalose levels, defect in glycogen metabolism, inability to cause rice blast disease	204
	<i>TRE1</i>	Cell wall-localized trehalase	Degrading trehalose into glucose	Ability to infect plants normally but decreased ability to colonize plant tissue	204
				Inability to grow on trehalose, no effect on virulence	204

^aAbbreviations: G6P, glucose-6-phosphate; T6P, trehalose-6-phosphate; ND, not described; GFAT, glutaminefructose-6-phosphate amidotransferase.

Trehalose also plays an important role as a free radical scavenger under oxidative stress conditions (71). This likely has direct relevance to human fungal pathogenesis, as reactive oxygen species (ROS) play a critical role in the immune system defense against fungi and other pathogenic microbes. A trehalose-deficient mutant of *S. cerevisiae* is more susceptible to hydrogen peroxide (H_2O_2) and accumulates more oxidized proteins than the wild type (71). A lack of trehalose in human-pathogenic fungi, e.g., *C. albicans* (16, 72), *C. neoformans* (19), and *A. nidulans* (65), also affects survival under oxidative stress conditions. For example, the *A. fumigatus* *tpsA/tpsB* null mutant has reduced viability after exposure to 100 mM H_2O_2 (68). Therefore, prevention of trehalose biosynthesis *in vivo* may enhance the efficacy of ROS-dependent host defense mechanisms that are often compromised in the setting of immune suppression therapies, such as treatment with corticosteroids. Consequently, a more thorough investigation of the role of fungal trehalose biosynthesis in immune cell interactions is warranted.

As an important component of the cell wall, trehalose-containing glycolipids, such as trehalose-dimycolate, are involved in virulence and cell wall homeostasis in *Mycobacterium* species (73, 74). However, to date, there are no robust reports of trehalose contributing directly to structural components of the fungal cell wall. Nonetheless, loss of *tps2* (called *orlA* in aspergilli) in *A. nidulans* and *A. fumigatus* affects cell wall homeostasis, likely through a reduction in chitin content leading to cell lysis at high temperatures (21, 75–77). This observation raises a critical point for consideration in identifying promising drug targets in the trehalose biosynthesis pathway. The canonical trehalose biosynthesis pathway utilizes substrates, i.e., glucose-6-phosphate and UDP-glucose, that intersect with glycolysis, the pentose phosphate pathway, and, consequently, cell wall biosynthesis. Therefore, disruption of this pathway leads to dysregulation of other key metabolic pathways in the cell, and as such, the overall consequences and impact of trehalose biosynthesis on each fungal pathogen remain to be fully defined. Given the importance of the cell wall in host-fungus interactions and fungal survival, the mechanistic connection(s) between the trehalose biosynthesis pathway, trehalose itself, and cell wall biosynthesis is an important area of ongoing research.

As suggested above, trehalose and associated molecules needed for its synthesis have impacts on central carbon metabolism. For example, disruption of *tps1* in *S. cerevisiae* results in a significant growth defect on glucose-containing media (64, 78). Ultimately, loss of TPS in *S. cerevisiae* results in altered energy homeostasis at the level of glycolysis, though the mechanisms remain to be fully elucidated. In *C. albicans*, loss of *tps1* does not affect growth on glucose at 30°C but does at 42°C (15). In *Cryptococcus neoformans* and *Cryptococcus gattii*, loss of Tps1p function impairs growth on glucose at 37°C (19, 20). In contrast, no defect in growth on glucose-containing medium was observed for the *A. fumigatus* *tpsA/tpsB* null mutant (68). For a pathogenic filamentous fungus such as *A. fumigatus*, accumulation of the signaling molecule T6P inhibits hexokinases and regulates the influx of glucose (21).

As discussed in part above, preliminary molecular genetic analyses have revealed important and complex roles for the trehalose biosynthesis pathway in fungal biology that extend beyond the biosynthesis of trehalose itself. However, the specific mechanisms remain to be elucidated and fully appreciated. Surprisingly, the amino acid sequences of the proteins involved in trehalose biosynthesis are highly conserved among TPS1, TPS2, and the regulatory subunits (TPS3/TSL1). In some pathogenic fungi, there is an expansion of the number of these genes, but their functions remain to be elucidated fully. A similar situation exists in *Arabidopsis thaliana*, which contains 11 putative AtTPSs and 10 putative AtTPPs (79–82). These observations highlight the importance of these gene products in metabolic homeostasis and fitness across a diverse array of organisms and environments. Consequently, they also provide support for the hypothesis that this pathway is an attractive antifungal drug target whose inhibition will severely diminish the fitness and virulence of pathogenic fungi.

TPS1 (TREHALOSE-6-PHOSPHATE SYNTHASE)

Tps1p, the trehalose-6-phosphate synthase, is found in both bacteria and eukaryotes. Tps1p converts UDP-glucose and glucose-6-phosphate (G6P) into trehalose-6-phosphate (T6P). For *S. cerevisiae*, *tps1* mutants were initially characterized in the late 1970s and were called by many names, e.g., *FDP1*, *CIF1*, *BYP1*, and *GLC6* (83–87). Later it was shown that these mutations are all allelic with the same gene, *tps1* (64, 88). *fdp1* and *cif1* mutants cannot grow on glucose and other fermentable sugars, such as fructose, mannose, and sucrose. Furthermore, these mutants show a defect in glucose-induced inactivation of fructose-1,6-bisphosphatase and have very low trehalose levels with a change in the regulation of glycogen synthesis (84, 85, 89, 90). The *byp1* mutant was previously believed to bypass glycolysis, and it shares similar phenotypes with *fdp1* and *cif1* mutants (86, 91, 92). The *byp1* mutant displays slow growth and is completely blocked for growth on both glucose and fructose (86, 91). There is also no growth on glucose of *byp1* mutants deficient in *pfk1* or *pfk2*, encoding phosphofructokinase enzymes catalyzing the phosphorylation of fructose-6-phosphate into fructose-1,6-bisphosphate or fructose-2,6-bisphosphate, respectively (86, 91). Because of the common involvement of these alleles in glucose-induced signaling, *FDP1* was renamed *GGI1* (general glucose sensor 1) (93).

GLC6 is related to glycogen accumulation and is allelic to *CIF1*, *FDP1*, *BYP1*, and *GGI1* (87). While *GGI1* was believed to function as a glucose sensor responsible for glucose influx, the same gene, called *tps1*, has been characterized as the smallest subunit of the trehalose synthase complex (64). Tps1p is an important component of trehalose-6-phosphate synthase, and deletion of *tps1* in *S. cerevisiae* causes a growth defect on glucose-containing medium similar to those of the *fdp1* and *cif1* mutants (64). Moreover, expression of *tps1* restores trehalose production in an *Escherichia coli otsA* mutant lacking a T6P synthase enzyme (the gene product of *otsA*) (88).

The growth defects of *tps1* mutants and mutants in associated alleles provide a rich opportunity to explore the critical role of the trehalose biosynthesis pathway in the regulation of carbon metabolism. A detailed understanding of the effects of Tps1p loss on fungal metabolism in each respective organism is critical for evaluating the impact of targeting Tps1p on therapeutic development. To this end, it seems clear that a loss of TPS1 has multiple effects on carbon metabolism that are organism specific. Extensive research with *S. cerevisiae* has been undertaken to understand the impact of *tps1* loss on regulation of glycolysis. To explain the growth defect on glucose-containing medium, three non-mutually exclusive hypotheses were initially proposed (89). The first hypothesis is that Tps1p regulates glucose influx to prevent an accumulation of sugar-phosphate intermediates upstream of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (93). In support of this hypothesis, deletion of *hxx2* decreased sugar-phosphate accumulation and restored *tps1* mutant growth on glucose (94). The second hypothesis proposes that trehalose synthesis provides a mechanism for free phosphate recovery (89). Reductions in intracellular phosphate after addition of glucose to the *tps1* mutant and inhibition of the trehalose synthase complex by free phosphate support this hypothesis (92, 95, 96). In further support of this hypothesis, stimulation of glycerol production, which increases phosphate recovery by reducing the accumulation of sugar-phosphate intermediates, restores the *tps1* mutant growth defect on glucose after a longer lag phase (89, 94). However, a *byp1 tps2* double mutant shows increased growth on glucose compared to that of the single *byp1* mutant, thereby contradicting the phosphate recovery hypothesis (97). Intriguingly, the *byp1 tps2* double mutant still accumulates sugar-phosphate intermediates. This observation is similar to data from a *tps1* mutant strain that expresses the *E. coli otsA* gene (98). These data argue for a model whereby T6P, trehalose, or Tps1p has additional roles in regulating the second half of glycolysis (83).

The third hypothesis proposes that T6P controls glucose influx through its inhibition of hexokinase activity (89). T6P competitively inhibits hexokinases in *S. cerevisiae* (99). However, overexpression of hexokinase activity does not result in a glucose growth

defect in wild-type *S. cerevisiae* cells (100). Recently, Walther et al. observed significant acidification of the cytosol in the absence of Tps1p due to loss of a plasma membrane H⁺ ATPase (101). In summary, the loss of Tps1p has significant effects on the regulation of glucose influx and glycolysis in yeast cells that affect cell fitness, which bodes well for targeting this protein for antifungal drug development. However, the complexity of Tps1p function and loss in yeast highlights a potential challenge in targeting this pathway for antifungal drug development. This challenge must be met with additional research to fully define Tps1p function in respective pathogenic organisms.

Importantly, while the function of Tps1p in human-pathogenic fungi is heterogeneous across species, studies to date suggest clear roles in fungal pathogenesis that bode well for therapeutic development. In *C. albicans*, *tps1* encodes a trehalose-6-phosphate synthase, and the promoter of this gene contains four copies of the stress response element (STRE). As stated above, the *tps1/tps1* null mutant grows normally at 30°C (15). However, at 42°C, the mutant cannot grow on glucose, but it is able to grow on glycerol or galactose at higher temperatures. The mutant has a defect in hyphal formation in serum-containing medium at 37°C. It also has decreased intracellular ATP levels and no trehalose accumulation at stationary phase or after heat shock. Importantly, the mutant is attenuated in virulence in a mouse model of systemic infection (15). The *tps1/tps1* null mutant displays a severe reduction in cell viability after exposure to high concentrations of hydrogen peroxide, while the wild type retains high cell viability associated with an increase in intracellular trehalose (16, 72). However, pretreatment of the *C. albicans* wild type or the *tps1/tps1* null mutant with a nonlethal concentration of hydrogen peroxide (0.5 mM H₂O₂) or mild heat stress (37°C) protects both strains against oxidative stress, to the same extent (16). From this result, it can be inferred that trehalose accumulation plays a major role in protecting yeast cells from an immediate direct exposure to severe oxidative stress. Direct exposure to oxidative stress occurs during interactions with immune cells. Other pathways related to the oxidative stress response, e.g., the HOG–mitogen-activated protein kinase (MAPK) pathway and enzymatic (catalase and superoxide dismutase) and nonenzymatic (glutathione and thioredoxin) components, may protect cells after mild stress exposure (16, 102). In contrast to oxidative stress, osmotic or heat stress has no effect on cell viability of the *C. albicans tps1/tps1* null mutant (72).

Perhaps consistent with the *in vitro* oxidative stress phenotype, the *tps1/tps1* null mutant is more susceptible to macrophage killing than the wild type (103). Intriguingly, this phenotype is dependent on the culture conditions and cell wall composition. Electron microscopy reveals a difference in the outer cell wall layer in the mutant compared to the wild type (103). In liquid culture medium, the mutant increases its β -mannosylation (overglycosylation) on the outer cell wall, leading to reduced hydrophobicity and increased resistance to macrophage killing, whereas on solid medium the mutant has no difference in overglycosylation on the cell wall and is more susceptible to macrophage killing (104). These observations have direct relevance to *C. albicans* Tps1p as a drug target. While the reduction in virulence of the *tps1/tps1* mutant in a systemic murine model is promising, the environment-specific impacts on the cell wall and phagocyte interactions warrant further investigation in *in vivo* models. The latter observation is important given the phagocyte resistance phenotype and altered cell wall observed under specific *in vitro* conditions. Importantly, this theme of trehalose biosynthesis affecting cell wall composition and integrity is found across the human-pathogenic fungi, although the mechanism(s) remains ill defined.

In *C. neoformans*, *tps1* encodes a 671-amino-acid trehalose-6-phosphate synthase with eight putative STREs in the promoter region. A *tps1* null mutant displays a prominent growth defect on glucose-containing medium at 37°C, but the growth defect is restored in a galactose-containing medium, similar to the case with *C. albicans* (19). The *tps1* null mutant grows in glucose-containing medium supplemented with 1 M sorbitol at 37°C, which suggests an impact on the fungal cell wall (19). Trehalose and T6P are not detected in the mutant (19). The *C. neoformans tps1* mutant is more susceptible to oxidative stress by hydrophobic peroxides, e.g., *t*-BOOH, to osmotic stress (1 M sorbitol), and,

importantly, to antifungal drugs (amphotericin B and caspofungin) (19). The increased susceptibility of the mutant to current antifungal drugs raises the possibility of a combination therapy approach targeting trehalose biosynthesis in addition to current antifungal drugs.

A critical relationship between trehalose and its synthesis through Tps1p and the virulence of *C. neoformans* is establishing Tps1p as a potential antifungal drug target in this important human pathogen (105–107). *tps1* was one of the most highly expressed transcripts in a global transcriptional analysis of yeast cells from cerebrospinal fluid (CSF) from rabbits with cryptococcal meningitis (107). Nuclear magnetic resonance (NMR) studies show that trehalose is one of the most abundant metabolites in cryptococcomas (105, 106). To further support the importance of trehalose in the virulence of *C. neoformans*, the *tps1* null mutant is avirulent in both rabbit and murine models of infection (19). Moreover, significant attenuation of the virulence of the *tps1* null mutant is also observed in *Caenorhabditis elegans* and zebrafish models (19, 108). These results in model organisms with body temperatures approximating room temperature reveal that the effect of Tps1p loss on *C. neoformans* virulence goes beyond growth inhibition at mammalian body temperature (19, 108). Additional research on the *C. neoformans tps1* null mutant in the context of interactions with the immune system is warranted.

Tps1p is also critical in the related pathogenic yeast, *Cryptococcus gattii*, which causes disease in immunocompetent hosts. However, there are important aspects of the *tps1* mutants of *C. neoformans* and *C. gattii* that differ (20). For example, growth of the *tps1* mutant in *C. gattii* is not restored at 37°C in galactose-containing media, and it exhibits significant defects in cell wall integrity and melanin and capsule synthesis that are not observed in *C. neoformans* (20). Importantly, the *C. gattii tps1* null mutant possesses profound virulence defects in both invertebrate and mammalian hosts. These species-specific differences in the trehalose pathway may affect the differences in disease presentation and/or ecological diversity of the *Cryptococcus* species complex. However, more studies are needed to determine the precise mechanisms and causality. A major future research direction alluded to above is interrogation of these mutants *in vivo* in the setting of active host responses under dynamic environmental conditions that occur in an active infection. Importantly, these data highlight and emphasize the importance of studying the trehalose pathway and its role in the biology of each fungal species. This conclusion is emphasized when one turns to studies of this pathway in the genus *Aspergillus*.

Unlike the yeast species studied to date, in the genus *Aspergillus* multiple copies of the *TPS1* gene exist in several species, with the relatively nonpathogenic species *A. nidulans* being an interesting exception. In *A. nidulans*, which has only one trehalose-6-phosphate synthase (*tpsA*), trehalose is found in conidia at high concentrations and is rapidly used during germination (65). *A. nidulans* also accumulates trehalose during conidial differentiation, nutrient starvation, heat shock, and oxidative shock (65–67). A *tpsA* null mutant lacks production of trehalose and shows reduced conidial viability during constant exposure to sublethal stresses, i.e., prolonged high temperature and oxidative stress (65). In *Aspergillus niger*, two copies of *tps1* exist and are named *tpsA* and *tpsB* (109). *TpsA* is the most important for trehalose production during conidiation and has increased expression on derepressing carbon sources (109). *tpsA* is expressed constitutively, while *tpsB* is induced by heat shock and has a role during differentiation (110). *A. fumigatus* also contains *tpsA* and *tpsB*, and loss of both genes eliminates trehalose production (68). Intriguingly, in this common human fungal pathogen of immunocompromised patients, two additional genes encoding proteins with amino acid sequence similarity to *TpsA* and *TpsB* are found in the genome and have been named *tpsC* and *tpsD* (68). However, mRNA levels of *tpsC* and *tpsD* are low under the *in vitro* environmental conditions examined to date. Consequently, their role in *A. fumigatus* pathobiology is not currently clear. Importantly, unlike *C. albicans*, *C. neoformans*, and *S. cerevisiae tps1* null mutants, the *A. fumigatus* double *tpsA/tpsB* mutant grows normally in the presence of glucose at 37°C (68). The ability to grow in the presence of glucose in the absence of genes encoding catalytic TPS may indicate a

regulatory or structural role for TpsC and TpsD in regulating glycolytic and/or carbon flux (68). Alternatively, or in addition, *A. fumigatus* contains a highly active glucokinase (*glkA*) that activates glucose and is likely resistant to inhibition by T6P (111). Despite the normal growth on glucose of the *tpsA/tpsB* null mutant, carbon metabolism is significantly altered, as clear changes in the cell wall of the mutant are observed (68). From transmission electron microscopy (TEM) images, the electron-dense outer layer on the cell walls of both hyphae and conidia of the *tpsA/tpsB* null mutant is absent, and conidia of the mutant have an enhanced electrolucent zone compared to that of the wild type (68). In addition to the abnormal cell wall structure from TEM images, the expression of *ags3*, encoding an α -glucan synthase, is significantly reduced in the double null mutant, and its reduction might contribute to the abnormal cell wall structure (68). Surprisingly, despite increased sensitivity to oxidative stress, the *A. fumigatus tpsA/tpsB* null mutant displays a modest increase in virulence in a cortisone acetate murine model of invasive pulmonary aspergillosis. While the mechanism behind this hypervirulence is not fully understood, cell wall changes in the null mutant may promote increased immunopathogenesis through activation of detrimental host immune responses. Alternatively, enhanced *in vivo* fitness of the null mutant due to alterations in carbon metabolism and stress responses cannot be ruled out. Regardless, the virulence persistence of the *tpsA/tpsB* null mutant of *A. fumigatus* is striking in comparison to the results of TPS studies of other pathogenic fungi and is worth further investigation in the context of therapeutic development.

On the surface, these results for *A. fumigatus* appear to argue against targeting Tps1p for antifungal drug development from the perspective of developing a broad-spectrum target. However, the latter conclusion must be tempered by the observation that two additional Tps1p-like proteins exist in *A. fumigatus* that remain to be characterized. While the loss of TpsA and TpsB is clearly not sufficient to negatively affect *A. fumigatus* virulence, elimination of all 4 TPS1-like proteins in a strain has yet to be investigated. Moreover, one cannot rule out that *in vivo*, at the infection site microenvironment, the *tpsA/tpsB* null mutant is somehow complemented by host or fungal factors to produce trehalose and/or other virulence factors. These results in conjunction with the yeast data suggest that TPS proteins play multiple roles beyond trehalose biosynthesis that warrant further investigation. *In vivo* analyses of fungal genetic null mutants remain a significant technical challenge that is beginning to be met with novel techniques, such as Nanostring nCounter gene expression analyses and conditional promoter systems, among other approaches (112–114). Thus, despite the above challenges, unraveling the differences in the functions of TPSs between *A. fumigatus* and pathogenic yeasts has the potential to yield new insights into the Achilles' heels of these important pathogens.

TPS2 (TREHALOSE-6-PHOSPHATE PHOSPHATASE)

Tps2p is the trehalose-6-phosphate phosphatase (TPP) that dephosphorylates T6P into trehalose and inorganic phosphate. The first described TPP was Tps2p of *S. cerevisiae* (115). Investigators observed that a *tps2* null mutant could not grow at temperatures above 34°C (116). There are now multiple reports that suggest T6P accumulation as the cause of the temperature-sensitive phenotype of the *tps2* mutant of *S. cerevisiae* (115, 117). Furthermore, T6P accumulates in this mutant during heat shock (115, 116). Thus, under stress conditions, it seems likely that accumulation of T6P in *tps2* mutants is toxic to cells (116). For human-pathogenic fungi, *tps2* null mutants consistently display severe virulence attenuation and other fitness defects that portend well for Tps2-targeted antifungal drug development.

In *C. albicans*, Tps2p contains 878 amino acid residues, with two phosphohydrolase domains, and shares 67% sequence similarity with *S. cerevisiae* Tps2p and 73% similarity with *A. nidulans* OrIA (17, 18). Van Dijck et al. observed that a *C. albicans tps2/tps2* null mutant accumulates T6P with a temperature-sensitive phenotype at 44°C (18). In contrast to the *C. albicans tps1/tps1* null mutant, the *tps2/tps2* null mutant does not have hyphal formation defects on glucose-containing medium at 30°C (18). The *tps2/*

tps2 null mutant has a significant decrease in growth rate at 42°C and aggregates in stationary phase, with a defect in cell wall integrity, when the pH is over 7.0 (17). Importantly, the mutant has a significant decrease in virulence, highlighted by a decrease in fungal dissemination to the kidneys and livers, in a systemic murine model (17, 18). The mechanism(s) for the decreased dissemination and virulence in the absence of Tps2p is currently unclear but is an important area of research for further validation of Tps2p as an antifungal drug target.

Along these lines, as mentioned earlier, the importance of trehalose biosynthesis in fungal stress responses and virulence suggests that therapeutic development of this pathway should also be considered in the context of combination approaches. For example, loss of *C. albicans* Gpr1p, a nutrient receptor activating cyclic AMP-protein kinase A (cAMP-PKA)-mediated signaling, results in a significant increase in trehalose levels and morphological defects on hypha-inducing media (118, 119). However, a *C. albicans* *gpr1/gpr1* null mutant displays only a slight decrease in virulence in a systemic murine model (120). In contrast, loss of *tps2* in the *gpr1/gpr1* null mutant completely attenuates virulence (120). The *gpr1 tps2* double null mutant accumulates very high levels of T6P under stress conditions, with a concomitant growth defect at high temperatures (120). These results support the observation that an increase in intracellular T6P levels has an enormous effect on the virulence and fitness of *C. albicans* under specific conditions, particularly those that promote TPS activity. Consequently, treatments that promote increased TPS activity may be synergistic *in vivo* with a drug targeting Tps2p to drive accumulation of toxic T6P levels. Given that TPS activity is induced by many host-associated stresses *in vivo*, Tps2p-targeting drugs may be expected to have increased potency *in vivo*, beyond *in vitro* MIC testing results.

In *C. neoformans*, *tps2* encodes a predicted 988-amino-acid protein with the presence of four STREs in the promoter region (19). *C. gattii tps2* encodes a 990-amino-acid protein with 89% sequence similarity to *C. neoformans* Tps2p (20). The *tps2* null mutants of both *C. neoformans* and *C. gattii* accumulate T6P and have severe growth defects at 37°C on glucose-containing medium, and in fact, they die at this temperature (19, 20). The growth and survival defects are rescued by growth on galactose- or sorbitol-containing medium at 37°C (19, 20). As in *S. cerevisiae*, *C. gattii* has a strong connection between the trehalose pathway and control of glycolytic fluxes via hexokinases. For instance, the loss of *hvk2* in a *tps2* null mutant background suppresses the high-temperature growth defect, possibly by reducing the pool of glucose-6-phosphate that is a building block for toxic T6P accumulation (20). From a therapeutic perspective, the toxic effect of Tps2p loss in *Cryptococcus* is tremendously exciting and further highlights the possibility of therapeutically targeting this important virulence-associated fungal enzyme. However, the *C. gattii tps2* null mutant has only a slight decrease in virulence in the *C. elegans* model, though a profound virulence defect in the murine inhalation model is observed (20). Consequently, the lack of full virulence attenuation in the *C. elegans* model warrants further investigation, as specific host conditions may feasibly inhibit or overcome the loss of Tps2p activity. However unlikely, this hypothesis can be tested experimentally with the development of Tps2-inhibiting small molecules in relevant animal models.

For the filamentous aspergilli, the story is somewhat different but no less impactful. For *A. nidulans*, the Tps2 gene ortholog has been characterized and was named *orlA* (osmotic-remediable lysis strain) (75–77). *orlA* encodes a 908-amino-acid protein that shares the same predicted protein domains as *S. cerevisiae* Tps2p (77). The *A. nidulans orlA* null mutant has a defect in chitin production and lyses when grown at 42°C, whereas growth is partially recovered on osmotic stabilizers or an *N*-acetylglucosamine-containing medium (75). Furthermore, glutamine:fructose-6-phosphate amidotransferase (GFAT), the first step in amino sugar synthesis, has reduced activity in the *A. nidulans orlA* null mutant at 28°C (75, 77). It is hypothesized that the lysis phenotype of the *A. nidulans orlA* null mutant occurs from a defect in chitin synthesis (76). The *orlA* null mutant accumulates significant levels of T6P but, surprisingly, still produces trehalose at both 32 and 42°C (77). For *A. niger*, a *tppA (tps2)* null mutant shows abnormal growth,

conidiation, and accumulation of T6P (110). The *A. fumigatus orlA* mutant exhibits defects in colony morphology and conidiation on glucose minimal medium (GMM) at 37°C (21). However, unlike that of the yeast *tps2* null mutants, *A. fumigatus* growth at 37°C is affected only minimally on GMM. A substantial accumulation of T6P and significant changes in cell wall integrity are observed in the *A. fumigatus orlA* mutant, but similar to *A. nidulans* and *C. albicans*, trehalose production is not lost. The mechanism behind the persistence of trehalose production in some fungal *tps2* null mutants remains enigmatic. Unlike *A. nidulans*, *A. fumigatus orlA* null mutant germlings do not lyse under temperature stress, possibly highlighting important cell wall differences between these two species. Importantly for therapeutic considerations, the *orlA* null mutant has a striking attenuation in virulence in a leukopenic invasive pulmonary aspergillosis murine model (21). Initial attempts to characterize metabolic defects in the *orlA* null mutant revealed a reduction in hexokinase activity along with a reduction in pyruvate decarboxylase activity required for ethanol fermentation (21). Intriguingly, loss of ethanol production through loss of the alcohol dehydrogenase *alcC* gene significantly reduces lung fungal burdens in murine models of invasive pulmonary aspergillosis (121).

In summary, loss of Tps2p consistently results in severe temperature-sensitive growth defects and/or attenuated virulence in human-pathogenic fungi. Thus, Tps2p orthologs in pathogenic fungi are potential broad-spectrum antifungal drug targets that are absent in humans. However, the mechanisms through which Tps2p mediates growth and virulence in pathogenic fungi still remain to be fully defined. While loss of Tps2p in yeast is conditionally lethal, the consequences of Tps2p loss are more nuanced in molds. For example, it is unclear if the reduction in chitin content in the *A. fumigatus orlA* null mutant is compensated by overproduction of the proinflammatory pathogen-associated molecular pattern (PAMP) beta glucan. If this is so, use of a Tps2p-inhibitory drug in certain patient populations may unexpectedly promote immunopathogenesis. These hypotheses remain to be tested experimentally. A major recent advance directly relevant to targeting Tps2p is the solving of high-resolution protein structures from three human-pathogenic fungi (discussed more below). The careful assessment of the germane protein structures, recently completed, is expected to catalyze the discovery of specific inhibitors of this phosphatase (122).

REGULATORY SUBUNITS

TPS3/TSL1

In *S. cerevisiae*, two additional proteins with high amino acid sequence similarity to Tps1p and Tps2p are found (96, 123–125). Tsl1p (trehalose synthase long chain) is found in the same protein complex as Tps1p and Tps2p (TPS complex) (96, 123), while Tps3p is also found to be a part of the complex (124). During heat shock, Tsl1p and Tps3p have similar regulatory roles in the TPS complex of *S. cerevisiae* (125). However, Tsl1p and Tps3p have different impacts on trehalose synthase activity under other conditions (124). While a *tsl1* null mutant has reduced trehalose synthase activity, deletion of *tps3* does not affect trehalose synthase activity (124). Tsl1p is important for TPS complex activity, while Tps3p is a target for phosphorylation that regulates Tps2 activity (126). It has been proposed that Tsl1 might also play a structural role in formation of the TPS complex, as no TPS activity is detected in the absence of Tsl1p (126). Consequently, these data strongly support a model in which trehalose-producing enzymes function as a complex in *S. cerevisiae*. Whether a similar or identical model occurs in pathogenic fungi is unclear, as biochemical work on the respective trehalose proteins in these fungi is thus far sparse. Given some of the divergent phenotypes associated with TPS/TPP mutants in pathogenic fungi and the presence/absence of specific genes, alternative models are possible, if not likely. It will be important to define these mechanisms and structures in the respective pathogenic fungi to maximize antifungal drug development opportunities. To this end, genetic analysis of regulatory subunit homologs has been conducted in some human-pathogenic fungi.

For *C. albicans*, a putative regulatory subunit called Tps3p is found encoded in the genome by amino acid sequence similarity searches, with 41% identity to *S. cerevisiae* Tps3p. However, it is still unclear how or whether *C. albicans* Tps3p regulates trehalose production in this important human-pathogenic yeast. The *tps3* promoter may contain a binding site for Cap1p, which is a transcription factor related to oxidative stress tolerance in *C. albicans*. *tps3* mRNA levels are induced significantly when *C. albicans* is exposed to hydrogen peroxide (127). *C. albicans tps3* is positively regulated by Efg1p, which is an APSES (Asm1, Phd1, Sok2, Efg1, and StuA family) transcriptional regulator involved in the yeast-to-hypha transition and cell shape during white-opaque switching, among other important phenotypes (128). A cell wall protein in hyphal cells, Tsa1p, is related to oxidative stress resistance and hyphal cell wall integrity, and a *tsa1* null mutant contains reduced *tps3* mRNA levels under oxidative stress conditions (129). Furthermore, *tps3* mRNA levels are reduced in *C. albicans* during biofilm formation (130). To date, a potential *C. albicans* Tsl1p homolog has not been identified by sequence comparison with BLAST algorithms. For *Cryptococcus gattii*, a putative Tps3p (CGB_I4320W) homolog has been identified by BLAST searches, with 33% identity to *S. cerevisiae* Tps3p, but it has not been characterized. Interestingly, a Tps3p homolog has not been identified in *C. neoformans* by sequence analyses.

In *A. niger*, TppB and TppC are the homologs of Tps3p and Tsl1p, respectively. The *tpbB* null mutant is more susceptible to thermal stress and has reduced internal trehalose levels, but it does not show increased sensitivity to oxidative stress, osmotic stress, or acidic stress (110). Because TppB and TppC share significant amino acid sequence similarity with the TPP TppA (Tps2/OrlA), it has been hypothesized that they may function as alternative phosphatases in the absence of TppA, which would explain the persistence of trehalose production in *tps2* null mutants (110). Importantly, however, the potential TPP activity of both TppB and TppC remains to be investigated to support this hypothesis. In *A. fumigatus*, as in *A. niger*, two proteins encoded in the genome share protein domains similar to those in OrlA and are the likely *A. fumigatus* Tps3p and Tsl1p homologs. The functions of these two proteins in trehalose biosynthesis and fungal metabolism are currently unknown but are under investigation in our laboratory (our unpublished data).

The catalytic residues required for TPP activity have been defined for the archaeon *Thermoplasma acidophilum* (TaT6PP) (aspartates 7, 9, 179, 180, and 183, threonines 11, 45, and 182, arginine 47, and lysine 161) (131) and the filariasis-causing parasite *Brugia malayi* (motif I active sites, aspartates 213 and 215; motif II active site, threonine 253; motif III active site, lysine 398; and motif IV active sites, aspartates 424 and 428) (132). These amino acid residues are found in the Mg²⁺-dependent haloacid dehalogenase (HAD) phosphatase domain superfamily (131). *S. cerevisiae* Tps3p and Tsl1p have a HAD-like domain but no known catalytic activity (133). In the plant *A. thaliana*, Tps3p also contains the HAD domain but also has no TPP activity (133, 134). Consequently, additional biochemical experiments are needed to clarify the roles of HAD domains and specific amino acid residues in regulatory-like proteins in the aspergilli and other human-pathogenic fungi. In summary, much remains to be learned about the functions of trehalose biosynthesis and associated proteins in human-pathogenic fungi. Additional genetic and biochemical investigations into potential complex formation and the function(s) of the putative regulatory proteins previously characterized for *S. cerevisiae* are needed to further our understanding of these proteins in human fungal pathogenesis. A particularly important area of future investigation is exploration of the so-called moonlighting roles of these proteins outside canonical trehalose biosynthesis that may directly affect virulence-associated functions, such as cell wall biosynthesis and stress responses to host defense mechanisms.

NONCANONICAL TREHALOSE BIOSYNTHESIS IN FUNGI

The persistence of trehalose production in the absence of TPP function in pathogenic fungi remains an enigma and potentially suggests the existence of a noncanonical trehalose biosynthesis pathway in some species. As discussed above, one hypoth-

esis is that other proteins with shared domains in the trehalose pathway contribute to dephosphorylation of T6P to produce trehalose. Van Dijck and colleagues suggested that nonspecific phosphatase activity may account for the persistence of trehalose in *tps2* null mutants (18). Yet there are hints that additional biosynthetic routes may exist in fungi that do not depend on the canonical TPS-TPP-mediated pathway. For example, in the model filamentous fungus *Neurospora crassa*, clock-controlled gene 9 (*ccg-9*) functions as a gene important for trehalose production and stress protection (48). The *ccg-9* gene product has high sequence similarity to a novel trehalose synthase (TSase) in the basidiomycete mushroom *Grifola frondosa*. The *G. frondosa* TSase functions as a trehalose phosphorylase that reversibly catalyzes glucose-1-phosphate and glucose into trehalose and inorganic phosphate (135). Sequence similarity is observed with trehalose phosphorylases in other basidiomycetes, including the mushrooms *Agaricus bisporus* (40) and *Schizophyllum commune* (42). Consequently, trehalose phosphorylase activity is found in many fungi within the Basidiomycota, Zygomycota, and Ascomycota (135). However, in the ascomycete yeasts, e.g., *S. cerevisiae* and *C. albicans*, TSase homologs have not been found by sequence-based comparisons to date. Based on the identification of two putative TSase proteins encoded in the *A. fumigatus* genome sequence, it has been proposed that this noncanonical pathway may be induced in this pathogen upon phosphate depletion caused by T6P accumulation in the absence of TPP activity to help to mitigate the accumulation of potentially toxic sugar phosphates and also to resupply the cell with inorganic phosphate (21). This model remains to be tested experimentally in *A. fumigatus*. Another potential explanation for trehalose production in the absence of detectable TPP activity is the presence of an unknown pathway for trehalose biosynthesis in these fungi. The pathway would be activated by loss of *Tps2* and/or T6P accumulation but not in the absence of trehalose *per se* because *tps1* mutants remain deficient in trehalose. These data illustrate a main theme of this review in that much remains to be learned about the complexity of the trehalose biosynthetic pathway and its impact on pathogenic fungal biology. Yet these studies are critical to realize the full therapeutic potential and challenges facing any attempts to target this pathway.

TREHALASES

Fungi utilize trehalose through trehalase-mediated degradation. In *S. cerevisiae*, there are three different trehalases: Nth1p, Nth2p, and Ath1p (136). Nth1p, the neutral trehalase, is located in the cytoplasm and functions at pH 7.0 (13, 136–138). Nth2p is also found in the cytoplasm but has no significant trehalase activity under conditions examined to date (137, 138). Nth1p has maximal activity during early growth stages. *NTH1* expression increases under stress conditions due to the presence of three STREs in its promoter (139). Nth1p is also regulated by PKA-mediated posttranslational modification (phosphorylation) (140, 141). Furthermore, Nth1p also needs Bmh2p (a 14-3-3 protein family member) and a Ca²⁺/calmodulin-dependent kinase II (Dsc1p) to control its activity (141). In contrast, the acid trehalase, Ath1p, functions at pH 5.0 and is important for utilizing trehalose as the sole carbon source (142). Both trehalose levels and its transport activity increase during glucose starvation, which suggests that trehalose transport and utilization are important under stress conditions that perturb carbon utilization. Crowe and Crowe (60) and Eleutherio et al. (143, 144) observed that trehalose is necessary at both intracellular and extracellular sites. The complexity of trehalase activity regulation in fungal cells is striking and further illustrates the central role of trehalose in fungal biology.

Similar to the situation for regulatory subunits, there are unfortunately, to date, relatively few studies on trehalases in pathogenic fungi. In *C. albicans*, there are two trehalase enzymes: Ntc1p (cytosolic trehalase) and Atc1p (cell wall-linked trehalase) (57, 145, 146). Ntc1p activity is not dependent on pH and is strongly inhibited by high ATP levels associated with glucose-replete conditions (145). Ntc1p activity is weakly activated by divalent cations (Ca²⁺ or Mn²⁺) but does not change in the presence of cAMP (145). While Ntc1p activity decreases during stationary phase, with growth on glycerol

media being an exception, Atc1p activity increases in resting cells and/or in trehalose- or glycerol-containing media (145). Atc1p activity is not increased in the absence of glucose and is not dependent on ATP, cAMP, divalent cations, or pH (145, 146). Importantly, the *C. albicans ntc1* null mutant has no virulence defect in a systemic murine model (147). However, in contrast to the *C. albicans Ntc1p* mutant, an *atc1* null mutant has a significant virulence defect in a systemic murine model that is associated with Atc1p roles in dimorphism and stress resistance (148). This promising result in the context of Atc1p loss warrants further investigation in *C. albicans* and perhaps other pathogenic yeast species.

For *C. neoformans*, only *nth1*, encoding an 826-amino-acid protein with identities to other fungal species proteins of between 45 and 62%, has been characterized (19). Nth1p in *C. gattii* has also been characterized and has approximately 89% sequence similarity to *C. neoformans* Nth1p (20). As with the *C. albicans ntc1* null mutant, the *nth1* null mutants of both *C. neoformans* and *C. gattii* have no apparent phenotypes related to virulence or *in vivo* fitness (19, 20).

In *A. nidulans*, an acid trehalase (*treA*) is essential for growth on trehalose but is not related to intracellular mobilization of the trehalose pool (67). However, the *S. cerevisiae* homolog of neutral trehalase in *A. nidulans* is involved in the mobilization of the intracellular trehalose pool (67). No studies to date have characterized the putative trehalases present in the *A. fumigatus* genome sequence and their potential role in host-pathogen interactions. Thus, with the exception of *C. albicans* Atc1p, fungal trehalases remain unclear as potential drug targets. Importantly, however, a trehalase inhibitor, validamycin, does exist and has been used to uncover a role for trehalose in the regulation of HSP90 function in *C. albicans* (149). As a critical chaperone involved in antifungal drug resistance, HSP90 is under active investigation as a therapeutic target (150, 151). Additional studies on the role of Atc1p in *C. albicans*-host interactions and in the context of HSP90-mediated drug resistance seem warranted. Along these lines, the role of fungal trehalases in shaping interactions with the microbiota and the subsequent impact on host immunity is still completely unexplored.

TREHALOSE TRANSPORTERS

In *S. cerevisiae*, Agt1p (also called Mal11p) is a high-affinity H⁺-trehalose symporter (152). The deletion of this gene causes decreased tolerance to peroxide and heat shock (153). Agt1p exports cytosolic trehalose to the extracellular space, and Ath1p degrades this trehalose pool during stress recovery (136). For pathogenic fungi, the trehalose transporter(s) has yet to be identified and characterized. However, from sequence-based analyses with BLASTp searches, many maltose permease transporters and other major facilitator superfamily (MFS) transporters that may potentially function as trehalose transporters are found in *C. albicans*, *C. neoformans*, *C. gattii*, and *A. fumigatus*. The relevance of trehalose transport in human invasive fungal infections is unclear but may conceivably affect potential interactions with the microbiota, where trehalose is produced by other resident microbes.

REGULATION OF THE TREHALOSE PATHWAY

Another viable strategy for targeting trehalose biosynthesis in pathogenic fungi is to identify conserved critical regulatory mechanisms. Studies examining the regulation of trehalose biosynthesis and its associated genes and encoded proteins remain in their infancy for pathogenic fungi. While early studies on trehalose biosynthesis in pathogenic fungi clearly revealed differences in model organisms, such as *S. cerevisiae*, we briefly review here what is known for this model system as a basis for future investigations of these pathogens.

The HOG-MAPK pathway in fungi consists of two important components: a two-component phosphorelay system and a MAPK module. The phosphorelay system contains hybrid sensor kinases, a histidine-containing phosphotransfer (HPt) protein, and response regulators that can sense and send an environmental signal to activate the MAPK pathway (154–156). For *S. cerevisiae*, there are many studies on the HOG-

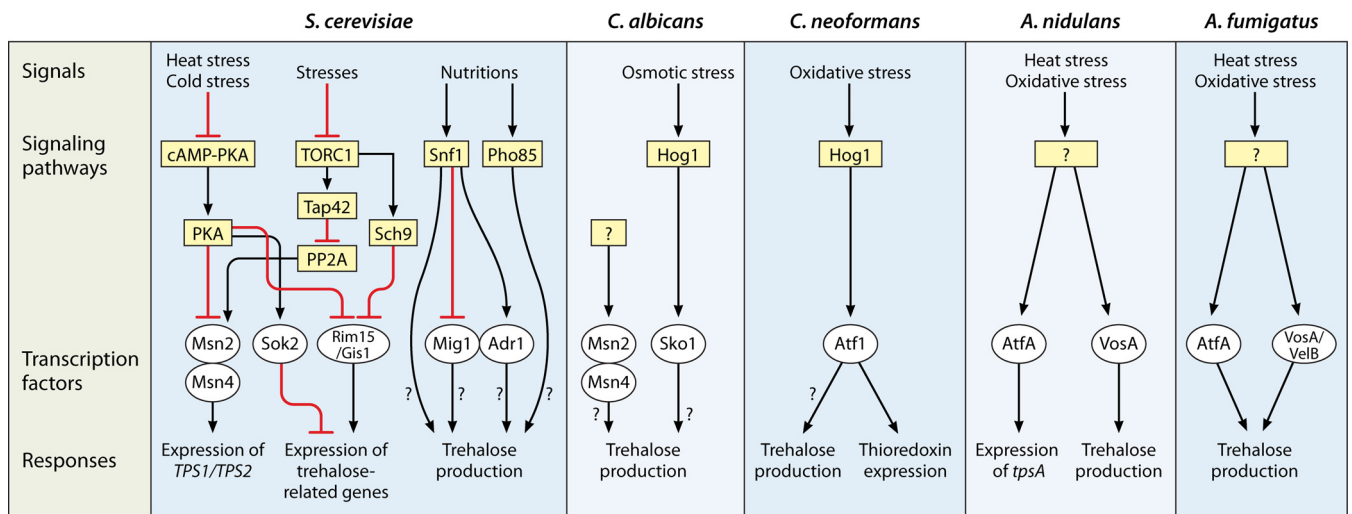


FIG 4 Comparison of mechanisms of regulation of the trehalose pathway in *S. cerevisiae*, *C. albicans*, *C. neoformans*, *A. nidulans*, and *A. fumigatus*.

MAPK cascade response to osmotic, oxidative, or heat stress (154, 155). Under these stress conditions, especially osmotic stress, the HOG pathway is important for yeast cells to grow and survive. The Hog1 MAPK is conserved in pathogenic yeasts and molds, i.e., *C. albicans* (Hog1p), *C. neoformans* (Hog1p), and *A. fumigatus* (SakA). While *hog1* mutants of *S. cerevisiae* and *sakA* mutants of *A. fumigatus* do not show decreased trehalose levels (157, 158), a *Cryptococcus hog1* mutant contains less trehalose than that in wild-type yeast cells (J. R. Perfect and R. G. Brennan, unpublished data). However, a clear link between trehalose metabolism and the SakA/MpkC pathway has been suggested for *A. fumigatus* and remains to be explored further (159).

S. cerevisiae requires the Msn2/4 transcription factors to express *tps1* and *tps2* at low temperatures (below 10°C) (160). These transcription factors are regulated via phosphorylation by the cAMP-PKA pathway, which plays an important role in the regulation of a wide range of stress conditions in addition to glucose signaling (161–166). Under stress conditions, e.g., heat and cold stresses, the Msn2/4p proteins localize to the nucleus and activate stress response genes that contain the canonical STRE sequence in their promoters, e.g., *tps1*, *tps2*, and *hsp12/26* (167). In the recovery stage or under nutrient-replete conditions, PKA-dependent phosphorylation of Msn2/4p restricts these proteins to the cytosol and prevents them from activating stress response genes (168). The PKA pathway also regulates the transcriptional repressor Sok2p, and intriguingly, trehalose-related genes also contain a binding site for Sok2p in their promoter regions (169). In addition, PKA can repress trehalose production through phosphorylation of the Rim15-Gis1p pathway, which binds to an upstream activating sequence (UAS_{pd_s}) in most trehalose-related genes (Fig. 4) (169).

In *C. albicans*, Msn2/4-like transcription factors do not appear to be directly related to stress responses (170). However, *C. albicans* Sko1p is involved in the osmotic stress response regulated by Hog1p and is also critical for the cell wall damage response (Fig. 4) (171, 172). A connection between the trehalose pathway and Sko1p or Msn2/4p is uncharacterized for *C. albicans*. Similarly, both *C. neoformans* and *A. fumigatus* lack apparent Msn2/4 and Sko1 homologs encoded in their genomes, though functional analogs may remain to be identified (173). *C. neoformans* does have the Atf1p transcription factor, which is not found in *C. albicans* (173, 174). *C. neoformans* Atf1p is transcriptionally regulated by Hog1p and is important for the oxidative stress response, in part through its regulation of thioredoxin expression (Fig. 4) (173, 174).

In *A. nidulans* and *Aspergillus oryzae*, the ATF/CREB-type transcription factor AtfA is a critical regulator of the conidial stress response (175–178). In *A. nidulans*, AtfA protects the fungus from oxidative and heat stresses (175). The expression of *tpsA* and a conidium-specific catalase gene, *catA*, in conidia is regulated by *A. nidulans* AtfA (Fig. 4) (175). *A.*

oryzae contains three ATF-like proteins, two of which (AtfA and AtfB) have been studied (177, 178). Conidia of *atfA* and *atfB* null mutants are susceptible to oxidative, UV, and heat stresses. Trehalose levels in conidia and the expression of *tpsA* and *tpsC*, which are homologs of *Sctps1* and *Sctps3*, respectively, are lower in both *atfA* and *atfB* null mutants than in the wild type (177, 178). Recently, Hagiwara et al. observed that AtfA in *A. fumigatus* is important for the fungus to survive under heat and oxidative stress conditions (158). As in other aspergilli, AtfA is involved in trehalose accumulation in the conidia of *A. fumigatus* (Fig. 4) (158). However, connections between AtfA and the virulence of *A. fumigatus* remain to be studied in an *in vivo* model.

TOR and TORC1 complexes in *S. cerevisiae* play a critical role in carbohydrate storage and metabolism in part by increasing the expression of glycogen- and trehalose-related genes (179, 180). TORC1 inhibits the Ser/Thr protein phosphatase 2A (PP2A) via phosphorylation of Tap42p. Therefore, inhibition of PP2A prevents dephosphorylation of Msn2p, which consequently remains in the cytosol in the phosphorylated form. While TORC1 can inhibit PP2A, it can also inhibit the Rim15-Gis1p pathway via activation of Sch9p to phosphorylate and sequester Rim15 in the cytosol (181). There is a connection between the PKA and TOR pathways through Msn2p, but the mechanisms that regulate and control the balance of these two pathways are unclear (168). Moreover, Pho85p and Snf1p also regulate trehalose and glycogen levels through transcriptional and posttranslational mechanisms (182, 183). It has been proposed that the Snf1 kinase regulates the transcription of Adr1p or Mig1p (a carbon catabolite repressor). However, the regulation of trehalose gene expression through Pho85p is unclear (Fig. 4). Determining the connections between these critical metabolism and fungal fitness regulatory pathways and trehalose biosynthesis in human-pathogenic fungi is a promising future research direction.

One family of regulatory proteins tied mechanistically to trehalose biosynthesis that is conserved in the pathogenic fungi is the velvet domain family. The velvet family proteins are conserved throughout the fungal kingdom, especially in the ascomycetes and basidiomycetes (184). This family is well characterized in *A. nidulans* and consists of four members, i.e., VelA, VelB, VosA, and VelC. VosA (viability of spores A) is a regulator of asexual development and is necessary for trehalose accumulation in *A. nidulans* conidia (184). Conidia of a *vosA* null mutant of *A. nidulans* display increased sensitivity to heat and oxidative stresses and lack trehalose (Fig. 4) (184). *A. fumigatus* velvet family *vosA* or *velB* null mutants also have reductions in conidial trehalose levels and a subsequent loss of conidial tolerance of oxidative and UV stresses (185). However, in contrast to those of *A. nidulans*, conidia of the *A. fumigatus* *vosA* mutant are not completely depleted of trehalose (Fig. 4) (184, 185). Further research is needed to investigate the impacts of velvet family-mediated trehalose biosynthesis and stress responses in the context of fungal pathogenesis.

In summary, the regulation of trehalose production in fungi is complex and involves multiple pathways, many of which remain to be investigated fully for human-pathogenic fungi. This complexity further reflects the importance of trehalose in the survival and adaptation of fungi in response to a wide variety of stresses. It is unclear if specific regulatory mechanisms that affect trehalose biosynthesis are promising antifungal drug targets, but it is clear that additional research into these mechanisms is warranted, with the potential to yield new insights into drug development.

FUTURE RESEARCH DIRECTIONS AND CLINICAL POTENTIAL

A brief review of the development of antifungal drugs highlights the challenges and paucity of current antifungal drug development. In the 1950s, the first systemic antifungal drug, amphotericin B deoxycholate, was licensed for clinical use (186, 187). After that important event, it took more than 2 decades to discover and use additional antifungal drug classes, including the azoles and echinocandins. In the 1980s, the discovery and development of the triazoles dramatically improved the treatment of invasive fungal infections, especially for polyene-refractory infections (188). In the 1990s, triazoles and amphotericin B formulations were further developed (188). Even

though the newest antifungal drug class, the echinocandins, was discovered in the 1970s, it took almost 30 years for these drugs to be used in the treatment of invasive fungal infections (approved by the FDA in 2002) (187, 188). The latest FDA-approved antifungal drugs are posaconazole (2006), anidulafungin (2006), and isavuconazole (isavuconazonium sulfate) (2015) (<http://www.accessdata.fda.gov/scripts/cder/daf>).

From this brief history of antifungal drug development, it is clear that there are many gaps in time between drug class discovery and development of a usable drug (189–192). Given the increasing number of fungal infections and the emergence of drug resistance and innate resistance of select fungal pathogens to some drugs, a potential shortage of effective antifungal drugs in the near future is a very real cause for concern. It remains a great challenge to identify and develop new fungicidal drugs that target these eukaryotic pathogens effectively, with minimal adverse host side effects.

A current approach for antifungal drug development is to identify and target essential processes in fungi. However, it is very difficult to discover targets that, when inhibited, have a killing effect only in the fungi, not against human cells. In addition to these challenges, it is argued that targeting essential genes may lead to rapid emergence of drug resistance, though this remains a topic for robust conversation (193). Another perspective is that organisms will be pathogens when they express virulence attributes that function to induce disease (194). Therefore, instead of targeting essential genes, targeting attributes involved in virulence is an alternative approach to the development of novel antifungal drugs specific to fungi but safe for humans (195). Additional approaches to drug development must be encouraged, as we need a continuous discovery of new agents to ensure that current antifungal drugs reach their full therapeutic potential against human fungal pathogens. Therefore, to keep up with drug resistance and new emerging causes of disease, we need to fully consider and appreciate a pathogenesis-based approach as an additional option for antifungal drug development (195).

Several reports suggest that targeting virulence factors can serve as a viable approach to the development of novel antifungal agents (195–197). For instance, an inhibitor of glycosylphosphatidylinositol (GPI) biosynthesis, which is important for GPI-modified proteins that provide cell wall integrity and membrane homeostasis (198), was discovered and developed into the oral broad-spectrum antifungal inhibitor E1210 (199, 200). E1210 is effective in murine models of oropharyngeal and disseminated candidiasis, pulmonary aspergillosis, and disseminated fusariosis (200). It has activities against fluconazole-resistant *Candida* strains, *Pseudallescheria boydii*, *Scedosporium prolificans*, and *Paecilomyces lilacinus* (201–203). While the GPI biosynthesis target is not a canonical virulence factor in the traditional definition of the term, and its inhibition directly affects fungal growth, its use highlights that studies on virulence-associated processes can identify targets that ultimately affect fungal fitness *in vivo*.

Along these lines, unique metabolic pathways that exist only in fungi and function when saprophytes cause disease should be a point of focus for therapeutic development. The trehalose pathway is one of these unique metabolic pathways. As discussed throughout this review and elsewhere, proteins involved in trehalose biosynthesis are critically involved in many aspects of the biology and virulence of pathogenic fungi (15, 18–21, 68, 204). Importantly, we are now beginning to develop the preliminary infrastructure in these major fungal pathogens to make insightful discoveries with therapeutic potential, with the trehalose pathway serving as one potential model (122). The search for inhibitors of this pathway as potential antifungal drugs has begun and represents both the synthesis and degradative portions of trehalose biology (205). There are already inhibitors of the trehalase enzymes, such as validamycin A, that have been identified (206). Many studies of plants and parasites have characterized the structure of trehalose-related enzymes to screen for small-molecule inhibitors (132, 207). For example, Xue et al. (207) characterized the structure of TPS1 from the plant pathogen *Magnaporthe oryzae* and screened for potential small-molecule inhibitors

by using a chemical database *in silico*. These studies are now possible for human-pathogenic fungi.

Recently, Miao et al. provided a new level of understanding of the major enzymes within the fungal trehalose pathway (122). In their studies, structures of the trehalose-6-phosphate phosphatase (Tps2) at different stages of the catalytic process were solved. These structures unveil the specific conformational changes and enzymatic components needed for substrate recognition and subsequent phosphate removal. Moreover, they reveal the essentially identical catalytic pockets, and therefore identical substrate recognition and enzyme mechanisms, of the *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* Tps2 proteins. Biochemical, *in vivo*, and structural investigations show how these enzymes are substrate specific (Fig. 5), thereby protecting the fungal cell from the potential cytotoxicity of T6P. The finding that fungal Tps2 proteins possess extreme substrate specificity as phosphatases for T6P bodes well for the creation of a specific inhibitor with limited “off target” phosphatase-inhibitory activity and thus toxicity. As an added “bonus” to this substrate specificity, structure-guided mutagenesis of residues involved in T6P binding and the very poor T6P phosphatase activity of the single-site mutants suggest that simple single-site Tps2 mutations within the catalytic pocket will not allow the generation of resistant strains. Although Tps2 mutants with changes within the catalytic pocket might avoid drug binding, they will not be able to produce trehalose from T6P, resulting in high levels of cytotoxic T6P. Finally, the structures of Tps1 proteins of several pathogenic fungi have been determined in complex with substrates and substrate analogs (Y. Miao, J. R. Perfect, and R. G. Brennan, unpublished data). Therefore, the high-resolution structures of the core targets of canonical fungal trehalose biosynthesis are now available such that aggressive drug discovery programs to identify novel inhibitors, and ultimately drugs, are able to commence, with the goal of disrupting this key pathway and thereby leading to novel, broad-spectrum fungicidal drugs. At minimum, the expected discovery of small-molecular inhibitors of these proteins will allow rigorous hypothesis testing in relevant animal models of infection to address many of the challenges facing therapeutic targeting of this pathway that are discussed throughout this review.

Consequently, the future of the trehalose biosynthesis pathway as a highly desirable antifungal drug target seems bright. Further mechanistic investigations into the functions of key proteins in the pathway are needed for the respective human fungal pathogens to help in determining additional therapeutic leverage points and to address concerns about the potential limitations of inhibitors of this pathway. With regard to the latter, in addition to some of the issues addressed throughout this review, because this pathway is found in a wide variety of organisms, inhibitors of this pathway may affect other human commensals, which should be investigated further. In fact, the role of trehalose biosynthesis in microbe-microbe interactions is an intriguing area of investigation. Depending on the specificity of the inhibitors’ structural design and routes of administration, it seems, however, that this potential limitation is addressable if needed. In addition, in filamentous fungi, such as *A. fumigatus*, redundant protein functions, including other alternative pathways or unknown phosphatases (21), may affect the efficacy of inhibitors, and this simply requires more investigation as highlighted by the example of the *A. fumigatus* *tpsA/B* null mutant (68).

One additional concern that is receiving more attention in the field is the efficacy of a given agent in the context of an established infection microenvironment. While the data discussed in this review suggest that trehalose biosynthesis proteins likely have roles in established infections, this remains to be fully validated experimentally. The use of conditional promoter systems *in vivo* may be a viable approach to confirm the role of these proteins in established infections, as will the identification of small-molecule inhibitors. All of these concerns emphasize one of the main themes of this review, i.e., that further studies are essential to move drug discovery forward to meet the challenges that await. A particularly attractive area worth further emphasis is the link between trehalose biosynthesis and fungal cell wall homeostasis given the critical importance of this fungus-specific structure to virulence and host immune responses.

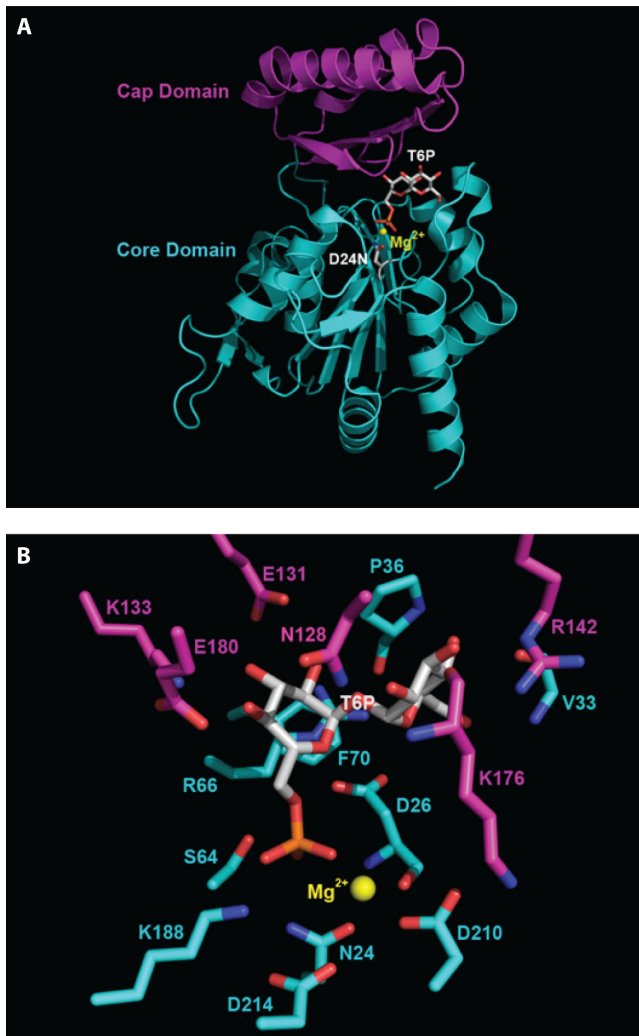


FIG 5 Structure of Tps2 in complex with its substrate, trehalose-6-phosphate. (A) Structure of *Cryptococcus neoformans* Tps2 (CnTps2), depicting the cap domain in magenta and the core domain in cyan. Substrate-bound Tps2 takes a “closed” conformation, bringing the cap and core domains closer, whereas substrate-free Tps2 assumes an “open” conformation whereby the cap and core domains are more splayed. Trehalose-6-phosphate (T6P) and residue N24 are labeled and depicted as atom-colored sticks, and the catalytically important magnesium ion (Mg^{2+}) is shown as a yellow sphere and labeled. In wild-type CnTps2, residue 24 is an aspartate and is responsible for the nucleophilic attack on the phosphate group of T6P. (B) View of the active site of Tps2 bound to T6P. All residues that interact with either T6P or the Mg^{2+} ion or play a key part in forming the substrate-binding pocket are shown as atom-colored sticks and labeled. The carbon atoms of residues from the cap domain are colored magenta, while those from the core domain are colored cyan. T6P is shown as atom-colored sticks, with its carbon atoms colored white, and the catalytically important Mg^{2+} ion is shown as a yellow sphere. All solvent molecules and dashes indicating protein-substrate or protein- Mg^{2+} interactions have been omitted for the sake of clarity. Note the tripartite stacking of the side chains of residues F70 and R66 and the glucose-6-phosphate ring of the T6P disaccharide.

In this arena, inhibitors of trehalose metabolism may prove to be highly effective adjunctive therapeutics in combination with existing cell wall-targeting drugs. These hypotheses and associated key *in vivo* experiments are on the cusp of being realized, as emphasized by the determination of key biosynthesis protein structures and the associated search for small-molecule inhibitors.

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