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**REVIEW**

# Thermotolerance in Fungi: The Role of Heat Shock Proteins and Trehalose

**V. M. Tereshina**

*Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*
*e-mail: V.M.Tereshina@inbox.ru*

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**Abstract**—The parallel synthesis of heat shock proteins and trehalose in response to heat shock did not allow the role of these compounds in the acquisition of thermotolerance by fungal cells to be established for a long time. This review analyses experimental data obtained with the use of mutant fungal strains and shows differences in the thermoprotective functions of trehalose and heat shock proteins in relation to cell membranes and macromolecules. The main emphasis has been placed on data demonstrating the thermoprotective role of trehalose in fungi, the present-day understanding of its biological functions, and mechanisms of trehalose interaction with subcellular structures and cell macromolecules.

*Key words*: heat shock, trehalose, heat shock proteins.

Fungal cells subjected to nonlethal heat shock acquire resistance to lethal heat shock. This phenomenon is referred to as acquired thermotolerance. For example, if yeast cells growing under optimal conditions (25°C) experience a temperature of 37°C, they acquire resistance to a lethal temperature of 50°C. The following main events can be identified as occurring in yeast cells under nonlethal heat shock [1]: synthesis of heat shock proteins (HSPs), trehalose synthesis (up to 0.5 M), maintenance of intracellular pH by regulation of the activity of membrane ATPase, a decrease in the activity of water and/or its redistribution in cytosol compartments, and detoxification of active oxygen species.

Of these events, two key processes are singled out: the synthesis of HSPs and the synthesis of trehalose. As they occur in parallel, an assessment of the role of these metabolites in the acquisition of thermotolerance by cells is problematic. In the last two decades, this problem has been studied in detail, and experimental data have been obtained that allow us, in the final analysis, to clarify the contradictory results of earlier investigations. First, the enzymatic (in particular, protease) and chaperone functions of many HSPs under heat shock were identified [2–5]. Second, trehalose synthetase was isolated [6, 7], which allowed not only its structure to be studied but also a new fact to be discovered: the regulation of hexokinase by trehalose-6-phosphate [8]. Thus, trehalose synthetase appeared to be the regulator of the involvement of glucose in the glycolytic pathway [9]. The data showing that two trehalose subunits are true HSPs was quite unexpected [10]. Using different molecular–biological approaches, the thermoprotective function of trehalose in vivo was demonstrated [11, 12]. Moreover, a hypothesis was proposed that molecular and chemical chaperones (i.e., HSPs and low-molecular-weight compounds, including those of a carbohydrate nature) interact in protein folding, which is a coordinated process of the antistress protection of cells and "a control system of protein quality" [13]. This review focuses on the regulation of trehalose metabolism and HSP synthesis, on the evidence of the thermoprotective function of trehalose in vivo, and on the present-day understanding of the functions of trehalose in cells.

## 1. HEAT SHOCK PROTEINS AND THEIR ROLE IN FUNGAL CELLS

According to Schlesinger's definition [14], HSPs are proteins whose synthesis is substantially stimulated by a temperature several degrees in excess of the optimum one. HSP genes contain, in the promoter region at the 5' noncoding end, a specific conservative 14-bp site whose presence is necessary for initiation of the transcription of HSP mRNA. In drosophilae, for example, HSP synthesis begins at 4°C higher than the optimum temperature, but the quickest response is observed when the temperature is increased by 10–12°C: after 4 min, the formation of mRNA molecules begins, and, after an hour, their numbers may reach several thousand per cell [15]. Simultaneously, the transcription of the so-called housekeeping genes, which were previously active, stops, as does the translation of their existing mRNA. During the entire period of elevated temperature, HSPs are the main proteins synthesized in the cell. Upon exposure to heat shock, the inhibition of vital

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activity occurs, which is primarily connected with rigorous inhibition of the synthesis of the proteins apart from the HSPs.

The synthesis of specific highly conservative proteins in response to heat shock is a universal reaction found in all living organisms. HSPs are divided into several families by their molecular mass: 100-, 90-, 70-, and 60-kDa HSPs [2, 14, 15]. In addition, small HSPs are synthesized. These have a molecular mass of 12–43 kDa and contain the so-called α-crystalline domain, a small (80–100 amino acids) conservative site at the *C* terminus [5]. In addition, some researchers believe that there exists another low-molecular-weight HSP, ubiquitin (8000 Da), characteristic of eukaryotic organisms [14]. The most universal HSPs in many organisms are HSPs 90, 70, and 20–30. Several types of HSPs are known in relation to their functions: chaperones (HSPs 70 and 60), proteins with catalytic activity (proteases, HSP 100 in particular; ubiquitin; tyrosine phosphatase), and proteins with an obscure function (α-crystallines, secreted glycoproteins) [3].

### *1.1. The Main HSP Families*

**HSPs 70** are a family of proteins possessing the highest degree of homology of their amino acid compositions: 70–80% among eukaryotic organisms and 50% homology of eukaryotic proteins with *Escherichia coli* proteins [14]. The genes encoding these proteins in *Saccharomyces cerevisiae* are divided into four subfamilies: *Ssa, Ssb, Ssc*, and *Ssd.* The expression of these genes is regulated in different ways. Thus, for example, protein SSA4p, a typical HSP with a low basal level, is substantially induced by shock, whereas proteins SSA1p and SSA2p are formed constitutively in small amounts [2]. These proteins are assigned to chaperones, a special class of proteins assisting in the process of proper folding of the polypeptide chain in the protein molecule [1, 2, 4]. HSPs 70 have ATPase activity and interact with the aggregates of denatured proteins, contributing to their deaggregation and further proper chain folding de novo.

**HSPs 90** are conservative chaperone-like proteins with weak ATPase activity. They are present in appreciable amounts in eukaryotic cytoplasm, and a small amount is found in the nucleus. In *S. cerevisiae* HSPs 90 may also function in the form of a macromolecular complex with HSPs 70 and 60. Two genes of these proteins are known in yeasts: *hsc* 83 and *hsp* 83. The expression of the former is constitutive, whereas the expression of the latter is induced by heat shock or upon transition to the stationary growth phase and sporulation [2].

**HSPs 100** are proteases with ATPase activity. HSP 104 in *S. cerevisiae* is synthesized under conditions of normal growth on fermentable sources of carbon. The expression of the *hsp* 104 gene occurs in the idiophase and at the initial stages of sporulation [16]. This protein is thought to be required for the induction of heat tolerance under lethal stress and for the general stability of aerobically growing cells. Deletion of the *hsp* 104 gene results in the loss of tolerance not only to heat but also to ethanol and arsenite and affects the viability of cells stored at low temperatures [3, 16].

**Small HSPs** are proteins with a chaperone function. They do not possess ATPase activity. Small HSPs are involved in protein folding under normal and, especially, extreme conditions and contribute to the elimination of completely denatured proteins and the renaturation of partially denatured ones, "passing" these proteins to chaperones possessing ATPase activity [5]. *S. cerevisiae* is known to have three small HSPs: 30, 26, and 12. HSP 30 is a stress-induced highly hydrophobic integral membrane protein whose main function seems to be the inhibition of the activity of the proton ATPase under heat shock conditions, which leads to energy conservation in the form of ATP [9].

**Ubiquitin** is a polypeptide with a molecular mass of 8 kDa consisting of 76 amino acids. It is involved in the universal nonlysosomal ATP-dependent degradation of polypeptides in eukaryotes. Ubiquitin has been shown to be activated by ATP via the carboxy-terminal glycine residue that is subsequently bound to the ε-lysine group of the protein that, in such a complex, undergoes proteolysis [14]. Ubiquitin is not required for vegetative growth at optimal temperatures, but it is essential for resistance to different kinds of stress, e.g., stress imposed by nonoptimal temperatures, starvation, or amino acid analogues. This protein was also found to be synthesized during sporulation and to be necessary for the maintenance of spore viability [3].

### *1.2. Regulation of HSP Synthesis*

Two main mechanisms of the induction of HSP gene expression are known: specific mechanisms, which operate in response to temperature stress, and general ones, occurring in response to different stresses (starvation, oxidative stress, osmotic stress, exposure to weak organic acids, ethanol, or low pH values). The specific mechanism is normally induced by an increase in temperature and is mediated by a transcriptional activator protein (heat shock factor (HSF)) that is capable, in the form of a homotrimer, of binding to a site in the HSP gene promoter—a short conservative DNA element (heat shock element (HSE)). A characteristic feature of the HSE is the presence of a repeating five-nucleotide motif that determines the HSF–HSE interaction. In the yeast, the expression of HSPs 104 and 70 is regulated in this way [18].

Several modes of HSF activation are thought to be possible: self-activation, activation with the involvement of HSP 70, and activation as a result of the release of a membrane thermosensor protein. Evidence in favor of the first mode is given by data indicating that, under in vitro heat shock, HSF monomers independently form

homotrimers, which bind to HSEs and initiate the expression of HSP genes [2]. In response to heat shock, HSF activity increases 30- to 100-fold and persists for 1 h [19]. The second mode of HSF activation, mediated by HSP 70 (negative modulation of HSF trimerization), is in agreement with the temporary character of the response to heat shock. At the beginning of the response, when numerous unfolded proteins appear, the HSF–HSP 70 complexes disintegrate, probably due to the higher affinity of HSP 70 to such proteins, and the HSF monomers thus released associate to form functionally active trimers. When the HSP 70 level increases in the heat shock process, the complex is restored and HSF trimer formation stops. This hypothesis is also supported by the fact that, in eukaryotes and prokaryotes, mutation in HSP 70 leads to HSP gene expression at temperatures that are optimal for growth [20]. The third mode of HSF activation suggests the following chain of events during heat shock: a decrease in membrane viscosity—the formation of domains with a nonbilayer phase—release of the thermosensor protein from these domains—interaction of the thermosensor protein with the HSF [21].

The second form of the activation of HSP expression is mediated by the regulatory protein Msn2/Msn4 (140 kDa). This protein recognizes a gene promoter site known as the stress response element (STRE), which possesses a characteristic nucleotide sequence, CCCCT (abbreviated as $C_4T$) [1, 2, 21]. It is by these means that, in *S. cerevisiae*, the induction of the expression of *cttl1* genes (catalase T genes), *trs1* and *trs2* genes (genes of the first and second trehalose synthetase subunits), and HSP 12 occurs. Gene activation via STRE occurs under different types of stress and accounts for the induction of HSP and trehalose syntheses under stresses caused not only by temperature but also by other factors.

In addition to the main mechanisms of HSP gene induction described above, other modes of regulation have also been revealed. Thus, *S. cerevisiae* has two protein factors, Yap1 and Yap2, regulating HSP 30 induction via an as yet unknown mechanism but not via HSE and STRE. The HSP 30 level is greatly increased under temperature stress or after treatment with ethanol and weak organic acids (e.g., sorbic acid). An increase in the amount of HSP 30 in the membrane is considered to decrease its fluidity; thus, it regulates the membrane function, since, under heat shock, the fluidity of the phospholipid bilayer considerably increases [22].

Evidently, the mechanism of transduction involving small GTPases of the RAS family and cAMP is also involved in the negative control of the work of the promoters of *hsp 70*, *ssa3, ub14*, *ctt1*, and *hsp 12* genes [23]. The STRE activity is also regulated by Yap1 (response to metabolic stress) and is subject to negative control by cAMP-dependent protein kinase. However, Yap1 does not influence the HSE–HSF mechanism [21]. The interaction of the regulatory factors involved in the process of HSP gene expression is still a poorly studied area.

## 2. THE METABOLISM OF TREHALOSE

The disaccharide α,α-trehalose, or α-1-D-glucopyranosyl-α-1-D-glucopyranoside (α,α-1,1-diglucose, or fungal sugar, mycose), belongs to the group of nonreducing sugars, as its molecule contains no free hemiacetal hydroxyls. Trehalose is the most widespread naturally occurring disaccharide. It has been revealed in all of the kingdoms: in fungi, myxomycetes, animals (insects, nematodes, crustaceans, and parasitic worms), plants (red algae, vascular cryptogams, and certain spermaphytes), and bacteria (cyanobacteria, mycobacteria, and actinomycetes) [24].

### *2.1. Biosynthesis of Trehalose*

The first in vitro synthesis of trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate was carried out in 1958 using a cell-free yeast extract [25]: UDPG + glucose-6-P ⟶ UDP + trehalose-6-P. The reaction required the presence of $Mg^{2+}$ ions and was irreversible; the pH optimum was 6.6. A partially purified enzyme preparation also revealed phosphatase activity, which explains the presence of trehalose itself, in addition to trehalose-6-P, in the end products.

Thirty years later, trehalose synthetase was isolated and purified [6, 7]. This is a multimer complex consisting of at least the following three subunits: TPS1, TPS2, and TSL1, which denote trehalose-6-P synthetase (56 kDa), trehalose-6-P phosphatase (102 kDa), and a regulatory subunit (123 kDa), respectively. It is thought that one more protein (TPS3), which has a high homology to TSL1, may be the fourth complex component. It performs the functions of complex stabilization and regulation of the activity of trehalose-6-P synthase under heat shock. A trehalose synthetase activator was also revealed. It is a protein dimer that consists of two 56-kDa subunits and exhibits the activity of a phosphoglucoisomerase, i.e., catalyzes the conversion of glucose-6-P to fructose-6-P. TPS1 and TPS2 were established as true heat shock proteins [10]; their genes have the $C_4T$ sequence characteristic of HSP genes. The expression of the trehalose synthase genes is regulated not only by heat shock but also by osmotic shock and starvation and is subject to negative control by the RAS–cAMP system [9]. The use of radioisotopic methods showed that the activity of trehalose-6-P synthetase is not directly stimulated by ATP and cAMP but is increased several times by cAMP-dependent protein kinase [27]. Trehalose-6-P phosphatase is highly specific to trehalose-6-P and requires the presence of $Mg^{2+}$. Its activity is not influenced by the levels of cAMP, ATP, or cAMP-dependent protein kinase. These investigations have led to a reconsideration of the data on the regulation of the activity of trehalose synthetase by means of ATP-dependent phosphorylation and dephos-

phorylation [28]. It should be emphasized that trehalose is synthesized in the form of trehalose-6-phosphate but stored in the form of a dephosphorylated carbohydrate.

It has been established that, in addition to UDP-glucose, there exist two more endogenous resources for trehalose synthesis—glycogen [29] and maltose [30].

### *2.2. Degradation of Trehalose*

The enzyme trehalase has a wider natural distribution than trehalose, as it is also present in organisms that do not contain this disaccharide. Two types of trehalases are known: regulated (neutral) and unregulated (acid). Regulated trehalases occur in cells an inactive form and require phosphorylation for their activation. Unregulated trehalases are present in cells in an active form, and their activity is controlled by compartmentalization of the substrate and the enzyme. In some fungi, only acid (unregulated) trehalase has been found (e.g., in *Aspergillus oryzae*, *Candida albicans*, *Schisophyllum commune*, and *Coprinus lagopus*), whereas, in others, only regulated trehalase was detected (*Mucor rouxii* and *Phycomyces blakesleeanus*). However, there are fungi possessing both types of trehalases (*Candida utilis* and *S. cerevisiae*) [31]. The attempts to reveal neutral trehalase in mycelial ascomycetes were, for a long time, unsuccessful, although it was shown in experiments with [$^{14}$C]glucose that acid trehalase is not involved in trehalose degradation in *Neurospora crassa* after the cessation of heat shock [32]. The neutral trehalase of *A. nidulans* and *N. crassa* is regulated by cAMP and $Ca^{2+}$ and is responsible for trehalose mobilization and glycerol accumulation during the germination of conidia [33].

There is extensive experimental material available that allows a conclusion to be made that trehalose is localized in the cytoplasm, where the inactive regulated form of trehalase, which requires activation by cAMP-dependent phosphorylation, is also present [26, 31, 34]. In the cytosol of *P. blakesleeanus* spores residing in a state of endogenous dormancy, cAMP-dependent trehalase, which is activated after heat shock or after chemical treatment of the spores with monocarboxylic acids, has been revealed [35]. Moreover, the spores have been found to contain a membrane-bound enzyme that seems to be an integral membrane protein (it does not pass to a 1 M NaCl solution and dissolves only when the membrane fraction is treated with neutral detergents, such as NP-40 and octylglucoside). This enzyme is inhibited by cAMP [36]. Active acid trehalase is localized separately from trehalose, being either in a vacuole, as, for example, in the yeast *C. utilis* [37], or in the cell walls of hyphae [31] and spores, e.g., the ascospores of *N. crassa* [38]. In yeasts, trehalose is likely to be transferred by active transport or pinocytosis into the vacuole, where its hydrolysis to glucose occurs [39]. In the vegetative cells of mycelial fungi, acid trehalase is a secreted enzyme that affords the utilization of exogenous trehalose. It has been shown that, in *Candida albicans* [40] and *N. crassa* [32], acid trehalase associated with the cell walls is not involved in the degradation of trehalose after the cessation of heat shock.

**Under heat shock,** the activity of neutral trehalase (NTH1) increases tenfold and a phosphorylated enzyme binds to the membrane [41]. Protein kinase A is the only enzyme that directly phosphorylates and activates NTH1. Purified NTH1 of *S. cerevisiae* is a homodimer consisting of two subunits with a molecular mass of 80–86 kDa; its $K_m$ for trehalose is 5–35 mM [29]. The expression of the *nth1* gene is induced by heat shock, which is confirmed by the presence of the $C_4T$ sequence for STRE-mediated regulation [42].

It appears that the activity of neutral trehalase may also be regulated by the cAMP-independent mechanism. It has been found that, when growing cells of the yeast *Pachysolen tannophylus* are subjected to heat shock, the activation of trehalase occurs without cAMP involvement both in the absence and in the presence of glucose [34]. In *C. utilis*, the trehalase activity is stimulated by nitrogen sources, inhibitors of protein synthesis, and ionophores (uncouplers of the respiratory chain), irrespective of the presence of glucose, and is not determined by cAMP-dependent phosphorylation [43]; in contrast, in *S. pombe*, it requires the presence of glucose and is connected with an increase in the cAMP level [44]. Another variant of dependence is observed in *S. cerevisiae*: for the transduction of a nitrogen-induced signal, the presence of glucose is required; however, it has been shown that trehalose phosphorylation occurs with the involvement of another, cAMP-independent, protein kinase [45].

There has been little study of the molecular structure of acid trehalase. It has been established that the acid trehalase isolated from vacuoles is a glycoprotein, which retains its activity after treatment with endo H, an enzyme hydrolyzing β(1–4) bonds in glycoproteins (thus, the activity is not related to the carbohydrate portion) [37]. In in vitro experiments, acid trehalase from the cell walls of *Aspergillus oryzae* conidia is strongly inhibited by mannitol [46].

### *2.3. The Biological Role of Trehalose*

**2.3.1. Energy function.** In the process of fungal cell differentiation, trehalose is accumulated in the idiophase, when the inhibition of growth processes is observed [47]. This disaccharide is referred to as the dormancy sugar, since its active synthesis occurs during sporulation and attains the highest level in resting forms. For example, in the ascospores of *N. tetrasperma*, and *Myrothecium verrucaria*, trehalose accounts for 14 and 20% of the dry spore mass, respectively [48]. For *Pichia pastoris*, evidence for the reserve function of trehalose in the process of germination of resting cells has been provided by the $^{13}$C NMR method [49]. It has been established that 80% of trehalose is

mobilized at the early stages of swelling, and 20% remains intact. A sharp reduction in the trehalose level has been shown for the early stages of the germination of *Cunninghamella japonica* spores [50] and *A. niger* conidia [51]. A common regularity is the rapid restoration of the trehalose level as soon as the spores are capable of utilizing exogenous glucose. In *P. pastoris*, endogenous trehalose makes up only 9% of the carbohydrates necessary for germination; thus, the main source of energy during germination is exogenous glucose, while trehalose fulfills the energy requirements at the initial stage of germination [49]. In the cycle of *S. cerevisiae* development, trehalase activity is dramatically increased at the beginning of budding, which is indicative of the use of trehalose as a reserve compound. At the stage of cell division, the activity of this enzyme decreases fivefold [52]. Mutants of *C. japonica* exemplify the direct correlation between the amount of trehalose in the spores and their capacity for germination [50].

It has been established that, in the process of spore storage, a slow utilization of trehalose occurs. This was shown for the ascospores of *P. pastoris* [49], basidiospores of *A. bisporus* [53], and sporangiospores of *Blakeslea trispora* [54]. Considerable fluctuations of the trehalose content in the process of storage are characteristic of the exogenously resting conidia of *A. niger*: its content decreases to 3–4% before subsequent trehalose resynthesis, probably from endogenous organic acids; thus, there exist mechanisms for the maintenance of a certain trehalose level in resting cells. Quite unexpected were the data obtained on the ability of *A. niger* conidia to change its trehalose and glycerol contents in response to heat and cold shocks, which is indicative of the existence, in resting cells, of adaptation mechanisms similar to those existing in vegetative cells [55].

However, there are conditions under which trehalose accumulates in the trophophase in actively growing cells: heat and osmotic shocks, drying, hydrostatic shock, and other stressors. It has been established that the activation of trehalose synthesis is a universal response to heat shock and does not depend on the temperature optimum of a fungus [54, 56–58]. In addition, the hypothesis that thermophilia may evolve in fungi on the basis of constitutive synthesis of trehalose in the trophophase has been proposed [59].

**2.3.2. The regulatory function.** Heat shock is known to cause a dramatic increase in the content of glucose in cells, since a partial inhibition of glycolysis occurs [1]. Trehalose synthesis may protect against the toxic and mutagenic effects of glucose, which may nonenzymatically react with proteins and nucleic acids under stress conditions [60]. One hour after an increase in temperature to 40°C, a 100-fold rise the trehalose concentration is observed in the cells of *S. cerevisiae* (from 0.01 to 1 g/g protein); a more than 50-fold increase occurs in the concentrations of glucose and ATP (at 37°C, 5 μmol/g of dry biomass is formed) [61]. It has been demonstrated that, under heat shock, a rapid exchange of the label between [$^{14}$C]glucose and trehalose occurs. The synthesis of one trehalose molecule from glucose requires three molecules of ATP, whereas no ATP is formed when trehalose degrades to glucose, which provides evidence for the existence in this case of a futile cycle whose purpose is probably to maintain constant levels of glucose and ATP.

Later, it was established that trehalose synthetase performs a double biological function: it catalyzes the trehalose synthesis proper and, via trehalose-6-P, controls the activity of hexokinase [8] and the involvement of glucose in the glycolytic pathway [9]. The latter conclusion is based on the fact that a mutation in the trehalose synthetase subunit trehalose-6-P-synthetase results in the inability of the yeast to grow on glucose.

**2.3.3. The protective function. In vitro evidence of the protective function of trehalose.** As early as 1971, Crowe suggested that trehalose is capable of substituting water around the polar groups in labile macromolecular systems [62]. The stabilizing effect of trehalose during membrane freezing and drying has since been established with respect to artificial membranes and membranes isolated from cells. For example, during lyophilization, trehalose (0.3 g/g of lipids) effectively protects frozen vesicles of the sarcoplasmic reticulum from lobster muscles, preserving not only their structure but also their function, namely, the capacity for ATP-dependent transport of $Ca^{2+}$ [63]. Fifty-nanometer monolayer phospholipid vesicles (liposomes) form, after lyophilization and subsequent rehydration, multilamellar structures of a significantly greater size. As a result, the membrane permeability is disrupted, and the liposome contents leak out. In the presence of trehalose, the membrane structure is retained, and membrane permeability is not impaired [64].

The most important requirement for membrane functioning is the maintenance of the mesomorphic structure of the lipid bilayer. In a hydrated state, one phospholipid molecule binds 10–12 molecules of water. Dehydration entails the loss of water bound to the polar groups of phospholipids, which results in a decrease in the distance between the phospholipid molecules, enhancement of the van der Waals interactions between their acyl chains, and, as a consequence, formation of a solid (gel) phase. During the gel–liquid crystal change of phase, the bilayer membrane structure is retained. Investigations using the method of differential scanning calorimetry of artificial dipalmitoyl phosphatidylcholine membranes dried in the presence of trehalose have shown that the temperature of their change of phase is 41°C, which is characteristic of a hydrated lipid bilayer, whereas the temperature of the change of phase of the membranes dried in the absence of trehalose is as high as 67°C [62].

In order to find out how trehalose interacts with the lipid bilayer, mathematical molecular modeling and

physicochemical studies using x-ray diffraction and solid-phase NMR have been carried out. The physicochemical methods showed that trehalose, similarly to water, forms hydrogen bonds with the polar heads of phospholipids. In so doing, the sugar intercalates into the membrane, performing the function of a spacer and increasing the distance between the phospholipid molecules [65]. Study of the crystalline structure of trehalose showed that the glucose rings in its molecule are located in different planes and, therefore, are not spatially equivalent [66]. Molecular modeling that assumed the formation of the maximum number of hydrogen bonds showed that, in this case, one glucose ring of trehalose remains on the membrane surface, forming five hydrogen bonds with the polar phospholipid heads, while the other glucose ring intercalates into the hydrophobic portion of the membrane between the phospholipid acyl chains.

Another important event influencing membrane functioning is the phase separation upon dehydration, since membranes contain 20–80% protein. As can be seen from the above example with the vesicles of the sarcoplasmic reticulum, the presence of sugar not only helps to keep the membranes intact but also retains the function of the enzymes involved in calcium ion metabolism [63]. Using phosphofructokinase, a very labile enzyme that is irreversibly deactivated on freezing or drying at room temperature, it has been established that many sugars, as well as proline, glycerol, and trimethylamine-*N*-oxide, stabilize this enzyme during freezing but are unable to protect it during drying. In the presence of 100 mM of trehalose, the enzyme retained its activity both during freezing and drying [62]. Thus, the use of membranes isolated from cells and model membranes consisting of pure phospholipids has shown that trehalose and some other carbohydrates are capable of membrane protection during dehydration; specifically, they prevent membrane mergence, change in the phase of the lipid bilayer, and phase separation, as well as being involved in the stabilization of membrane-bound enzymes. The attempt to understand why many low-molecular-weight organic compounds protect enzymes in aqueous solutions has led to the suggestion that they are involved in the formation of the solvate envelopes of protein macromolecules. It should be emphasized that no covalent bonds between a protein molecule and a protective compound are formed.

**Evidence of the thermoprotective function of trehalose obtained in in vivo experiments.** In the phase of active growth (trophophase), neutral trehalase is in an active phosphorylated state, and the activity of the trehalose-6-P-synthetase complex constitutes only 20% of the enzyme activity in the stationary phase. Therefore, this stage is characterized by a very low trehalose level. However, under temperature shock, the trehalose concentration quickly increases to a level of 0.5 M [67], which is determined not only by the activation of the trehalose-synthase complex but also by temperature-induced deactivation of trehalase. Thus, the affinity of calcium ions to the activated cAMP–proteinase–trehalase complex decreases 20-fold at 40°C compared to the affinity at 30°C, while the activity of the trehalose-6-P-synthase–phosphatase complex increases threefold at 40°C [68]. These data show how the growth temperature of fungi produces an in vivo effect on the activity of trehalose synthetase and trehalase.

The first evidence of in vivo membrane stabilization by trehalose has been obtained in vivo. Using the NMR method on intact cells, it was established that the trehalose level in *S. cerevisiae* correlates with the membrane viscosity, whereas the HSP 104 content correlates with the content of free cell water [69].

Attempts to vary the trehalose level in a wild strain by applying different stressors that did not induce HSP synthesis have shown that there exists a direct correlation between the trehalose level and thermotolerance. The use canavanine, an inducer of HSP synthesis, was not found to lead to an increase in cell thermotolerance nor influence trehalose synthesis. These results led to the suggestion that it is trehalose, rather than HSPs, that is responsible for the thermotolerance of yeast cells [70].

Another approach has been used in studies conducted not with mutants but with genetically engineered related diploid strains of *S. cerevisiae* that differed in their capacity for trehalose synthesis under nitrogen source deficiency. It was established that strains whose trehalose content was more than 5% of the dry mass were much more tolerant of heat shock and freezing–thawing than strains whose trehalose content was less than 4% [71]. An important methodical subtlety of this study should be emphasized: the strains were subjected to heat shock (52°C, 5 min) without preliminary maintenance at a supraoptimal temperature, when increased synthesis of not only trehalose but also HSPs begins. Thus, in this experiment, direct correlation between the trehalose concentration and the cell thermotolerance was observed. We found the same relationship when we studied the thermotolerance of *Blakeslea trispora* spores from stylosporangia, formed at 29–30°C, and from sporangioles, formed at 25–26°C. The spores from stylosporangia were shown to contain significantly more trehalose, which correlated with their higher thermotolerance [57].

In order to explore the relationship between trehalose and HSP syntheses and cell thermotolerance, mutants defective in (1) pathways of the cAMP–proteinase cascade, (2) HSP synthesis, (3) trehalase activity, and (4) structure of trehalose synthetase have been used. *S. cerevisiae* mutants with an impaired RAS–adenylate cyclase system constitutively were found to have a high cell level of trehalose. Thus, strain *cyr1-2*, with a mutation in the gene of structural adenylate cyclase and constitutively synthesizing several HSPs, had a low cAMP level, exhibited equally high trehalose contents at 27 and 40°C, and was highly thermotolerant at both temperatures. *S. cerevisiae* mutant *bcyl-1*, which is incapable of HSP synthesis, has a defect in the regula-

tory subunit of cAMP-dependent protein kinase, i.e., a defect that does not affect cAMP. It exhibits a low trehalose level at 27°C, the inability to synthesize trehalose in response to heat shock, and low thermostability [70]. As follows from the above examples, the correlation between the HSP content, trehalose content, and cell thermostability is retained. Similar results have been obtained with the use of mutants of other fungi. For example, it was shown in experiments with *N. crassa* mutant *cr-1*, which was deficient in cAMP-dependent adenylate cyclase, that a low cAMP level correlated with a constitutively high cell thermostability against the background of a high cell content of trehalose. This effect was brought to an end when cAMP was added to the growth medium [72]. Mutants of the yeast *S. cerevisiae* that were deficient in the adenylate cyclase pathway and exhibited a low cAMP level accumulated different amounts of trehalose after a temperature shift from 23 to 36°C: strain AM9-88 accumulated up to 3% of the dry mass, and strain 131, up to 10% of the dry mass. Trehalose accumulation was accompanied by a parallel increase in the activity of trehalose synthetase. The enzyme synthesis was inhibited by cycloheximide, demonstrating the necessity of protein formation de novo. Strain 131, characterized by the highest trehalose content, was the most thermotolerant. However, another *S. cerevisiae* mutant, *klg102*, which was incapable of synthesizing trehalose, was also highly thermotolerant [73]. These contradictory results do not allow researchers to explain the development of cell thermotolerance by the accumulation of only trehalose or HSPs.

Thus, it follows from the above examples that the study of mutants with impaired RAS–adenylate cyclase systems provides highly contradictory information. One thing is clear: the acquisition of thermotolerance requires a low level of cAMP, which leads to a decrease in the trehalase activity but does not affect the activity of trehalose synthetase.

A different approach to the study of the relationship between trehalose and thermotolerance has been used in investigations involving mutants incapable of synthesizing HSPs. Thus, in *S. cerevisiae* mutants deficient in the synthesis of HSP 104 or unable to synthesize it at 40°C, heat shock caused an increase in the activity of trehalose synthetase, accumulation of a normal amount of trehalose, and development of a normal level of cell thermotolerance [74]. On the other hand, mutant *ts-187*, which was incapable of HSP synthesis, was less tolerant than the wild strain and accumulated a lesser amount of trehalose. These results show that the synthesis of shock proteins may modulate the acquisition of thermotolerance but is not necessary for the attainment of this property. In another fungus, *Schizosaccharomyces pombe*, heat shock was found to cause the induction of the trehalose metabolism enzymes, trehalose accumulation, and the development of cell thermotolerance even in the presence of cycloheximide, which supports the hypothesis that trehalose plays a thermoprotective role [75].

It was noted that the parallelism of trehalose and HSP accumulation observed in the wild strain of *S. cerevisiae* under heat shock is disturbed in mutants. Thus, despite the high trehalose level, a mutant deficient in the function of neutral trehalase exhibited low thermotolerance upon reverse transfer from 40 to 30°C. Another mutant, *ubs4ubs5*, synthesized an insignificant amount of trehalose in the exponential growth phase but demonstrated constitutive HSP synthesis and was highly thermotolerant [76]. Based on the results obtained, the authors proposed the hypothesis of the "fire-brigade" function of trehalose, which is quickly synthesized in response to heat shock, whereas HSPs are endowed with the role of long-term thermoprotectants.

New evidence of the involvement of trehalose in the mechanism of thermotolerance in fungi appeared after the structure and function of the enzymes operating in the metabolism of this disaccharide were studied. In *S. cerevisiae* mutants with deletions in genes of individual trehalose synthetase subunits, the inability to accumulate trehalose in cells and low cell thermotolerance were observed, whereas a mutant with a deletion in the *nth1* gene and incapable of trehalose degradation retained high thermotolerance, even after the cessation of heat shock [11].

A temperature-sensitive mutant of *S. cerevisiae* with defective trehalose-6-P-phosphatase accumulated trehalose-6-P in response to an increase in growth temperature. Only trehalose, but not trehalose-6-P, induced cell thermotolerance. Interestingly, the kinetics of trehalose-6-P accumulation after a temperature shift from 25 to 37°C repeated the kinetics of inhibition observed in RNA and protein synthesis; i.e., a high level of this metabolite inhibited growth [77].

A mutation that caused hyperinduction of the protein factor YAP1, involved in the regulation of HSP synthesis, was found to determine the development of tolerance to many metabolic inhibitors by *S. cerevisiae* cells. This mutation was neutralized by a mutation in the *trs2* gene, which encodes trehalose-6-P phosphatase, the enzyme catalyzing the second step in trehalose synthesis: the *trs2* mutant lost the property of thermotolerance. Another conclusion that can be drawn from these data is that syntheses of HSPs and trehalose are conjugated: the transcription of the *trs2* gene is induced by many stressors via the promoter element $C_4T$ and requires the functioning of YAP1 [78].

Additional evidence of the thermoprotective role of trehalose in vivo and the relationship between trehalose and HSP syntheses has been obtained in experiments with *S. cerevisiae* mutants deficient in TRS1, HSP 104, and TRS1 × HSP 104 and bearing an introduced plasmid that carried a gene of a bacterial temperature-sensitive luciferase, which allowed the observation of the state of this protein in the cell [12]. It was established

in the experiments using this model that trehalose not only stabilizes luciferase during heat shock but also prevents the protein macromolecules from aggregating and maintains them in a conformational state from which they can be reactivated by molecular chaperones; thus, trehalose performs the function of a chemical chaperone additional to HSPs. At the same time, high trehalose concentrations inhibit the reactivation of denatured proteins by molecular chaperones, which agrees with the properties of chemical chaperones and explains the biological sense of an immediate reduction in the trehalose level after the cessation of heat shock. In the stationary phase, the cells of both the HSP 104- and TRS2-deficient mutants are less thermotolerant than cells of the wild strain, whereas in double mutant TRS1 × HSP 104, cell thermotolerance is completely absent, which provides evidence for the synergistic relationship between the syntheses of HSP 104 and trehalose [79]. In the HSP 104-deficient mutant, the activity of the enzymes involved in trehalose metabolism decreases, which, in the authors' opinion, is indicative of the direct (as a molecular chaperone) or indirect (via cAMP-protein kinase) influence of HSP 104 on the activity of neutral trehalase and trehalose synthetase [80].

It should be noted that the function of chemical chaperones, i.e., the stabilization of the structure of protein macromolecules, is inherent in certain amino acids; trimethylamine; and polyols, including glycerol. In addition, there is evidence that they are involved in protein folding. Defective protein folding due to point mutations or deletions is known to be the molecular basis for many diseases, e.g., familial cholesterolemia, emphysema, and cirrhosis. It has been established that glycerol contributes to the proper folding of polypeptide chains with point mutations into protein molecules. Such a restoring capacity is related to the increase in the hydrophobic effect, i.e., an enhancement of the interaction between the internal protein domains, which increases protein rigidity and thermostability. Chemical chaperones are thought to be involved in protein folding in combination with molecular chaperones, forming a unified system of the antistress protection and quality control of protein molecules [13]. The functions of trehalose in cell thermotolerance are also related to its properties as a chemical chaperone, its ability to stabilize proteins under heat shock, and participation in their folding [79].

**2.3.4. Other functions of trehalose.** In mycelial fungi, trehalose is a transported form of carbohydrate [44]. In yeasts, exogenous trehalose is transferred by pinocytosis to the vacuoles where acid trehalose is localized, which demonstrates the additional function of trehalose as a source of glucose in the cells [39].

There is evidence that certain trehalose derivatives are involved in cell protection against oxidative stress [81]. It is possible that the mechanism of antioxidant protection under heat shock includes not only the stimulation of desaturase activity but also the stimulation of trehalose synthesis, as was shown for *C. japonica* [82], as well as trehalose functioning as an antioxidant [83]. The latter suggestion was put forward in a study involving *S. cerevisiae* cells subjected to nonlethal heat shock or treatment with a proteolysis inhibitor. Such cells had a high trehalose level, contained less defective proteins, and were considerably more tolerant to reactive oxygen species, whose generation was induced by $H_2O_2/FeCl_3$. The authors suggested that, under oxidative stress, trehalose protects proteins against oxidative degradation, acting, among other things, as a trap for free radicals; i.e., it reveals antioxidant properties. However, this study did not even provide a hypothetical explanation of the mechanism of this putative function of trehalose, which seems to be a questionable idea considering that trehalose is a nonreducing, i.e., difficult-to-oxidize, disaccharide.

## CONCLUSION

This analysis of the relevant publications allows preliminarily conclusions to be drawn from the many years of discussion on the biological functions of HSPs and trehalose in fungal cells. According to the present-day concepts, trehalose performs multiple functions, playing the roles of (a) a reserve carbohydrate used during germination and in the process of storage of fungal spores, (b) a membrane protectant under various types of stress (thermal, oxidative, and osmotic stresses and stresses caused by heavy metals, drugs, and metabolic inhibitors), (c) the regulator of the process of glycolysis and of the cell concentrations of glucose and ATP, (d) a transported carbohydrate form, and (e) a chemical chaperone involved in protein stabilization and folding.

Under heat shock, HSPs perform the equally important, but quite different, function of molecular chaperones, i.e., they are involved in protein stabilization and adaptive modification of cell protein composition, which includes identification of defective proteins and their partial proteolysis and refolding as well as control of the folding of newly synthesized polypeptides. Trehalose synthetase and trehalase, the enzymes involved in trehalose metabolism, may well be attributed to HSPs. The roles of HSPs and trehalose in the acquisition of thermotolerance can vary, depending on the developmental stage and the rate of protein synthesis connected with it. Thus, for example, in spores, which are more thermotolerant dormant structures where protein synthesis is absent, HSPs were not revealed, but trehalose accumulation is observed. The thermoprotective function of trehalose should be emphasized. Owing to the characteristic features of its structure and the ability to form intermolecular hydrogen bonds, trehalose stabilizes proteins and membranes.

The aggregate knowledge of the mechanisms of action of trehalose allows us to conclude that this compound plays an important role in fungal cells during heat shock, performing protective and chaperone functions as well as a regulatory function (by controlling the

involvement of glucose in glycolysis and the activity of the membrane-associated ATPase). The other aspect of the thermoprotective function of trehalose is connected with the specific features of regulation of trehalose synthesis by the induction of the expression of the gene of trehalose synthetase under heat shock, as well as by the direct activation of this enzyme by increased temperature. Thus, trehalose acts both in an emergency and performs long-term protection of the subcellular structures during exposure to stressors, participating, together with molecular chaperones, in a unified process of antistress protection and quality control of proteins.

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