

COLD STRESS IN ANTARCTIC FUNGI TARGETS ENZYMES OF THE GLYCOLYTIC PATHWAY AND TRICARBOXYLIC ACID CYCLE

Nedelina Kostadinova, Spassen Vassilev, Boryana Spasova, Maria Angelova

Bulgarian Academy of Sciences, The Stephan Angeloff Institute of Microbiology, Sofia, Bulgaria

Correspondence to: Maria Angelova

E-mail: mariange_bg@yahoo.com

ABSTRACT

*To evaluate the concept of metabolic cold adaptation in Antarctic fungi, we compared the activities of several key enzymes of the glycolytic pathway and the TCA cycle (hexokinase, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, succinate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase) in psychrotolerant *Penicillium* sp. 161 and mesophilic *Aspergillus glaucus* 363 during both the stress exposure (6 h) and recovery phases. Mycelia of the Antarctic strains, grown until middle exponential phase at optimal temperature, were shifted to colder temperatures, i.e., 4 and 10 °C. Our investigations showed a re-routing of carbon metabolism away from glycolysis into the pentose phosphate pathway (PPP), which serves as a cellular stress-resistance mechanism under cold stress conditions. Moreover, the data clearly suggest strain-dependent differences in cold stress response concerning TCA enzyme activities between both fungi. The psychrotolerant strain induces glyoxalate cycle activities and the mesophilic strain uses a reduction of respiratory activity. A recovery response after removal of the stress factor was observed.*

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Abbreviations:

ATP: adenosine-5'-triphosphate;

CAT: catalase;

DNA: deoxyribonucleic acid;

EMP: Embden-Myerhoff pathway;

GAPDH: glyceraldehyde-3-phosphate dehydrogenase;

GPDH: glucose-6-phosphate dehydrogenase;

HK: hexokinase;

ICDH: isocitrate dehydrogenase;

MDH: malate dehydrogenase;

NADPH: nicotinamide adenine dinucleotide phosphate;

PPP: pentose phosphate pathway;

ROS: reactive oxygen species;

SDH: succinate dehydrogenase;

SOD: superoxide dismutase;

TCA cycle: tricarboxylic acid cycle

Introduction

The basic features which underlie the cold adaptive physiology of microorganisms are of immense value to human knowledge (34). While, for commercial reasons, the heat shock response and high temperature biology have been well studied, cellular response to low temperature and cold temperature biology have been studied mainly in mesophilic bacteria, yeast and fungi (11, 17, 29). Association between the decrease in environmental

temperature and increase in the intracellular oxidative stress was first indicated by studies on various plant systems and subsequently also in microorganisms. The intensive stress response results in generation of reactive oxygen species (ROS), e.g. superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^\bullet), which cause damage of cellular components such as DNA, proteins and lipids and can play an important role in cell injury (16). The metabolic network of microbial cells must, therefore, be reconfigured both to allow the maintenance of metabolism and to produce reducing agents for the scavenging of ROS and thus ameliorate the damage they can do (32).

Microorganisms have a well-developed biochemical defense system, which includes enzymatic and non-enzymatic reactions. Several studies have evidenced that exposure to low temperatures results in compensatory changes taking place in the antioxidant defence system in temperate strains (1, 44). Similar response has been demonstrated also in microorganisms which live and grow permanently in a cold environment. Castellano et al. (10) suggested that S-glutathionylated superoxide dismutase (SOD) from psychrophilic eubacterium *Pseudoalteromonas haloplanktis* could represent a further cold-adaptation strategy to improve the antioxidant cellular defence mechanism during cold shock. Low temperature treatment of Antarctic bacterium *Pseudomonas fluorescens* MTCC 667 caused increase in the level of ROS and antioxidant enzyme activities (12). Our previous investigations showed a relationship between cold stress and oxidative stress in filamentous fungi isolated from different regions of Antarctica (18, 19, 39). Low temperature treatment enhanced ROS levels in the Antarctic fungi and

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cell response against this situation includes alteration in the activities of SOD and catalase (CAT).

On the other hand, oxidative stress response includes metabolic components such as stress related proteins (heat or cold-shock proteins and chaperons). Several enzymes of the central carbon metabolism are also altered in abundance, suggesting a rearrangement of carbon distribution (27). In the presence of oxidants glucose is channeled towards the oxidative pentose phosphate pathway (OPPP) with simultaneous suppression of glycolysis and downstream pathways such as the tricarboxylic acid (TCA) cycle. The stimulation of the OPPP enhances the production of NADPH to support antioxidant enzyme activity. Lehmann et al. (27) proposed that a combination of transcriptional changes (induction of antioxidant enzymes and defenses) and post-translational control of carbon flow through central carbon metabolism are required to cope with oxidative stress conditions. However, the changes in metabolic components to the oxidative stress response are much less well understood in microorganisms isolated from extreme cold habitats.

With the goal to better understand the processes involved in the cold stress adaptation, we investigated the metabolic response of two Antarctic fungal strains (psychrotrophic *Penicillium* sp. 161 and mesophilic *Aspergillus glaucus* 363) to short-term temperature downshift. The present study demonstrated changes in activity of several key enzymes of the glycolytic pathway and the TCA cycle (hexokinase, HK; glucose-6-phosphate dehydrogenase, GPDH; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; isocitrate dehydrogenase, ICDH; succinate dehydrogenase, SDH and malate dehydrogenase, MDH) during both the stress exposure (6 h) and recovery phases.

Materials and Methods

Fungal strains, culture media and cultivation

The fungal strains *Penicillium* sp. 161 and *Aspergillus glaucus* 363 (with optimal growth temperature 20 and 25 °C, respectively) isolated from Livingston Island (South Shetlands archipelago, Antarctica) were used for the experiments. The strains belong to the Mycological collection of the Institute of Microbiology, Sofia, and are maintained at 4 °C on beer agar, pH 6.3.

Composition of the seed and production media was as described previously (3). Cultivation was performed in a 3 L bioreactor, ABR-09 developed and constructed by the former Central Laboratory for Bioinstrumentation and Automatisations (CLBA) of the Bulgarian Academy of Sciences. The bioreactor was equipped with temperature, pH and automatic dissolved oxygen (DO) monitoring equipment and a control system.

For the submerged cultivation, 74 ml seed medium was inoculated with 6 ml spore suspension at a concentration of 2×10^8 spores/ml in 500 ml Erlenmeyer flasks. The cultivation was performed at 20 °C for 48 h for the psychrotolerant strain and at 25 °C for 24 h for mesophilic strain on a shaker (220 rpm).

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For bioreactor cultures, 200 ml of the seed culture was brought into the 3 l bioreactor containing 1800 ml of the production medium. The cultures were grown at optimal temperature with a stirrer speed of 400 rpm air flow, 0.5 v.v.m. In the time of the middle exponential phase (24 h for *Penicillium* sp. 161 and 18 h for *A. glaucus* 363), the temperature was reduced to 4 or 10 °C. This downshift was reached approx. in 40 min. After an incubation of 6 h under cold stress conditions, temperature was up-shifted to the optimal value. The control variants were grown at optimal temperature during the whole period.

Cell-free extract preparation and isolation of cytosolic and mitochondrial fractionations

The cell-free extract as well as cytosolic and mitochondrial fractionations were obtained as previously described in detail (24). All steps were performed at 0–4 °C.

Enzyme activity determination

The cytosolic HK (EC 2.7.1.1.), GPDH (EC 1.1.1.49.), GAPDH (EC 1.2.1.12) and LDH (EC 1.1.1.27) activities were determined according to Bergmeyer et al. (8) and Bergmeyer and Moellering (9), respectively. SDH (EC 1.3.99.1.), MDH (EC 1.1.1.37.) and IDH (EC 1.1.1.41.) as mitochondrial matrix enzymes were measured by the method of Veeger et al. (41), Smith (38) and Kornberg (23), respectively. One unit equals to 1 μ mol of substrate reduced per min. Specific activity is given as U/mg protein.

Other analytical methods

The amount of cellular protein was estimated by the Lowry procedure (30) using bovine albumin as a standard. The dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman (Clifton, USA) № 4 filter. The separated mycelia were washed twice with distilled water and dried to a constant weight at 105°C.

Results and Discussion

The present study is the first to identify alteration in key metabolic enzymes in response to cold stress in Antarctic fungi. Sardesai and Babu (36), the closest to the current paper, study the effect of low temperature on switchover of respiratory pathway to lactate glycolysis in psychrotrophic *Rhizobium* strains. Our investigation added more information on eukaryotic microorganisms such as filamentous fungi isolated from Antarctica. We compared the metabolite response to cold stress between two Antarctic fungi, belonging to different thermal classes (psychrotolerant and mesophilic) assaying enzyme activities of the glycolytic pathway and the TCA cycle. The duration of temperature downshift was chosen to be 6 h since this range was found to be wide enough to give a clear contrast between the control and stressed cultures. In the present study, we chose a temperature shift from the optimal temperature of the model strains to 4 or 10°C. The optimal growth temperature for *Penicillium* sp. 161 (20 °C)

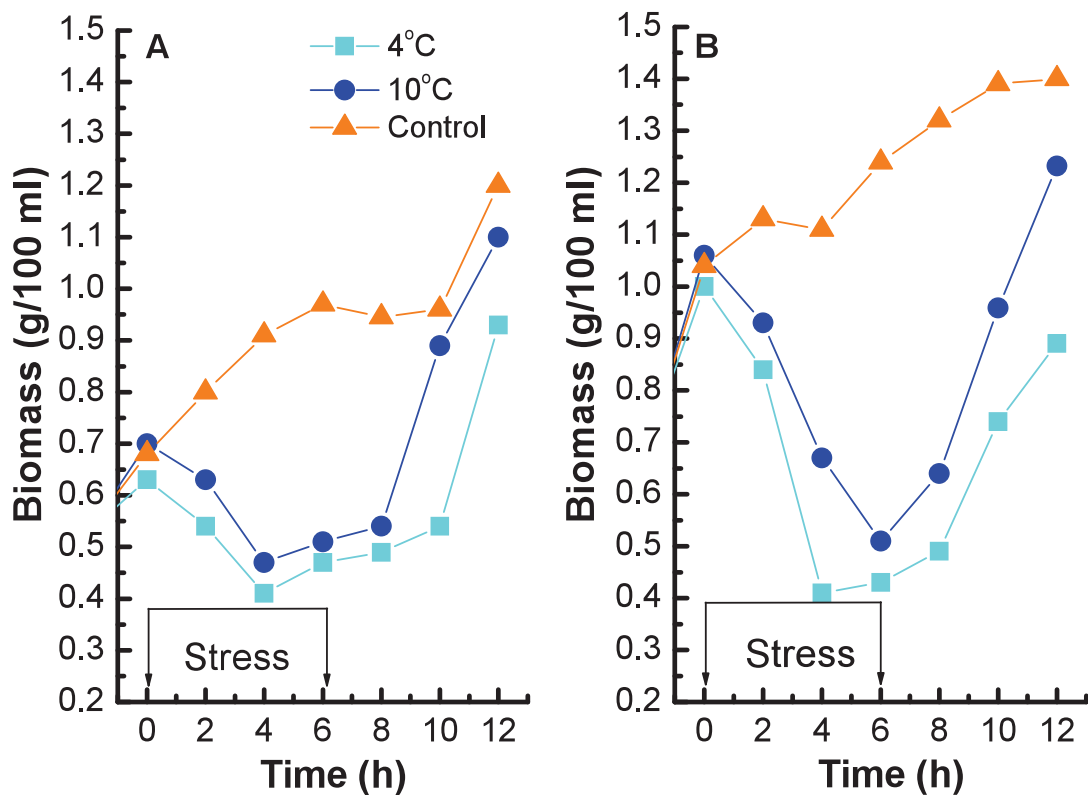


Fig. 1. Effect of cold shock on biomass production by *Penicillium* sp. 161 (A) and *A. glaucus* 363 (B): ▲ – growth at optimal temperature; ■ – downshift from optimal temperature to 4 °C; ● - downshift from optimal temperature to 10 °C.

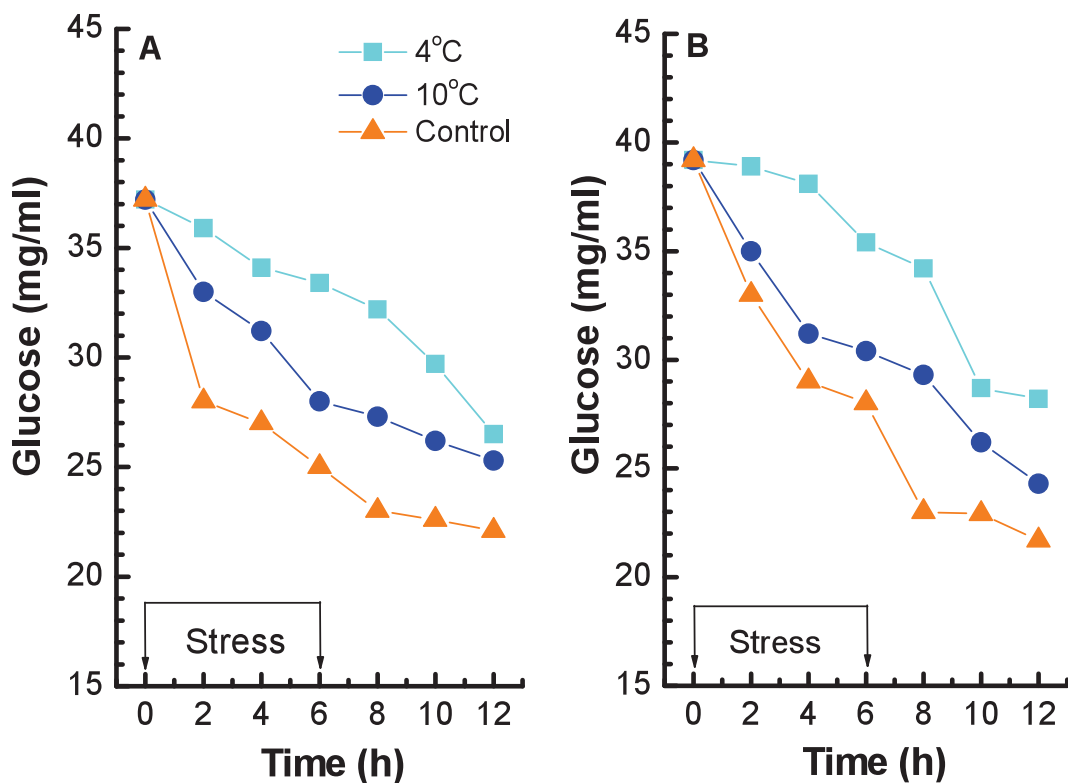


Fig. 2. Glucose consumption in *Penicillium* sp. 161 (A) and *A. glaucus* 363 (B) during cold shock and following recovery period: ▲ – growth at optimal temperature; ■ – downshift from optimal temperature to 4 °C; ● - downshift from optimal temperature to 10 °C.

and for *Aspergillus glaucus* 363 (25 °C) were established in our previous study.

Fungal growth and glucose consumption under short-term cold stress

Mycelia of the Antarctic strains grown until middle exponential phase at optimal temperature were shifted to colder temperatures, i.e. 4 and 10 °C. **Fig. 1** shows the growth curves of the model strains after temperature downshift exposure and subsequent restoration of the normal conditions. Within the first 4 h of the beginning of the stress, the growth of the psychrotolerant strain *Penicillium* sp. 161 ceased and biomass as measured by dry weight decreased sharply in comparison to the control at lower temperatures (**Fig. 1A**). In the next 2 h the growth was resumed at both temperatures and the return to optimal 20 °C after 6 h allowed biomass to increase relative to the control levels. A similar trend was demonstrated for the Antarctic mesophilic strain *A. glaucus* 363 after shift from 25 to 10 or 4 °C (**Fig. 1B**), but the difference in biomass production between the control and treated mycelia was more significant in comparison with the psychrotolerant strain. Moreover, after 6 h recovery from either temperature treatment, the biomass production was restored and the dry weight reached its level to that before the stress.

The concentration of glucose in the culture medium was measured throughout the experiment (**Fig. 2**). Maximum glucose consumption occurred in cultures incubated at optimal growth temperature compared to the stressed cultures. A comparison of the model strains also showed that the consumption of glucose by the mesophilic strain (**Fig. 2B**) was faster than that of the psychrotolerant fungus (**Fig. 2A**). Comparing the curves in **Fig. 2A** and **Fig. 2B**, it was possible to verify that the downshift of temperature from optimal to 10 or 4 °C caused significant decrease in glucose consumption. This tendency continued even after return to optimal temperature.

Our previous report demonstrated that the growth at low temperature does clearly induce oxidative stress events in the Antarctic and temperate fungal strains, this being an induction of ROS generation, enhanced level of oxidative damaged proteins, accumulation of reserve carbohydrates and increased activity of antioxidant enzyme defense (18, 19, 39). On the other hand, it was demonstrated that organisms can overcome oxidative stress by reconfiguration of their metabolite pathways (20). In the present study, exposure of both Antarctic strains to low temperatures caused significant reduction of biomass production and glucose consumption in comparison to the control variant. Similar results have been noted for different organisms including fungi and yeasts (17, 28, 44).

Effect of temperature downshift on activity of glycolytic enzymes

During stress, mycelites use large quantities of adenosine-5'-triphosphate (ATP) to maintain their ion gradients (5, 33). In fungi, a major source of ATP is glycolysis, which therefore should be very active during stress (43). The effect of temperature downshift on the level of three key

glycolytic enzymes (HK, GPDH and GAPDH) was evaluated (**Fig. 3**). Changes in activity of the first enzyme of hexose monophosphate pathway (HK) in cultures of *Penicillium* sp. 161 and *A. glaucus* 363 under low temperature exposure are demonstrated in **Fig. 3A** and **Fig. 3B**, respectively. The specific activity of HK in mycelia of Antarctic fungi grown at optimal temperature was 3.7-fold higher in the mesophilic strain than that in the psychrotolerant strain. Short-term exposure (6 h) to cold stress led to a general decrease in the HK activity of both strains. The reduction in activity was more pronounced at 4 °C than at 10 °C. The cell response to 4 °C included a 2.6- and 1.5-fold diminished activity for cultures of *Penicillium* sp. 161 and *A. glaucus* 363, respectively during the first 2 h of the treatment. In contrast, the key enzyme in pentose phosphate pathway, GPDH, showed notable increase especially at 4 °C (**Fig. 3C** and **Fig. 3D**). As is shown, there is about 1.5-fold increase in GPDH activity after 4 h cold treatment. The changes of HK and GPDH activities coincided with the beginning of treatment and the observed tendency continued even after return to the optimal temperature.

Because glycolysis is a critical energy pathway during stress conditions, we further investigated the importance of GAPDH as a target for inactivation by low temperature. As shown in **Fig. 3E** and **Fig. 3F**, temperature downshift to 4 and 10 °C had a significant effect on GAPDH activity in cultures of both Antarctic strains. Immediately after cold-stress initiation GAPDH activity increased sharply (about 1.6-fold at 4 °C and 1.2-fold at 10 °C). This tendency continued even after cessation of the stress.

The main finding of the above mentioned investigation is the re-routing of carbon metabolism away from glycolysis into the PPP, which serves as a cellular stress-resistance mechanism under cold stress conditions. HK catalyzes the primary step in the glycolytic pathway, i.e. phosphorylation of glucose to form glucose-6-phosphate. The cold stress response includes reduction in HK activity in both Antarctic strains. Interestingly, the activity of the first enzyme in the psychrotolerant strain *Penicillium* sp. 161 was less affected by low temperature than is that of the mesophile *A. glaucus* 363. Contradictory data have been published about the effect of cold stress on HK activity. Expression of NbHxk1, a *Nicotiana benthamiana* hexokinase gene, was stimulated by treatment with methyl viologen, an oxidative stress agent (37). In roots of plants *Echinochloa phyllopogon* and *Echinochloa crus-galis*, chilling stimulated HK activity (15). In contrast, the present study demonstrated a reduction in activity, which coincides with the results of other published research. Decreased HK activity during stress conditions was reported for cultures of shrimp *Litopenaeus vannamei* (21). Michael and Valerie (31) found that the HK activity of *Modiolus modiolus* at low temperatures was significantly lower than that at high temperatures. A possible explanation could be related to the inhibition of HK by trehalose accumulated in the cytosol during low temperature exposure as a stress protectant (18, 19, 35, 42). Antarctic strains *Penicillium* sp. 161 and *A. glaucus*

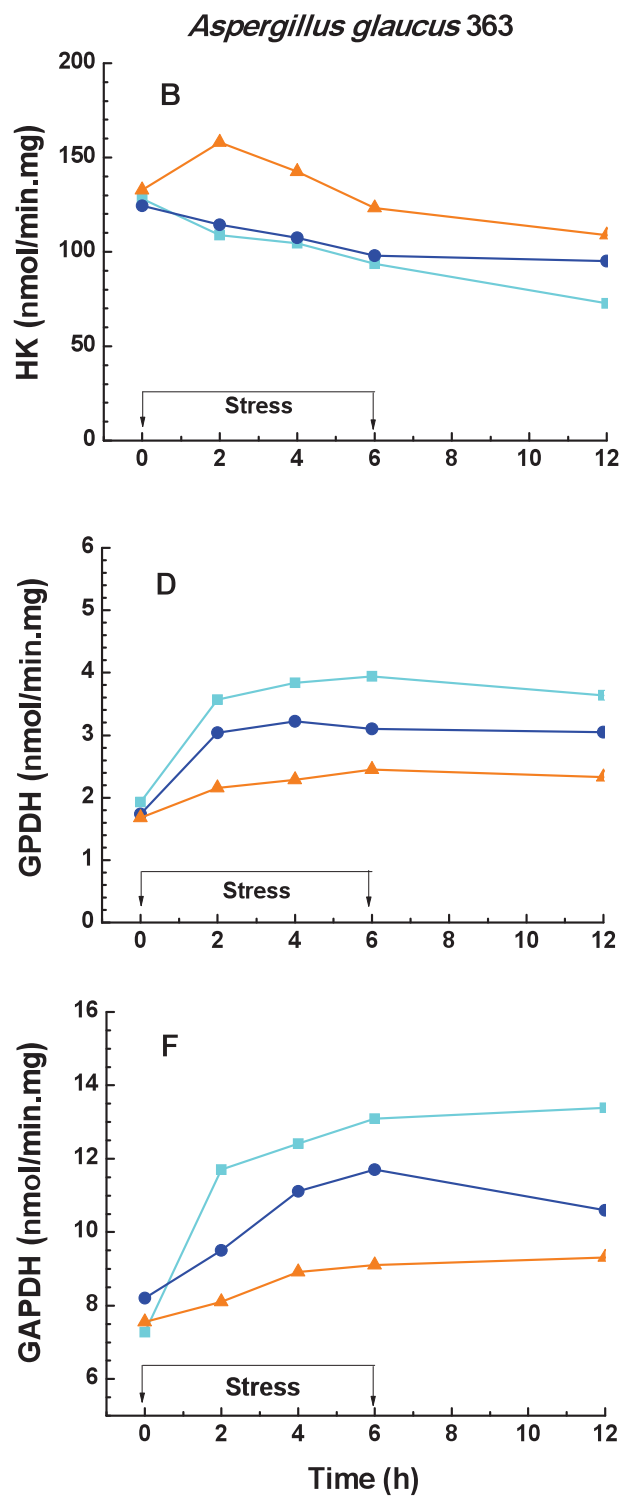
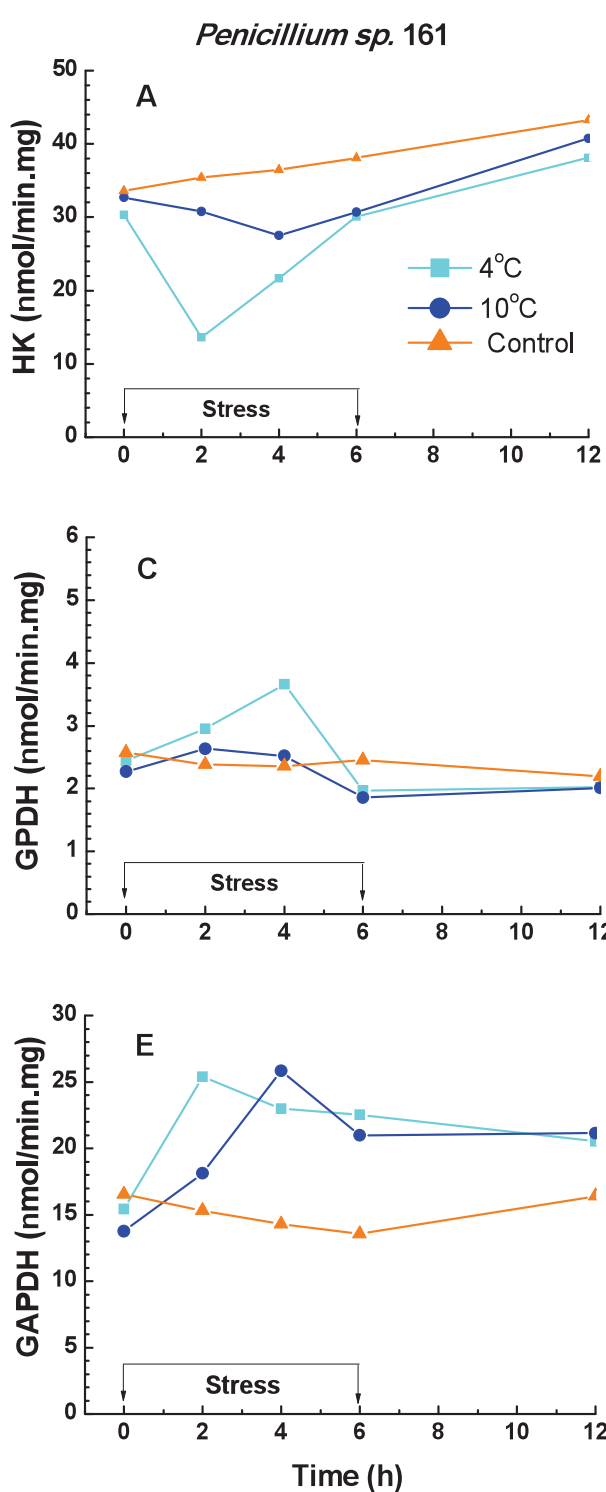


Fig. 3. Activity of key glycolytic enzymes in psychrotolerant *Penicillium sp. 161* (A, C, E) and mesophilic *A. glaucus* 363 (B, D, F) at optimal temperature (▲) and during downshift from optimal temperature to 4 °C (■) and downshift from optimal temperature to 10 °C (●): HK (A, B), GPDH (C, D), GAPDH (E, F).

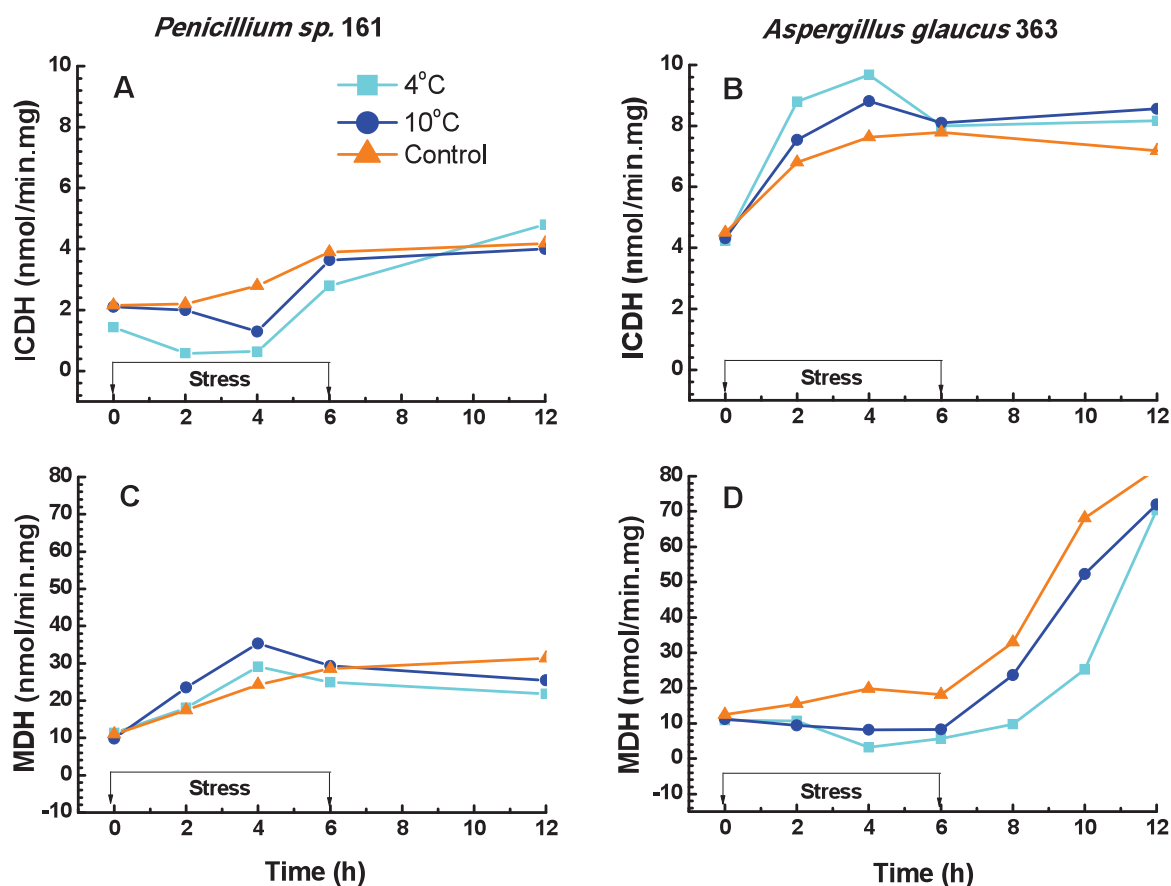


Fig. 4. Activity of key enzymes of the TCA cycle in psychrotolerant *Penicillium* sp. 161 (A, C) and mesophilic *A. glaucus* 363 (B, D) at optimal temperature (▲) and during downshift from optimal temperature to 4 °C (■) and downshift from optimal temperature to 10 °C (●): ICDH (A, B), MDH (C, D).

363 also accumulated trehalose during temperature downshift (Kostadinova et al., in preparation). According to published data, trehalose 6-phosphate is a physiological inhibitor of hexokinase in fungi and yeast grown under stress conditions (5, 14).

We also found significant increase in GPDH activity during cold stress exposure. In recent years, different studies have shown that GPDH has a relevant role in the mechanism of protection against oxidative stress (4). This enzyme participates in maintaining the balance of nicotinamide adenine dinucleotide phosphate (NADPH), an important source of reducing power for antioxidant enzymes. The increased activity of GPDH after 6 h treatment at 4 or 10 °C points to the involvement of the PPP in cold adaptation. The role of GPDH as enzyme involved in the protective mechanisms of microbial cells against oxidative stress is well recognized (4, 28, 44) but, to our knowledge, there are a few data on the effects of cold exposure.

GAPDH catalyzes a reversible oxidation and phosphorylation reaction in glycolytic metabolism. Our results show that increase in GPDH activity is accompanied by a

rise in GAPDH in both Antarctic strains. The changes in up-regulation occur in a temperature dependent manner. Previous studies on the activity of GAPDH in microbial cultures under oxidative stress conditions have shown contradictory results. Associated with oxidative stress, the activity of GAPDH was greatly reduced in *Aspergillus niger* (28). On the other hand, protein level of GAPDH in plant cells increased to different types of abiotic stress (6) as a result of GAPDH mRNA accumulation (7). Up-regulation of GAPDH activity could be explained by its importance especially in stress conditions, where glycolytic flux may be strongly deviated to accumulation of reserve carbohydrates (2).

Changes in activity of TCA cycle enzymes

We next measured the activities of key enzymes associated to TCA cycle, ICDH, SDH and MDH, since changes in these may account for the rapid changes in metabolite abundance (Fig. 4). Among all tested enzymes, SDH did not show changes in activity during the stress conditions (data not shown). For the other two enzymes, ICDH and MDH, the experiments demonstrated strain-dependent differences in response to temperature downshift. ICDH activity of psychrotolerant

strain was strongly reduced following 4 h of stress application and recovered to the control value after return to optimal temperature (**Fig. 4A**). In contrast, the activity of ICDH in the mesophilic strain increased significantly following 4 h of cold stress, sharply recovered during the next 2 h and then reached the level measured for the non-stressed culture (**Fig. 4B**). Cold stress exposure altered also the activity of the other investigated enzyme, MDH. In the culture of psychrotolerant *Penicillium* sp. 161, MDH activity increased to peak in 1 - 4 h and then declined to the control level (**Fig. 4C**). At the same time, the enzyme activity in the mesophilic strain fell by 55 and 70% at 10 and 4 °C, respectively during 6 h stress and slowly recovered after return to the optimal temperature, but did not achieve the control level (**Fig. 4D**).

The changes in the enzymatic activity of the TCA cycles evidenced that this crucial metabolic pathway appears to be an important supplier of the precursor that plays an important role in the adaptation to cold stress. Temperature downshift did not alter SDH in both strains. On the other hand, ICDH decarboxylates isocitrate with the concomitant formation of α -ketoglutarate and the reducing factors NADPH or NADH. A reduction in the activity of ICDH would target isocitrate to isocitrate lyase and lead to the production of glyoxylate (22). In the psychrotolerant strain temperature downshift elicited namely this response. Environmental stresses such as nutrient stress, heavy metal treatment and temperature change have been shown to decrease ICDH activities in bacteria (22). In contrast, the mesophilic strain demonstrated enhanced ICDH level, probably to support the regulation of mitochondrial energy and redox status.

MDH is involved in reversible conversion of L-malate and oxaloacetate. In the present study, its activity in the psychrotolerant strain was found to be higher in the temperature stressed cultures. This increased activity could be used to consume the product/substrate (oxaloacetate) for production of more energy (ATP) which may be utilized for other physiological activities (26). Moreover, considering that this enzyme is involved in the respiratory process, such increase in activity during the exposure to low temperatures would allow higher energy availability for cellular metabolic processes. Enhanced level of MDH has been reported for plant cells under dark chilling (40) and heavy metal stress (13). It is suggested that up-regulation of individual TCA enzymes, including MDH is involved in stress tolerance. Unlike the above mentioned changes, MDH in the mesophilic strain demonstrated a reduction in activity under cold stress. It can be assumed that decreased activity under cold stress might result from the balance between inactivation by free radicals and synthesis of new molecules. The inhibition of MDH activity in cold sensitive strain could be due to reduced respiratory activity. Kumar et al. (25) showed a similar effect in MDH activities of salt-sensitive rice.

Conclusions

Taken together, our results indicated that temperature downshift, especially to 4 °C activated alternative pathway of carbohydrate metabolism and accelerated energy generation in both Antarctic fungi. The data clearly demonstrate strain-dependent differences in cold stress response concerning TCA enzyme activities between *Penicillium* sp. 161 and *A. glaucus* 363. While the psychrotolerant strain induces glyoxalate cycle activities, the mesophilic strain uses a reduction of respiratory activity. Moreover, most of the tested enzymes showed a recovery response after the removal of the stress factor. This suggests that the cold-induced oxidative stress did not kill the fungi after 6 h and that a large proportion of this response must be actively regulated and furthermore not be the effect of irreversible changes driven by oxidative damage.

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