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ORIGINAL PAPER

Temperature downshift induces antioxidant response in fungi isolated from Antarctica

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Abstract Although investigators have been studying the cold-shock response in a variety of organisms for the last two decades or more, comparatively little is known about the difference between antioxidant cell response to cold stress in Antarctic and temperate microorganisms. The change of environmental temperature, which is one of the most common stresses, could be crucial for their use in the biotechnological industry and in ecological research. We compared the effect of short-term temperature downshift on antioxidant cell response in Antarctic and temperate fungi belonging to the genus *Penicillium*. Our study showed that downshift from an optimal temperature to 15° or 6°C led to a cell response typical of oxidative stress: significant reduction of biomass production; increase in the levels of oxidative damaged proteins and accumulation of storage carbohydrates (glycogen and trehalose) in comparison to growth at optimal temperature. Cell response against cold stress includes also increase in the activities of SOD and CAT, which are key enzymes for directly scavenging reactive oxygen species. This response is more species-dependent than dependent on the degree of cold-shock. Antarctic psychrotolerant strain *Penicillium olsonii* p14 that is adapted to life in extremely cold conditions demonstrated enhanced tolerance to temperature downshift in comparison with both mesophilic strains (Antarctic *Penicillium waksmanii* m12 and temperate *Penicillium* sp. t35).

Keywords Antarctica · Fungi · Temperature downshift · Antioxidant enzymes · Carbonyl proteins · Trehalose · Glycogen

Abbreviation

SOD	Superoxide dismutase
CAT	Catalase
ROS	Reactive oxygen species
NBT	Nitro-blue tetrazolium
DNPH	2, 4-Dinitrophenylhydrazine

Introduction

Temperature is one of the most important parameters that affect the growth and survival of microorganisms (Deegenaars and Watson 1998). Most of the known cultivable microorganisms are mesophiles and occupy temperature niches that are not regarded as extreme. Others that are especially adapted to low-temperature habitats have been described to be either psychrotrophic or psychrophilic (Russell 2006). Microbes growing at low temperatures encounter a number of growth constraints: enzyme reaction rates decrease, the efficiency of uptake and transport systems decreases, membranes become less fluid and nucleic acid structures become stabler (Thomas and Cavicchioli 2000). Their ability to thrive at low temperatures requires many adaptations to maintain metabolic rates and sustained

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growth that is compatible with life in the cold. Cells have developed programmed responses to stress, which include rapid changes in processes such as protein phosphorylation and degradation, and longer term effects involving transcriptional changes that become manifested in altered cell states (Chattopadhyay 2006). Within the possible effects, activation of antioxidant defense is one of the most important mechanisms responsible for growth and survival at low temperatures, although little has been elucidated about this mechanism.

Several recent studies provided convincing evidence that cold stress induces production of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^·$), and singlet oxygen ($^1O_2$). Under low temperature conditions, the cytochrome pathway of respiration is repressed and ROS are produced (Sugie et al. 2006; Mizuno et al. 2008). Enhanced ROS levels were demonstrated in different microorganisms (Smirnova et al. 2001; Zhang et al. 2001, 2003) and plants (Chinnusamy et al. 2006; Sugie et al. 2006). Sub-tropical species are particularly sensitive to ROS when they are exposed to temperatures below 10°C (Zubini et al. 2005). Similar data were published about animals living at low temperatures (Selman et al. 2002). In this case, the increased polyunsaturation of mitochondrial membranes should raise the rates of mitochondrial respiration, which would in turn enhance the formation of ROS (Guderley 2004).

ROS are highly reactive and can damage many important cellular components such as lipids, proteins and nucleic acids in living cells (Sies 1985). Aerobic organisms have developed enzymatic and nonenzymatic scavenging systems to quench ROS. Cold exposure significantly increased activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in Antarctic plants (Arora et al. 2002), algae (Collén and Davison 2001) and animals (Ansaldo et al. 2000; Abele et al. 2001; Heise et al. 2006; Şahin and Gümüşlü 2004). Modulation in antioxidant defense has been established also in bacteria and yeasts (Smirnova et al. 2001; Zhang et al. 2001, 2003).

Although investigators have been studying the cold-shock response in a variety of organisms for the last two decades or more, comparatively little is known about the difference between antioxidant cell response to cold stress in Antarctic and temperate microorganisms. At the same time, there is a growing interest in various applications of Antarctic bacteria, yeasts and fungi. The change in environmental temperature, which is one of the most common stresses, could be crucial for their use in the biotechnological industry and in ecological research (Vishniac 1996; Narinx et al. 1997; Nichols et al. 2002; Michaud et al. 2004).

Our present study was designed to compare the effect of short-term temperature downshift on antioxidant cell response in Antarctic and temperate fungi, belonging to genus *Penicillium*. Two mesophilic (Antarctic and temperate) and one Antarctic psychrotolerant strains were used to obtain information on the growth of mycelia, content of oxidative damaged proteins, synthesis of reserve carbohydrates (glycogen and trehalose) and changes in activities of both antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT).

Materials and methods

Fungal strains, culture media and cultivation

The fungal strains, *Penicillium olsonii* p14 and *Penicillium waksmanii* m12, isolated from Antarctic soils (Gocheva et al. 2005), as well as strain *Penicillium* sp. t35, member of *P. frequentans* series isolated from temperate Bulgarian soil samples were included in the experiments. All strains belonged to the mycological collection of the Institute of Microbiology, Sofia, and they were maintained at 4°C on beer agar, pH 6.3. The composition of the culture medium AN-3 used for submerged cultivation was as follows (g/l): sucrose, 20.0; ammonium citrate, 7.5; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$; $MnSO_4$, 0.0025; CuSO4, 0.0011. Cultivation was performed in a 3 l bioreactor, ABR-09 as described in Gocheva et al. (2006).

For the submerged cultivation, 74 ml medium AN-3 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 for 48 h on a rotary shaker (220 rpm) at an optimal temperature (20°C for *P. olsonii* p14 and 30°C for *Penicillium waksmanii* m12 and *Penicillium* sp. t35, respectively). For bioreactor cultures, 200 ml of the seed culture was brought into the 3 l bioreactor, containing 1,800 ml of the medium AN-3. The cultures were grown at optimal temperature with a stirrer speed of 400 rpm air flow, 0.5 vvm. In the time of the middle exponential phase (0.260, 0.340 and 0.460 g dry weight/100 ml for *P. olsonii* p14, *P. waksmanii* m12 and *Penicillium* sp. t35, respectively), the temperature was reduced to 6 or 15°C. This downshift was reached approximately in 40 min. After an incubation of 6 h under cold stress conditions, the temperature was up-shifted to the optimal value. The control variants were grown at an optimal temperature throughout the whole period.

Cell-free extract preparation

The cell-free extract was prepared as previously described (Angelova et al. 1995). Briefly, mycelium biomass was harvested by filtration, washed in distilled H_2O and then in cold 50 mM potassium buffer (pH 7.8) and resuspended in

the same buffer. The cell suspension was disrupted by homogenizer model ULTRA Turrax T25 IKA WERK. The temperature during treatment was maintained at 4°–6°C by chilling in an ice–salt bath and by filtration through a Whatman filter No.4 (Clifton, USA). Cell-free extracts were centrifuged at 13,000*g* for 20 min at 4°C.

Enzyme activity determination

SOD activity was measured by the nitro-blue tetrazolium (NBT) reduction method of Beauchamp and Fridovich (1971). The reaction mixture contained 56 μM (NBT), 0.01 M methionine, 1.17 μM riboflavin, 20 μM NaCN and 0.05 M phosphate buffer at pH 7.8. Superoxide was measured by the increasing amount of the absorbance at 560 nm at 30°C after 6 min incubation from the beginning of illumination. One unit of SOD activity was defined as the amount of enzyme required for inhibition of the reduction of NBT by 50% (A_{560}) and was expressed as units per mg protein (U/mg protein). Catalase activity was determined by monitoring the decomposition of 18 mM H_2O_2 at 240 nm (Beers and Sizer 1952). One unit of activity is that which decomposes 1 μmol of H_2O_2 min^{-1} mg $protein^{-1}$at 25°C and pH 7.0. Specific activity is given as U $(mg\ protein)^{-1}$. Protein was estimated by the Lowry procedure (Lowry et al. 1951) using a solution of bovine serum albumin as the standard.

Analytical methods

Glycogen and trehalose contents were determined following the procedure of Becker (1978) and Vandecamen et al. (1989) and modified by Parrou et al. (1997). Soluble reducing sugars were determined by the Somogy–Nelson method (Somogy 1952).

Protein oxidative damage was measured spectrophotometrically as protein carbonyl content using the 2,4-dinitrophenylhydrazine (DNPH) binding assay (Hart et al. 1999) slightly modified by Adachi and Ishii (2000). The cell-free extracts were incubated with DNPH for 1 h at 37°C, proteins were precipitated in 10% cold TCA, washed with ethanol: ethylacetate (1:1), to remove excess of DNPH and finally dissolved in 6 M guanidine chloride, pH 2. Optical density was measured at 380 nm, and the carbonyl content was calculated using a molar extinction coefficient of 21 mM^{-1} cm^{-1}, as nanomoles of DNPH incorporated (protein carbonyls) per mg of protein.

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) No 4 filter. The separated mycelia were washed twice with distilled water and dried to a constant weight at 105°C.

Statistical evaluation of the results

The results obtained in this investigation were evaluated from at least three repeated experiments using three or five parallel runs. The statistical comparison between controls and treated cultures was determined by the Student's *t*-test for MIE (mean interval estimation) and by one-way analysis of variance (ANOVA) followed by Dunnet's post test, with a significance level of 0.05.

Results

The range of temperature shift (6 h duration) was chosen since this range was found to be wide enough to give a clear contrast between the control and stressed cultures. Our previous study on the model strains indicated that the optimal growth temperature was 20°C for Antarctic psychrotolerant *Penicillium olsonii* p14 and 30°C for Antarctic mesophilic *Penicillium waksmanii* m12 and temperate mesophilic *Penicillium* sp. t35, respectively (Gocheva et al. 2006). In the present study, we chose a temperature shift from the optimal temperature to 6° or 15°C.

Response of fungal growth to temperature shifts

Figure 1 shows growth curves of the model strains after temperature downshift exposure and subsequent restoration of the normal conditions. A shift from 20° to 15° or 6°C in Antarctic psychrotolerant strain (*P. olsonii* p14) resulted in a lag period of 2 h before growth resumed at a lower temperature (Fig. 1 a). The return to optimal 20°C after 6 h allowed the biomass to increase relative to the control levels. A similar trend was demonstrated for the Antarctic mesophilic strain, *P. waksmanii* m12, after a shift from 30° to 15° or 6°C (Fig. 1 b), but the difference in biomass production between the control and treated mycelia was more significant in comparison with the psychrotolerant strain. While the psychrotolerant strain reached 75–85% of the control's biomass, the mesophilic fungus *P. waksmanii* m12 formed about 65–70% of the untreated mycelium. By comparison, *Penicillium* sp. t35 exhibited a longer lag phase (about 4 h) when shifted to cold temperatures, 6° or 15°C (Fig. 1c). After this acclimation phase, the growth of the temperate strain at 15°C resumed at a rate similar to that observed in Antarctic mesophilic cultures grown under the same conditions, but cultures stressed by shift from 30° to 6°C were able to support a minimal level of growth.

Effect of temperature downshift on protein oxidation

Reaction of proteins with oxygen radicals leads to the appearance of carbonyl groups in polypeptide chains

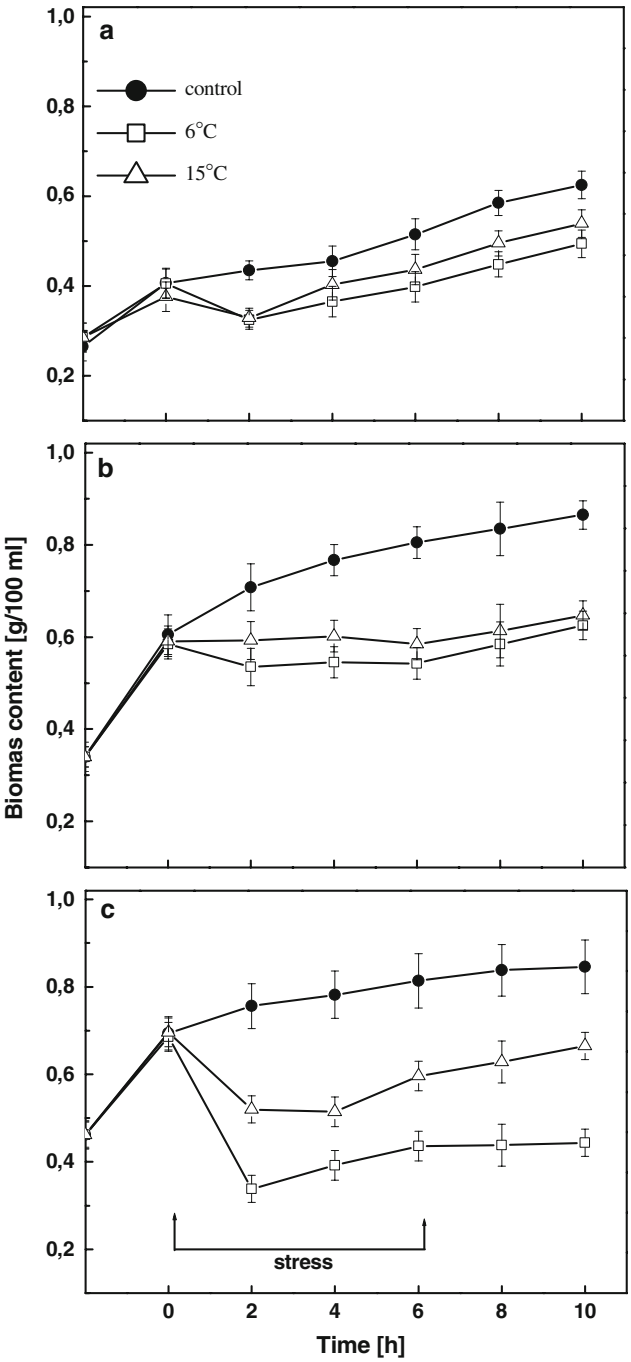

Fig. 1 Effect of temperature downshift on biomass production by *P. olsonii* p14 (**a**), *P. waksmanii* m12 (**b**), and *Penicillium* sp. t35 (**c**). *Filled circles*, growth at optimal temperature; *unfilled squares*, downshift from optimal temperature to 6°C; *unfilled triangles*, downshift from optimal temperature to 15°C. *Bars* represent SD of means. The effect of treatment was significant for the temperature treatment ($P \leq 0.05$)

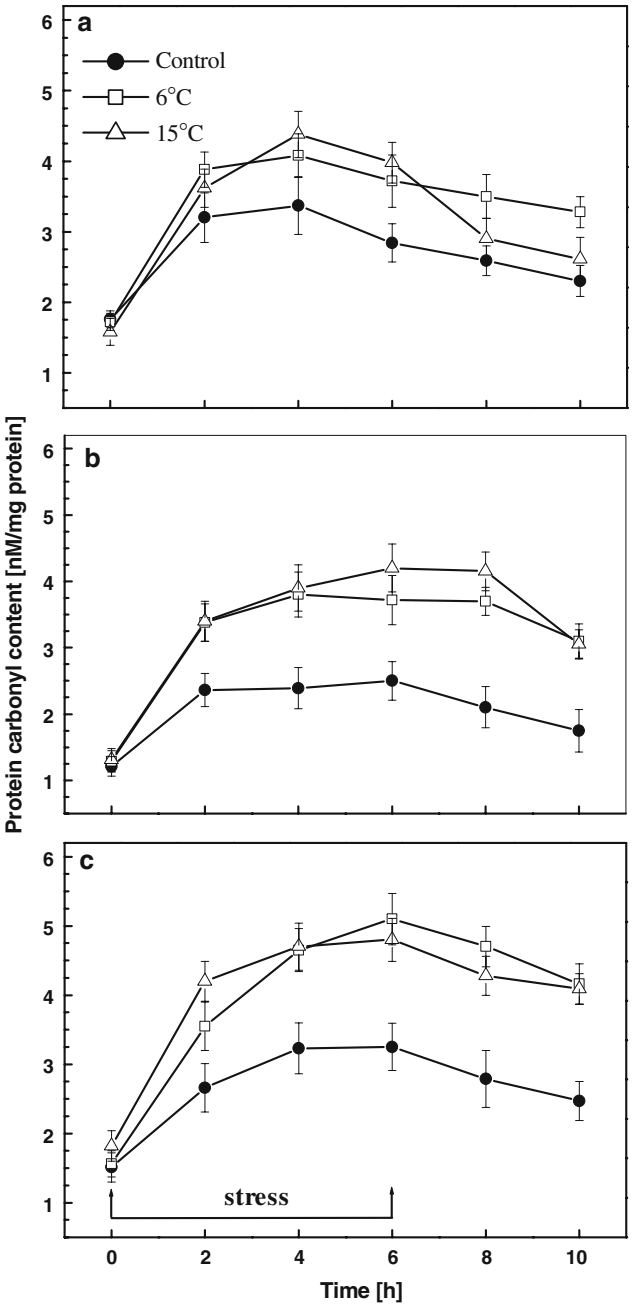

Fig. 2 Oxidative damage of proteins in *P. olsonii* p14 (**a**), *P. waksmanii* m12 (**b**), and *Penicillium* sp. t35 (**c**) at optimal temperature (*filled circles*) and at temperature downshift from optimal to 6°C (*unfilled squares*) or 15°C (*unfilled triangles*). *Bars* represent SD of means. The effect of treatment was significant for the temperature treatment ($P \leq 0.05$)

(Davies and Goldberg 1987). Measuring the content of these groups in intracellular proteins is one of accepted assays for oxidative damage in microbial cells. We investigated whether the exposure of Antarctic and temperate *Penicillium* to temperature downshift causes oxidative damage to proteins (Fig. 2). Exponentially growing cultures were shifted from an optimal temperature to 6° and 15°C as described above. When fungal strains were exposed to cold temperatures, the amount of carbonyl groups in cell proteins increased with the duration of the exposure in comparison with the control. This tendency

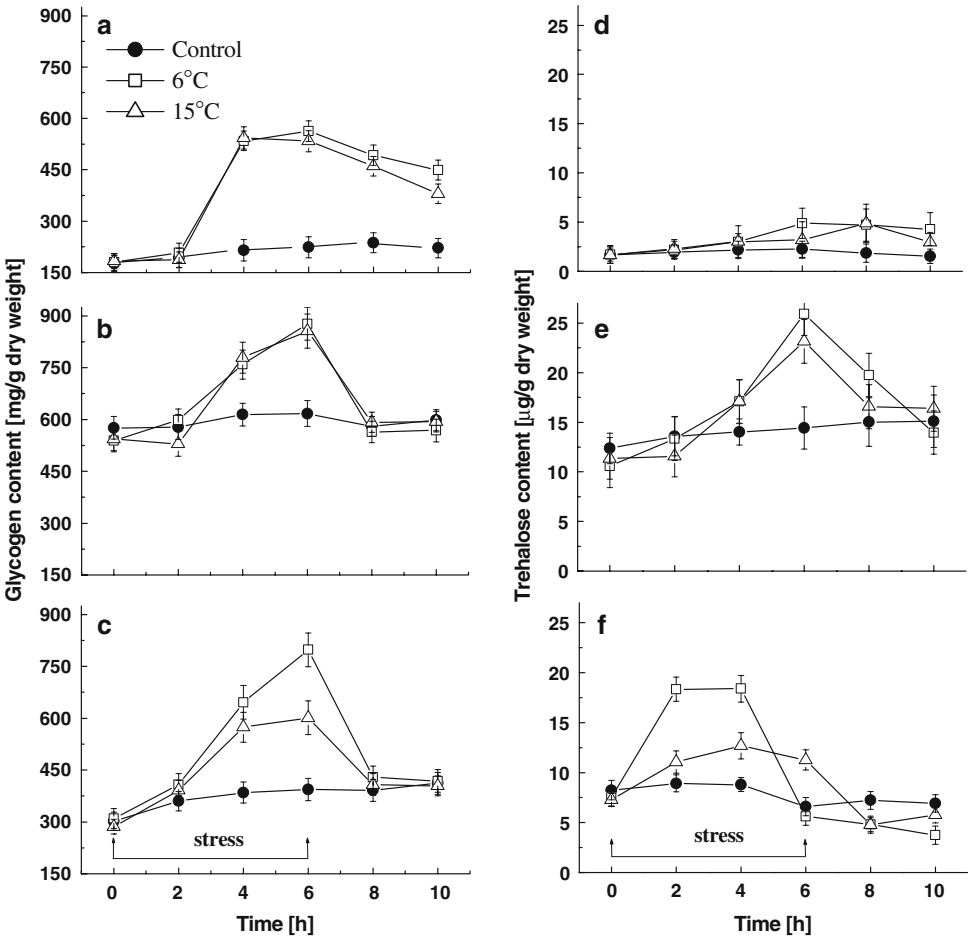

Fig. 3 Glycogen (**a**, **b**, **c**) and trehalose (d, e, f) accumulation in *P. olsonii* p14 (a, d), *P. waksmanii* m12 (b, e), and *Penicillium* sp. t35 (**c**, **f**) at optimal temperature (*filled circles*) and at temperature downshift from optimal to 6°C (*unfilled squares*) or 15°C (*unfilled triangles*). *Bars* represent SD of means. The effect of treatment was significant for the temperature treatment ($P \leq 0.05$)

continued even after return to optimal temperature. The increase was dependent on temperature requirements of the strains and less on the grade of temperature shift used in this experiment. The results concerning enhancement in protein carbonyl in all tested fungi showed that there are no significant differences between means for both temperature drops ($P \leq 0.05$; from optimal temperature to 6°C or 15°C). Temperature downshift caused more pronounced elevation of damaged protein content in the mesophilic *Penicillium* strains (*P. waksmanii*. m12 and *Penicillium* sp. t35) than in psychrotolerant ones. While mesophilic strains demonstrated 60–70% higher carbonyl protein content, psychrotolerant *P. olsonii* p14 showed comparably lower sensitivity to cold temperatures.

Temperature downshift induces accumulation of reserve carbohydrates

The production of reserve carbohydrates, glycogen and trehalose after temperature downshift to 6° or 15°C, was detected. As shown in Fig. 3, there is no accumulation in response to cold during the first 2 h, but a reproducible increase in glycogen and trehalose content was observed after 4–6 h of cold treatment. In the experiments with the psychrotolerant *Penicillium* strain, glycogen was accumulated up to 2.0–2.5 times the basal level accompanied by a 2.5-fold increase in trehalose content (Fig. 3a, d). The induction of both carbohydrates did not depend on the downshift level. A similar trend was also observed for the Antarctic mesophilic strain (Fig. 3b, 3e). In contrast to this situation, the temperate *Penicillium* (Fig. 3c, f) demonstrated clear dependence on the degree of cold shock. While the downshift from 30° to 15°C caused about a 1.5-fold increase in glycogen and trehalose content, treatment with 6°C induced more than twofold accumulation of these carbohydrates. However, after 4 or 6 h, the glycogen and trehalose content decreased to the control levels in all tested strains.

Changes in antioxidant enzyme activity

When the mycelia, taken from the middle exponential phase at optimal temperature, were subjected for 6 h to 6° or 15°C stress, an activation of antioxidant enzyme defense was established. As shown in Fig. 4, SOD activity in the cold stressed cultures was not different from the control

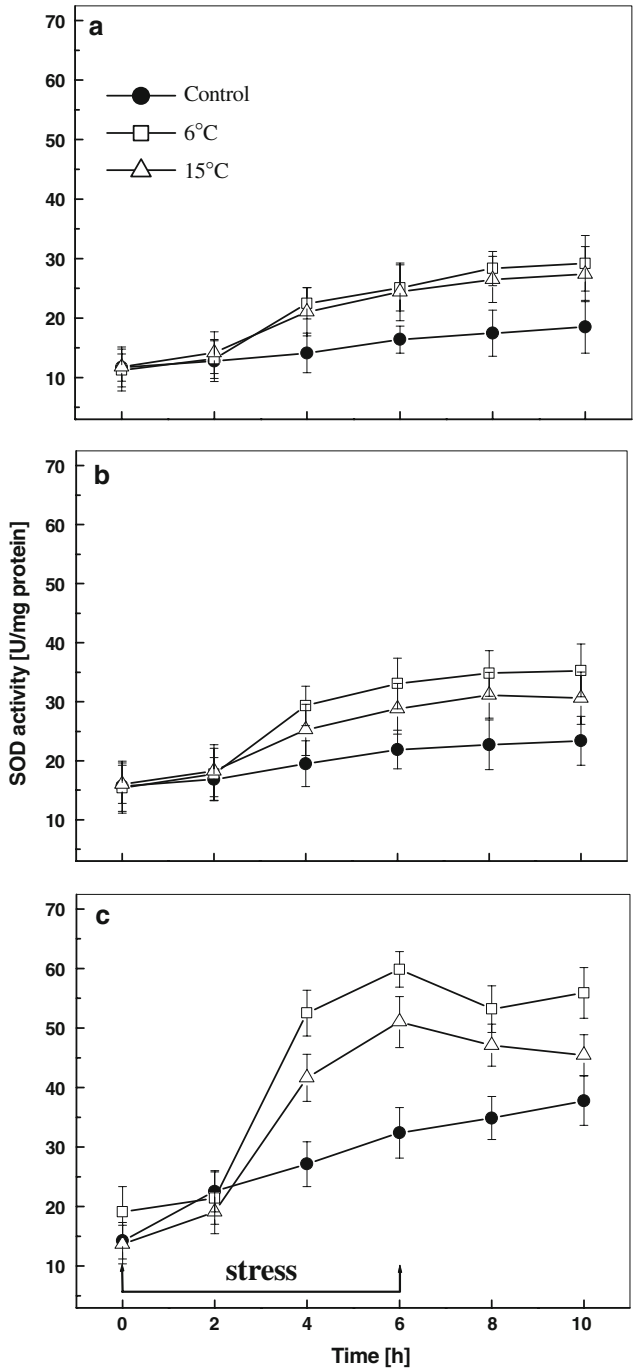

Fig. 4 SOD activities in cultures of *P. olsonii* p14 (**a**), *P. waksmanii* m12 (**b**) and *Penicillium* sp. t35 (**c**) at optimal temperature (*filled circles*) and at temperature downshift from optimal to 6°C (*unfilled squares*) or 15°C (*unfilled triangles*). *Bars* represent SD of means. The effect of treatment was significant for the temperature treatment ($P \leq 0.05$)

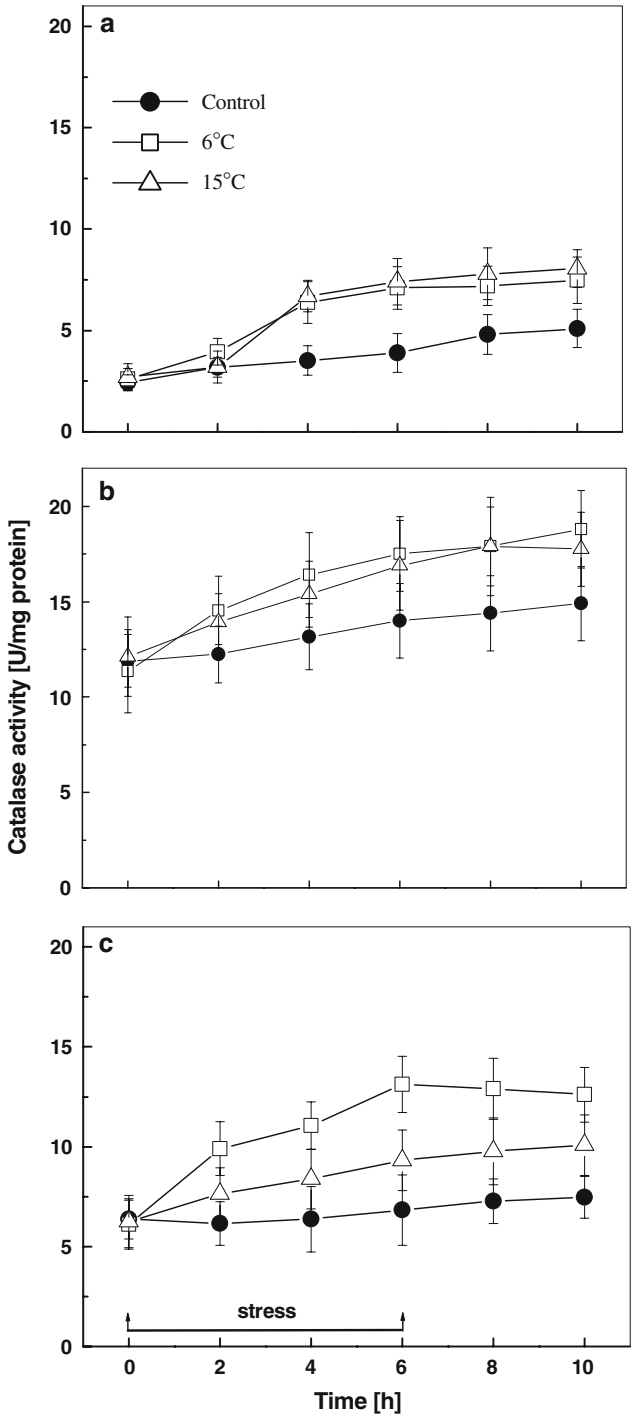

Fig. 5 CAT activities in cultures of *P. olsonii* p14 (**a**), *P. waksmanii* m12 (**b**), and *Penicillium* sp. t35 (**c**) upon optimal temperature (*filled circles*) and upon temperature downshift from optimal to 6°C (*unfilled squares*) or 15°C (*unfilled triangles*). *Bars* represent SD of means. The effect of treatment was significant for the temperature treatment ($P \leq 0.05$)

variants during the first 2 h, whereas during the following 4–6 h, significant enzyme production was observed. The antioxidant cell response of Antarctic strains included a twofold increase in SOD activity, independent of the degree of cold stress. In contrast, the temperate strain demonstrated a temperature-dependent response that was higher at 6°C in comparison with the condition at 15°C.

Similar trends were demonstrated in relation to the CAT activity (Fig. 5.). In all the variants, the enzyme level

exceeded the control value by approximately twofold. The highest percentage of increased CAT activity (approximately twofold) was found in the temperate strain after a temperature downshift from 30° to 6°C. It is noteworthy that the tendency of increased SOD and CAT activities in comparison to the control variant continued even after return to the optimal temperature.

Discussion

Our previous report demonstrated that growth at low temperature does clearly induce oxidative stress events in the Antarctic and temperate fungal strains, belonging to the genus *Penicillium* (Gocheva et al. 2006). The primary focus of this study was to determine whether evolution at a permanent cold temperature resulted in metabolic adaptation of cell response to cold shock. To this end, we compared the specific biomarkers for oxidative damage and antioxidant defense in these strains cultivated under condition of temperature downshift.

The main findings from this study are the following. First, our study showed that downshift from an optimal temperature to 15° or 6°C led to a cell response, which is typical of oxidative stress. Exposure of *Penicillium* strains to low temperatures caused statistically significant reduction of biomass production and increase in levels of oxidative damaged proteins in comparison to the control variant. Similar results have been noted for yeasts (Zhang et al. 2003), plants (Prasad 1997) and animals (Selman et al. 2002). Increased protein carbonyl in all tissues of cold stress rats was reported by Şahin and Gümüşlü (2004). Low temperature stress was shown to induce ROS accumulation in cells (Suzuki and Mittler 2006; Ouellet 2007), which leads to a production of oxidized proteins. Oxidative damaged proteins tend to aggregate, presumably because the oxidative modification of critical residues causes protein unfolding, and these abnormal proteins are selectively degraded in cells (Davies and Goldberg 1987).

At the same time, trehalose and glycogen demonstrated drastic enhancement under cold shock in comparison to the almost constant level produced by the cells cultivated at optimal growth temperature (Fig. 3). Both carbohydrates are important storage compounds in fungal vegetative cells and spores (Silljé et al. 1999; Rúa et al. 2008). According to several authors (see Robinson 2001; Ray 2006), trehalose and glycogen appear to be general stress protectants in the cytosol. It has also shown accumulations of these carbohydrates in the fungal hyphae in response to low temperature (Voit 2003; Şahin and Gümüşlü 2004; Gocheva et al. 2006). Kandror et al. (2002) demonstrated the important role of trehalose in cold adaptation of *E. coli*. On the other hand, at the end of the stress period, glycogen and trehalose quickly decreased reaching the control levels. One possible explanation could be related to the finding that the genes involved in glycogen and trehalose synthesis and degradation were up-regulated (Sahara et al. 2002; Voit 2003).

Secondly, our results suggested that the growth of tested *Penicillium* strains under cold shock conditions shows clear signs of increased oxidative stress. Cell response against this stress includes alteration in the activities of SOD and CAT, which are key enzymes for directly scavenging ROS. Our results showed that acute cold shock exposure from an optimal temperature to 15° or 6°C was also accompanied by typical oxidative stress reaction, an enhanced expression of both antioxidant enzymes. Similar data were reported by Smirnova et al. (2001) about *E. coli*, Zhang et al. (2003) about *Saccharomyces cerevisiae* and Şahin and Gümüşlü (2004) about animals.

Thirdly, our results revealed that the fungal cell response against cold shock is more species-dependent than dependent on the degree of cold stress. Antarctic psychrotolerant strain *P. olsonii* p14 that is adapted to life in extremely cold conditions demonstrated enhanced tolerance to temperature downshift in comparison with both the mesophilic strains (Antarctic and temperate). Increase in the levels of evaluated stress biomarkers (carbonyl groups, trehalose and glycogen) and antioxidant enzyme activities were lower than those in mesophilic strains. The clearest oxidative stress response to a sudden drop in temperature was expressed in temperate *Penicillium* sp. t35. In the experiments the highest amount of reserve carbohydrates and oxidative damaged proteins was measured as well as the highest levels of antioxidant enzyme defense. Studies on bacteria indicated that the key problems that occur after a cold shock are a lowering in the rates of enzymatic and transport processes (Cavicchioli et al. 2000). The situation promotes a decrease in the demand for ATP and an accumulation of electrons in certain points of the respiratory chain, leading to a sudden increase in the production of a number of ROS, which can damage lipids, proteins and DNA (Chattopadhyay 2002). All of these problems are also faced by cells living permanently in cold environments (i.e., not just after "cold shock") and as such must be overcome in the evolutionary process of low temperature adaptation (Cavicchioli et al. 2000). In response, microorganisms have evolved various ways to adapt, i.e., effective antioxidant enzyme defense.

In conclusion, our results demonstrated that growth at low temperatures does clearly induce oxidative stress events in all fungal strains tested, this being an enhanced level of oxidative damaged proteins, accumulation of reserve carbohydrates and increased activity of antioxidant enzyme defense.

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