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Trehalose accumulation in *Baudoinia compniacensis* following abiotic stress

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ABSTRACT

Baudoinia compniacensis is a microfungus recently described as the principal agent of fouling known as "warehouse staining", affecting building exteriors, fixtures and vegetation surfaces in areas proximate to distillery aging warehouses, commercial bakeries and other areas subject to low-level ethanol vapour exposure. The surfaces most affected tend to be highly exposed and undergo extreme diurnal temperature fluctuations. In previous work, we have demonstrated the existence of heat-inducible putative chaperone proteins that may also be induced by low-level exposures to ethanol vapour (e.g., <10 ppm). The present study investigated the cellular accumulation of trehalose, a disaccharide identified in some microorganisms to be important in the protection of cell components during adverse stress conditions, such as thermal stress. Following heat shock at 45 °C, we observed a 2.5-fold accumulation of trehalose relative to unheated controls maintained at 26 °C. Peak trehalose concentrations of 10 mg g^{-1} dry wt were seen at 90 min after heat treatment, followed by a gradual return to post-treatment by 150 min. Exposure of *B. compniacensis* cells to ethanol resulted in a similar increased accumulation of trehalose compared to unexposed controls. These findings imply that trehalose may be important in the tolerance of this fungus to abiotic stresses, such as heat and solvent exposure, and suggest future research directions for the control and prevention of warehouse staining.

1. Introduction

Baudoinia compniacensis (Richon) J.A. Scott & Unter. (≡ *Torula compniacensis* Richon) is a strictly filamentous, non-lichenized, highly melanized microfungus allied with the family, Teratosphaeriaceae Crous & U. Braun (Capnodiales: Ascomycota). Morphologically similar, closely-related genera include *Capnobotryella* Sugiyama and *Devriesia* Seifert & N.L. Nick. Species of *Baudoinia* J.A. Scott & Unter. are slow growing cosmopolitan colonists of exterior surfaces, such as masonry, metal, paint, plastics and wood, that are subject to episodic exposure to high relative humidity, extreme diurnal temperature fluctuations and low-levels of ethanol vapour. They have been identified as the lead causative agents or "founding colonists" of a dense, darkly coloured "sooty mould" biofilm that develops on outdoor surfaces associated with distillery maturation buildings and commercial bakeries (Richon and Petit, 1881; Kjøller, 1961; Scott et al., 2007). This growth, termed "warehouse staining" has been known anecdotally in the spirits industry for many years; however recent mounting government and community concerns in numerous jurisdictions in North America and Europe have led to studies that have addressed taxonomic, physiological and ecological aspects of this phenomenon (Dixon, 2009). These studies have had the dual goals of elucidating the interesting biology of this fungus as well as providing possible avenues for the minimization of its growth in areas subject to neighbourhood complaints of aesthetic damage.

In previous physiological investigations, we have shown that exposure of dormant cells to airborne ethanol at concentrations of 5–10 ppm is strongly stimulatory to germination (Ewaze et al., 2007). Following germination, *B. compniacensis* is able to utilize a range inorganic and organic carbon sources, including ethanol, and nitrogen in the form of nitrate and ammonium. The substrata that become colonized by *B. compniacensis* are characteristically highly exposed, subjected to numerous environmental stresses including high temperatures, desiccation and variations in water activity. Previously we have shown that pre-conditioning of *B. compniacensis* cells to either high temperature or low-level ethanol vapour for short-duration induces the production of putative stress-response or "heat shock" proteins (Ewaze et al., 2007).

Modifications in protein expression are a common mechanism of inducible resistance to environmental stress. Another such mechanism is the cellular accumulation of carbohydrates, such as

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trehalose. In the present study, we were interested to investigate the potential role of the disaccharide trehalose in stress-response, and to determine the extent to which trehalose may support thermotolerance and cellular survival in this recently rediscovered extremophilic fungus.

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-gluco-pyranoside) is a non-reducing disaccharide that is absent in vertebrates but found in a number of prokaryotic and eukaryotic organisms including, yeasts and filamentous fungi (Elbein et al., 2003). Originally identified as a carbohydrate storage compound and an energy source for resting cells or germinating fungal conidia (Van Laera, 1989; Jorge et al., 1997), trehalose has been shown to be important in metabolic signalling and the regulation of carbohydrate metabolism during periods of environmental stress (Arguelles, 2000). Fungal cells accumulate this disaccharide, reaching levels up to 30% of the cell dry mass in response to stressful abiotic conditions, such as high heat, dehydration, ethanol exposure and oxidative stresses (Parrou et al., 1997, 2005). The synthesis of trehalose has recently been recognized as an important cellular protective mechanism by acting to stabilize the native conformation of proteins and cellular membranes during periods of stress (Singer and Lindquist, 1998). Given the foregoing, coupled with our knowledge of the ability of *B. compniacensis* to survive high temperatures, we hypothesized that trehalose functions in the stress-tolerance machinery of the warehouse staining fungus.

2. Materials and methods

2.1. Fungal isolates

Voucher materials from this study have been deposited in the Canadian National Mycological Herbarium, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada (DAOM). Living cultures have been deposited in the University of Alberta Microfungus Collection, Edmonton, Alberta, Canada (UAMH). Isolates of *B. compniacensis* used in this study included UAMH 10762 and UAMH 10764, obtained from tree bark near a spirit aging warehouse in Ontario, Canada, and from a concrete wall near a distillery warehouse in Kentucky, USA, respectively. Commercial baker's yeast, *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, was used as a control for all experiments.

2.2. Cultivation media

Modified Leonian's medium (Malloch, 1981), was used as a general purpose cultivation medium for this study. This medium was prepared by adding the following to 1.0 L distilled water; 6.25 g maltose, 6.25 g malt extract (BioShop, Burlington, Ontario, Canada), 0.63 g $MgSO_4 \cdot 7H_2O$, 1.25 g KH_2PO_4, 0.63 g Bacto peptone (Difco, Becton Dickinson and Co. Sparks, Maryland, USA), 1.0 g yeast extract (Difco), and 15 g agar (Sigma–Aldrich, St Louis, Missouri, USA) for solid media.

2.3. Preparation of inoculum

Stock cultures of *B. compniacensis* were grown in 90 mm Petri dishes containing Modified Leonian's Agar (MLA; Malloch, 1981). Plates were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, Wisconsin, USA) and incubated inverted for 14 days at 26 °C. These stock plates were used for the preparation of mycelial inocula.

Mycelial suspensions for liquid cultures were made by immersing log-phase agar cultures in sterile water and gently scraping surface mycelia into suspension. Suspensions were axenically filtered to remove large mycelial aggregates by passage through gauze in a thistle funnel covered with aluminum foil. Mycelial fragments in the filtered suspension were enumerated using a haemocytometer and their concentration adjusted with distilled water to 10^6 fragments mL^{-1}. The fungal suspensions were stored at 4 °C for 4–24 h until use. Fresh material was prepared in the same manner for all experiments.

2.4. Effect of ethanol on trehalose accumulation

Log-phase cultures of *Baudoinia* were prepared for ethanol treatment experiments by combining a 1.0 mL aliquot of aqueous mycelial suspension (10^6 fragments mL^{-1}) with 50 mL Modified Leonian's Broth (MLB; Malloch, 1981) containing either 0.05% or 0.25% ethanol. Control mycelia suspensions in 50 mL MLB without ethanol were included. Flasks were inoculated in duplicate, sealed with Parafilm and incubated at 26 °C on an orbital shaker at 120 rpm for 12 days. Following treatment, cells were harvested, washed twice with ice-cold distilled water and filtered via water-suction, weighed and then divided into two portions, one portion (fresh extract) was analysed directly for trehalose and the other portion was dried to constant weight at 70 °C in pre-weighed aluminum dishes. Trehalose was detected in the fresh extracts as described below (Lillie and Pringle, 1980).

2.5. Effect of heat treatment on trehalose accumulation

A 1.0 mL aliquot of aqueous mycelial suspension (10^6 cells mL^{-1}) was taken from the stock cultures, mixed with 50 mL MLB and incubated with continuous shaking on an orbital shaker for a period of 7 days at 26 °C. Duplicate flasks were transferred to 50 mL MLB medium pre-warmed to 45 °C, and incubated in two parallel series on a shaking water bath at 45 °C for periods of 0, 30, 60, 90, 120 and 150 min. The first parallel series of the temperature-treated suspensions for the desired incubation time were harvested using a filtration method above, and the second series were transferred to 50 mL MLB medium at 26 °C and incubated on an orbital shaker for additional incubation periods of 90, 120 and 150 min prior to harvesting. Controls at 26 °C were included for all the tested time intervals. Trehalose was extracted and measured as described previously. For each treatment, an aliquot of fresh cells was maintained for trehalose extraction. All treatments and controls were carried out in duplicate.

2.6. Trehalose extraction

For each replicate, a weighed quantity of freshly harvested cells was extracted with two volumes of 3.0 mL ice-cold 0.5 M trichloroacetic acid (TCA) with agitation for 40–60 min at 26 °C, followed by centrifugation at 4000 × *g* for 10 min at 5 °C (Lillie and Pringle, 1980). The supernatants from each extraction were pooled, and the trehalose concentration was determined by the anthrone method of Trevelyan and Harrison (1956). Because this procedure detects both trehalose and glucose, the amount of trehalose was determined after subtracting the value of glucose or any other reducing sugar(s) quantified by the *o*-toluidine method of Winckers and Jacob (1971). An equivalent weight of freshly harvested cells was washed and dried to constant weight at 70 °C in a pre-weighed aluminum dish. Trehalose content was calculated as a function of mycelial dry weight. Percentage increase of trehalose content was calculated according to the control value for each experiment.

The identity of trehalose was verified as the only carbohydrate extracted in 0.5 M TCA using thin-layer chromatography TLC on Silica Gel 60 F-254 plates (Merck, KGaA, Darmstadt, Germany). Plates were developed in n-butanol–pyridine–water (15:3:2), and carbohydrate spots were visualized by treatment of the plates with alkaline silver nitrate (Trevelyan and Harrison, 1956).

3. Results

The accumulation of trehalose was determined in mycelial cells of *B. compniacensis* under ambient, heat shock and heat shock followed by ambient temperatures (Fig. 1). After return to ambient conditions following heat shock there was a rapid accumulation of trehalose between 0 and 90 min to a maximum of 10 mg g^{-1}, approximately 2.5-fold higher than the trehalose content seen in the absence of heat. A gradual decline in trehalose content was observed after 90 min (Fig. 1).

The thin-layer chromatography profile of the TCA extract revealed a putative trehalose spot that comigrated with a purified trehalose standard (Fig. 2).

As in heat treatments, a concentration dependent relationship was observed, with increased trehalose detected in mycelial cells of two *B. compniacensis* isolates following ethanol treatments, which exceeded control values by up to 1.7–3.6-fold (Table 1).

4. Discussion

The habitat of *B. compniacensis* is restricted to humid environments exposed to low concentrations of ethanol vapour (e.g. 1–10 ppm) and extreme diurnal temperature fluctuations from sun-heating of colonized surfaces of building exteriors and roofing (Scott et al., 2007). As mentioned previously, earlier studies conducted by our group have shown that exposure to these abiotic stresses induces the expression of several putative heat shock proteins (Ewaze et al., 2007). Other mechanisms of stress tolerance, such as trehalose, a disaccharide recognized in many microorganisms as a protectant of proteins and nucleic acids against degradation during periods of stress, have not previously been studied in *Baudoinia*.

An increase in mycelial trehalose following heat shock has been reported for a number of fungal species including *Botrytis cinerea* Pers. (Doehlemann et al., 2006), *Pisolithus* sp. (Ferreira et al., 2007), *Chaetomium thermophilum* var. *coprophilum* Cooney & R. Emerson (Jepsen and Jepsen, 2004), *Cryptococcus neoformans* (San Felice) Vuill. (Petzold et al., 2006) and *Talaromyces macrosporus* (Stolk & Samson) Frisvad, Samson & Stolk (Dijksterhuis et al., 2002). Trehalose has been shown to be an intracellular protective response, enabling certain fungi to tolerate otherwise lethal temperatures for extended times (Voit, 2003; Ferreira et al., 2007) by stabilizing membranes and native protein confirmation, and suppressing aggregation of denatured proteins (Tereshina, 2005; Doehlemann et al., 2006; Petzold et al., 2006). Trehalose content has been reported as low or absent under non-stressed conditions in taxa such as *Candida albicans* (C.P. Robin) Berkhout (Arnold and McLennan, 1975), *Neurospora crassa* Shear & B.O. Dodge (Eilers et al., 1970) and *Saccharomyces cerevisiae* (Rousseau et al., 1972). The overall increase in trehalose we observed in *B. compniacensis* following heat shock is consistent with that seen in previous studies of other fungi (Jepsen and Jepsen, 2004; Doehlemann et al., 2006), although concentrations have been seen to vary substantially between taxa (Tereshina, 2005).

Our results showed a rapid decline of cellular trehalose levels in *B. compniacensis*, returning to negligible levels after 160 min at ambient temperature (e.g., 26 °C) following heat shock. We postulate that this decline in trehalose concentration reflects the reactivation of trehalase, although this was not directly measured in our study. Previous studies have shown that heat shock intervals as little as a 1 min in duration within the temperature range of 40–50 °C can activate trehalose synthesis up to three-fold (Thevelein, 1984). However, cAMP-proteinase–trehalase complex activity in *S. cerevisiae* increases up to 20-fold at temperatures below 40 °C (Panek et al., 1987).

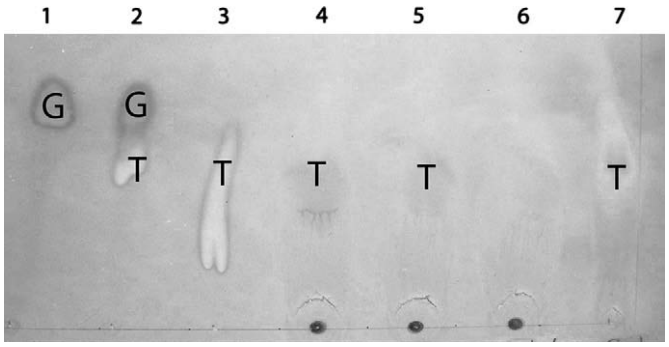

Fig. 2. Thin-layer chromatographic separation of mycelial extracts from 10 day old cultures of isolate UAMH 10764 compared to a trehalose standard. Lane 1. Glucose standard (12 μL). Lane 2. Standard mixture of glucose and trehalose (12 μL). Lane 3. Trehalose standard (20 μL). Lane 4. Mycelium treated with 0.25% EtOH (v/v) (20 μL). Lane 5. Mycelium treated with 0.05% EtOH (v/v) (20 μL). Lane 6. Untreated mycelium (20 μL). Lane 7. Extracted cells of *Saccharomyces cerevisiae* (20 μL).

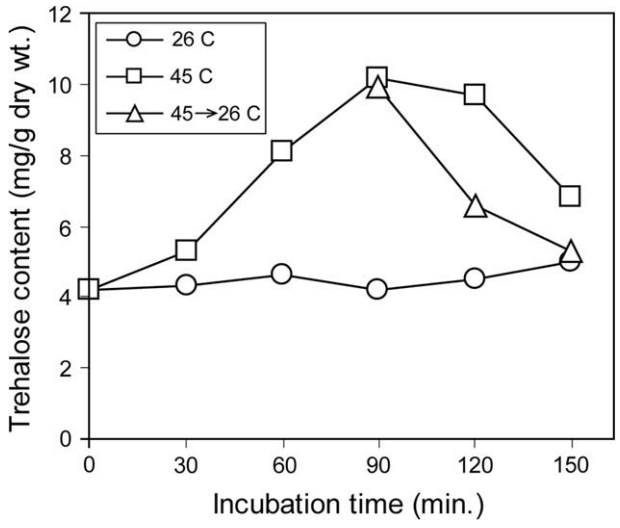

Fig. 1. Trehalose content of mycelium of *B. compniacensis* isolate UAMH 10764 under different temperature regimens following growth in MLB for 7 days.

Table 1
Trehalose concentration in two *B. compniacensis* strains following exposure to ethanol concentrations required for initial germination.

Incubation days with ethanol (%)	Trehalose content (mg g^{-1} dry wt)			
	UAMH 10764	% Increase	UAMH 10762	% Increase
6 d				
Control (0%)	1.4	–	2.1	–
0.05%	4.3	207	3.7	76
0.25%	5.1	264	5.1	143
8 d				
Control (0%)	2.0	–	2.4	–
0.05%	5.0	150	4.0	67
0.25%	5.6	180	7.8	225
10 d				
Control (0%)	2.4	–	4.0	–
0.05%	5.8	142	6.5	63
0.25%	7.0	192	6.8	70

Cultures of strain UAMH 10764 and UAMH 10762 were grown in MLB for 12 d. Trehalose was extracted by use of 0.5 M TCA at 26 C and quantified with anthrone reagent as described in the Materials and Method. Results are expressed as mg g^{-1} trehalose dry wt. Values are mean of two replicates.

The presence of exogenous or endogenous ethanol has been shown to directly affect fungal growth in a variety of ways, including the reduction of solution or substrate water activity, the disruption of hydrophobic regions of membranes, the inhibition and denaturation of glycolytic enzymes and metabolic effects associated with the accumulation of acetaldehyde (Hallsworth, 1998; Hallsworth et al., 1998). These effects have mostly been demonstrated using exposure to ethanol concentrations greater than 5% w/v (Hallsworth, 1998; Utama et al., 2002). Ethanol concentrations in this range have been shown previously to inhibit *B. compniacensis* growth completely (Ewaze et al., 2007). However, lower ethanol concentrations (e.g. 1–10 ppm) produce strongly stimulatory effects on cell germination (Ewaze et al., 2008). Our present results indicate that these similarly low-levels of ethanol are sufficient to stimulate the synthesis of trehalose, thereby enhancing the tolerance of log-phase cells to abiotic stress, such as high heat. However, it currently remains unknown whether trehalose accumulates in the hyphal tips and regions of apical growth or in the established mycelium of *B. compniacensis*.

Our data suggest that low-level exposure to environmental ethanol vapour function importantly in the colonization biology of *B. compniacensis* through the pre-conditioning of vegetative cells to withstand extreme environmental stress. The possible role of trehalose in ethanol-induced environmental stress resistance may provide a lucrative direction for future research on the prevention and control of this conspicuous and damaging colonist of outdoor walls and other surfaces. Further investigation is necessary to elucidate the complex nature of interrelationships between environmental exposures to ethanol and other organic vapours and the molecular regulation of *B. compniacensis* metabolism and germination.

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