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Physiological studies of the warehouse staining fungus, *Baudoinia compniacensis*

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ABSTRACT

Baudoinia compniacensis, the fungus responsible for highly conspicuous black growth on walls and other surfaces in the vicinity of distillery warehouses and commercial bakeries, has been little studied, in part because its isolation and cultivation have long been considered difficult. In the present study, basic details regarding the physiology of this organism are elucidated for the first time. It is able to utilize ethanol as a carbon source, but not other simple alcohols; glucose is also well utilized, as is the ethanol breakdown product acetate. Inorganic and many organic nitrogen sources support growth well, but urea does not. Though strongly inhibited by salt concentrations over 2 M, *B. compniacensis* can survive considerably higher concentrations. The fungus does not ordinarily survive temperatures of 52 °C or higher when moisture is present, but can be pre-adapted to survive this temperature by prior heat or ethanol exposure. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of cellular proteins reveals that heat and ethanol pre-adaptation appear to induce formation of putative heat shock proteins.

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Introduction

The black fungus responsible for 'warehouse staining' in the spirits industry was described as *Torula compniacensis* in 1881 (Richon & Petit 1881), but since then its nondescript morphology and the great difficulty of isolating it onto artificial media have resulted in an almost complete lack of study. Recently, it has been isolated in pure culture and studied from a modern standpoint, resulting in redescription as the type species of the new genus *Baudoinia* (Scott *et al.* 2007). This fungus is of interest in part because of its unusual habitat: it appears to grow outdoors only where the environment is exposed to ethanolic vapours that transpire from stored wooden barrels of distilled spirits or arise from commercial baking. Another interesting feature is that the fungus is black, giving the walls, tree trunks, fences, etc, that it grows on a scorched or blackened, mouldy appearance that, although regarded as a traditional aspect of cognac production in France, is looked upon with public suspicion in some other areas such as North America. Thus there is an impetus to both understand and control the growth of this organism.

The object of the present study was to determine the effect of physiological and physical factors on the growth of *Baudoinia*, in particular, nutritional factors, such as culture medium constituents, and environmental factors, such as medium pH and temperature. This information is important for improving the *in vitro* cultivation of this fungus, enabling determination of the best medium and conditions for optimal growth and reproduction. A better understanding of the growth conditions required for the fungus enables controlled experiments to

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discover potential factors to control the organism. It also enables better understand of the physiology of an ecologically unique organism. The salient environmental factors to be considered include the organism's apparent growth response in the presence of ethanol, its exposed, relatively xeric habitat on plant trunks and stems, as well as human building structures, cement walls, etc, the likelihood of its regular exposure to very high (up to 65 °C on asphalt roofing, a common habitat) and, in some areas, very low temperatures based in these exposed sites, and the probable requirement in many of its otherwise biota-poor habitats to utilize simple nitrogen sources made available through rainfall. The present study investigates responses of *Baudoinia compniacensis* to ethanol and other carbon sources, various nitrogen sources, solutes, and high and low temperatures.

Materials and methods

Isolates used

Isolates UAMH 10762 (University of Alberta Microfungus Collection, Edmonton, AB, Canada) and UAMH 10839 were isolated from tree bark and concrete walls, respectively, near a bond warehouse in Windsor, ON. Isolate UAMH 10764 was from a concrete wall near a distillery warehouse in Loretto, KY. Voucher materials from this study have been deposited in DAOM. Living cultures have been deposited in UAMH.

Preparation of inoculum

Stock cultures of the isolates were grown on 90 mm Petri dishes containing modified Leonian's agar (MLA; Malloch 1981). Plates were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI) and incubated inverted for 14 d at 26 °C. These stock plates were used for the preparation for mycelial inocula. Mycelial suspensions for liquid cultures were made by flooding the plate surface with 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. The short mycelial fragments in the resulting suspension were enumerated and their concentration adjusted in distilled water until haemocytometer counts of 5×10^5 fragments ml^{-1} were obtained as a standard to be used for all experiments. 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.

Media

MLA was used as a general purpose cultivation medium. This medium was prepared by adding the following ingredients to 1 l distilled water: 6.25 g maltose, 6.25 g malt extract (Bioshop, Burlington, ON), 0.63 g $MgSO_4 \cdot 7H_2O$, 1.25 g KH_2PO_4, 0.63 g Bacto peptone (Difco, Becton Dickinson, Sparks, MD), 1 g yeast extract (Difco), 15 g agar (Sigma–Aldrich, St Louis, MO) for solid media. The initial pH of MLA was adjusted to 6.8 by drop-wise addition of 5 N NaOH or 5 N HCl.

A completely defined medium was used for physiological studies. This medium was prepared by the addition of carbon and nitrogen stock solutions to a basic stock solution of trace elements. The composition of the stock solutions is given below.

Stock solution A: trace elements (×100 concentration)
A mixture was made consisting of 50 ml each of the following stock solutions: 50 $mg\,l^{-1}$ $CoCl_2$, 40 $mg\,l^{-1}$ $CuSO_4 \cdot 5H_2O$, 12 $mg\,l^{-1}$ $FeCl_3 \cdot 6H_2O$ chelated with 17 $mg\,l^{-1}$ Na_2EDTA, 620 $mg\,l^{-1}$ $MnCl_2 \cdot 4H_2O$, 30 $mg\,l^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, and 920 $mg\,l^{-1}$ $ZnSO_4 \cdot 7H_2O$. The mixture was made to 1 l total volume with DDI water. It was added to all growth media at a rate of 10 $ml\,l^{-1}$ before autoclaving.

Stock solution B: (Nitrogen source)
The medium was amended with different nitrogen sources, including NH_4Cl, NH_4NO_3, KNO_3, $NaNO_3$, alanine, glutamine, glutamate, asparagine, asparagine/phenylalanine, casamino acids, and urea. A range of concentrations from 2.5–10 mM was tested for each compound.

Stock solution C: (carbon source)
A stock solution of 95 % (v/v) ethanol was added to cooled, autoclaved culture medium under axenic conditions immediately before inoculation to a final concentration governed by the particular experiment. A similar method was used for the testing of other alcohols. Acetate was also tested as a carbon source: this two-carbon compound is normally formed as a breakdown product on the pathway to acetyl coenzyme A in fungi utilizing ethanol as a carbon source. In addition, glucose was tested. When acetate or glucose was used as a carbon source, it was added at the desired concentration to growth media before autoclaving. Concentrations ranging from 0.5–10 mM were used. Acetate was added in the form of ammonium acetate (CH_3COONH_4), and served as sole carbon and nitrogen source. Media were dispatched into sterile 125 ml Erlenmeyer flasks.

Growth measurement

For fungal growth measurement in different nutrient solutions, cultures were incubated at 26 °C unless otherwise stated, on a rotary shaker operating at 120 rev min^{-1}, using Gyro and Recipro Shake (Model:SK-300/SK-600). At the end of the incubation period, the contents of each 50 ml Erlenmeyer flask were filtered through pre-weighed, 0.8 mm Gilson A/F glass fibre filter membranes. The filter membranes bearing mycelium were washed thoroughly with distilled water and oven-dried at 60 °C for 24 h. Dry weight was measured using an ultra microbalance (AD-6, Perkin Elmer, Waltham, MA) equipped with a polonium-210 antistatic ionizing strip. Each nutrient variable was run in two to three replicates.

Tolerance of pH effect resulting from use of ammonium as sole nitrogen source

Isolate UAMH 10762 was grown in 50 ml flasks of defined medium with NH_4Cl as the sole nitrogen source and 0.25 % ethanol as the sole carbon source. At days 2, 4, 6, 8 and 10, two

flasks per treatment were removed. The pH of the medium was measured using an Accumet Basic AB15 pH meter (Fisher Scientific, UE Tech Park, Singapore) and the mycelium was filtered washed and weighed as detailed in the previous section.

Heat treatment effect on viability

Mycelial suspensions were prepared as described under 'Preparation of inoculum', above, and incubated for 6 d in modified Leonian's broth (MLB) incubated at 26 °C on a rotary shaker to obtain exponential growth. Large mycelial aggregates were removed by filtering the suspension through sterile gauze placed in a thistle funnel. Inoculum was adjusted to 5×10^5 fragments ml^{-1} using a haemocytometer. Aliquots of 1 ml of well-shaken mycelial suspension were transferred to similar test tubes holding 9 ml MLB prewarmed to different temperatures (0, 2, 20, 26, 32, 37, 39, 45, 52, 55 and 65 °C). Flasks were held for a brief (30 min) period of incubation at those temperatures. At the end of the heat incubation, the tubes were well shaken and 0.25 ml aliquots, expected to contain approximately 500 CFU, were removed and plated on MLA, three plates per temperature. The plates were incubated 14 d at 26 °C and the number of outgrowing colonies was counted. It should be noted that *Baudoinia compniacensis* colonies are very restricted, slow-growing, and black, facilitating accurate counting.

Effect of prior heat shock on viability after high temperature exposure

To determine the effect of prior heat shock on the viability of *Baudoinia compniacensis* exposed to different high temperature conditions, 200 µl of filtered exponential-phase mycelial fragments of UAMH 10762, prepared as in the previous experiment, were incubated in two parallel series of 50 ml pre-warmed MLB flasks at 26, 37, 39, 45, and 52 °C for 30 min. The temperature-treated suspensions were then centrifuged at 7000 rev min^{-1} for 5 min, and supernatants were discarded. With material from the first parallel series, each pellet was resuspended in 50 ml sterile distilled deionized (DDI) water. After these treatments, 0.25 ml mycelial suspension was removed from each treatment tube and plated on MLA, and this procedure was replicated twice again. With material from the second parallel series, the mycelial fragments were resuspended in 50 ml fresh MLB pre-warmed to 52 °C. Incubation was continued at 52 °C for 15 min. A 0.25 ml sample of suspended mycelial fragments was then plated onto MLA, again with threefold replication per temperature. Once inoculated, the plates were incubated and counted as described above.

Effect of NaCl pre-treatment on growth

Inoculum of UAMH 10762, prepared as for the temperature experiments, was transferred to 50 ml Erlenmeyer flasks with different concentrations of NaCl (control, 0.5, 1, 2, 3, 4, and 6 M). Aliquots of 200 µl suspended mycelial fragments were transferred from each of the treatment flasks after 18, 24, and 72 h and inoculated onto MLA. The plates were incubated at 26 °C and the number of the colonies was counted after 14 d.

Preparation of cell-free extracts

A weighed quantity of mycelial tissue was ground with a small quantity of fine perlite in an ice-cold mortar and pestle in 100 mM Tris–HCl extraction buffer. The crude extract was filtered through filter paper (Whatman no.1) and maintained at 4 °C prior to use.

Gel electrophoresis

In order to examine the effect on protein expression of protective pre-incubation before ethanol shock or heat shock, mycelium of isolates used for ethanol and heat shock studies were grown on MLB at 26 °C for 6 d on a rotating shaker at 120 rev min^{-1}. The growth medium containing mycelium was transferred to a sterile centrifuge tube and mycelium was concentrated by centrifugation. The supernatant was discarded and fresh medium pre-warmed to one of several experimental temperatures was added, after which mycelium was resuspended. The tubes were transferred immediately to water-baths heated to the experimental temperature in an appropriately heated incubator. Control mycelia were treated in the same manner as heat-treated mycelia, but handled at 26 °C throughout the procedure.

Heat effects on UAMH 10762 protein synthesis were studied by comparing proteins produced by untreated control mycelium to proteins produced under conditions of (1) heat shock, in this case, mycelium incubated at 40 °C for 30 min; (2) heat stress at near-lethal temperature, in this case, mycelium incubated at 55 °C for 15 min; and (3) potentially protective exposure to heat shock followed by heat stress at near-lethal temperature, in this case, mycelium incubated at 40 °C for 30 min, transferred to pre-warmed MLB at 55 °C for 15 min. For heat effects on isolate UAMH 10764, an additional test was done with mycelium incubated at 40 °C for 30 min, then transferred to a pre-warmed medium at a highly lethal temperature of 66 °C for 15 min.

For study of the potential protective effect of ethanol-induced shock response proteins, extracted proteins from control mycelium were compared with proteins from mycelium incubated with 7 % ethanol for 30 min at 26 °C, and mycelium incubated with 7 % ethanol for 30 min at 26 °C and then transferred to pre-warmed MLB at 55 °C for 15 min.

For electrophoresis, 60 µl of treated cell-free extract was loaded per well. Protein bands were determined using discontinuous SDS-PAGE as described by Laemmli (1970), using 12 % resolving gel and 4 % stacking gel with Miniprotean® 3 Cell (BioRad, Hercules, CA). All steps were carried out at 4 °C at 200 V for 40 min. Gels were stained for 2 h at room temperature in a solution modified from that of Laemmli (1970), containing 50 % (v/v) methanol and 0.1 % (w/v) Coomassie Brilliant Blue to which 10 % (v/v) acetic acid was added immediately before use. Gels were destained overnight in 5 % (v/v) methanol and 7.5 % (v/v) acetic acid. A detailed analysis of the bands produced was obtained by scanning the gels and analysing the images with the software Scion Image for Windows (Scion Corporation, Frederick, MD). Bands were scored as present/absent and strong/weak based on the graphical readouts produced by this reading method (not shown).

Table 1 – Effect of alcohols as carbon sources on the mycelial growth of *Baudoinia compniacensis* in shaken flasks

Strain	Conc. (%v/v)	Average dry weight (mg) (±average deviation)			
		Methanol	Ethanol	Propanol	Butanol
UAMH 10839	0.1	0.48 (0.08)	2.48 (0.13)	0.31 (0.06)	0.3 (0.03)
	0.2	–	3.08 (0.08)	0.51 (0.1)	0.41 (0)
	0.25	0.43 (0.08)	3.88 (0.13)	0.6 (0.03)	0.6 (0.03)
	0.5	0.1 (0.01)	2.89 (0.16)	0.31 (0.03)	0.4 (0.03)
	1	–	1.91 (0.21)	0.21 (0)	0.2 (0.02)
UAMH 10762	0.1	0.5 (0.1)	1.81 (0.11)	0.3 (0.03)	–
	0.2	–	2.98 (0.28)	0.5 (0.06)	–
	0.25	0.4 (0.05)	3.4 (0.1)	0.5 (0.1)	–
	0.5	0.2 (0.03)	2.6 (0.25)	0.41 (0.06)	–
	1	–	1.41 (0.11)	0.2 (0.01)	–
UAMH 10764	0.1	0.41 (0.07)	0.91 (0.09)	<LOD	<LOD
	0.2	–	1.4 (0.05)	<LOD	<LOD
	0.25	0.31 (0.01)	1.7 (0.05)	<LOD	<LOD
	0.5	0.1 (0.02)	1.19 (0.14)	<LOD	<LOD
	1	–	1.11 (0.09)	<LOD	<LOD

Five millimolar NH_4Cl was used as a nitrogen source for all alcohols; Initial pH of all media was adjusted to pH 6.1 with 5 N HCl/NaOH. LOD, limit of detection; –, not tested.

Protein determination

For standardization of SDS-PAGE, cell-free extract was analysed for protein quantity using the method described by Lowry *et al.* (1951).

Results

Effect of carbon sources

With ammonium chloride as the nitrogen source for growth in shaken flasks, several non-carbohydrate compounds were tested as carbon and energy sources (Table 1). Methanol, propanol, and butanol scarcely supported fungal growth. Ethanol was suitable as a carbon and energy source; the concentration that supported the greatest amount of growth was found to be 0.25 %. The optimal concentration of ammonium acetate for growth was 1 mM, whereas that of glucose was between 1 and 10 mM within the range tested (Table 2).

Effect of nitrogen sources

Cultures grown on 0.25 % ethanol as the sole carbon source were capable of utilizing a wide variety of sources (Table 3). Comparable levels of growth were observed on both ammonium and nitrate-based nitrogen sources. No growth was recorded for the three isolates when urea was used as a nitrogen source. All the individual amino acids tested, as well as the casamino acids preparation, supported appreciable growth (Table 4).

Effect of pH changes with ammonium as sole nitrogen source

When isolate UAMH 10762 was grown on a medium containing NH_4Cl as an nitrogen source, the pH of the culture medium dropped from 5.9 to 3.8 over 6 d of culture and then remained at 3.8 (Fig 1). This decrease in pH value is most likely due to the uptake of ammonium ion, though some additional secretion of acidic material cannot be ruled out. The pH changes did not appear to affect the formation of a normal exponential growth curve for *Baudoinia compniacensis*, though it should be stressed that no comparison test was done with buffered media.

Heat treatment

The optimum temperature for survival of isolate UAMH 10762 inoculum was 26 °C (Fig 2). Values for 2 and 20 °C were similar to those obtained at 26 °C. Survival sharply decreased at 30 °C and above, and 52 °C was completely lethal. Exposure to 0 °C

Table 2 – Effect of different carbon sources on the mycelial growth of *Baudoinia compniacensis* in shaken flasks

Strain	Conc. (mM)	Average dry weight (mg) (±average deviation)	
		Glucose	Ammonium acetate
UAMH 10839	0.5	2.7 (0.2)	4.4 (0.1)
	1	4.6 (0.1)	13.2 (0.2)
	5	5.85 (0.15)	3.18 (0.03)
	10	6.53 (0.07)	2.3 (0.3)
UAMH 10762	0.5	2.18 (0.18)	1.95 (0.15)
	1	3.7 (0.05)	3.7 (0.2)
	5	4.1 (0.1)	2.5 (0.1)
	10	4.6 (0.2)	1.7 (0.1)
UAMH 10764	0.5	2.35 (0.15)	1.9 (0.1)
	1	2.6 (0.2)	2.3 (0.1)
	5	2.9 (0.1)	2.1 (0.1)
	10	3.5 (0.2)	1.75 (0.05)

Five millimolar NH_4Cl was used as a nitrogen source for the glucose test. The ammonium moeity of ammonium acetate served as the sole nitrogen source in the acetate test. Initial pHs of the media were adjusted with 5 N HCl/NaOH as follows: glucose pH 6.5; ammonium acetate pH 5.9.

Table 3 – Effect of different nitrogen sources on the mycelial growth of *Baudoinia compniacensis* in shaken flasks

Strain	Conc. (mM)	Average dry weight (mg) (±average deviation)				
		Urea	NH_4Cl	NH_4NO_3	KNO_3	$NaNO_3$
UAMH 10839	2.5	<LOD	2.1 (0.1)	2.4 (0.1)	–	1.6 (0.1)
	5	<LOD	3.4 (0.15)	2.9 (0.2)	2.9 (0.2)	2.2 (0.05)
	10	<LOD	3.55 (0.15)	2.3 (0.05)	–	1.5 (0.05)
UAMH 10762	2.5	<LOD	2.2 (0.05)	3.2 (0.1)	–	1.7 (0.2)
	5	<LOD	3.23 (0.23)	3.55 (0.2)	3.2 (0.1)	2.7 (0.15)
	10	<LOD	2.43 (0.08)	3.1 (0.3)	–	1.6 (0.05)
UAMH 10764	2.5	<LOD	1 (0.25)	1.7 (0.2)	0.6 (0.05)	–
	5	<LOD	1.43 (0.08)	1.4 (0.05)	1.2 (0.2)	–
	10	<LOD	0.53 (0.08)	0.9 (0.1)	0.3 (0.05)	–

Point two five percent (v/v) ethanol was used as the sole carbon source for all media. Initial pHs of the media were adjusted with 5 N HCl/NaOH as follows: urea pH 7.2, NH_4Cl pH 6.2, NH_4NO_3 pH 5.9, KNO_3 pH 6.1, $NaNO_3$ pH 6.4. LOD, limit of detection; –, not tested.

yielded a value of 74 % of the level of growth recovery seen at the optimal temperature. Pretreatment of inoculum at 37, 39 and 45 °C allowed a degree of survival at the normally lethal temperature of 52 °C (25, 6 and 3 %, of control values, respectively; Fig 2).

Effect of NaCl pre-treatment on viability

Exposure of UAMH 10762 inoculum to various NaCl concentrations for different time periods (Fig 3) showed that for all concentrations, there was little difference in the effects of 18 and 24 h exposures. However, an exposure of 72 h yielded a strongly negative effect at all concentrations. Even at the shorter exposure times, concentrations of 2 M or above caused a sharp decline in survival. Interestingly, however, no concentration completely eliminated viable cells, and colony levels of *ca* 2.5 % of control levels were seen even after 18–24 h exposure to 6 M NaCl.

Effect of heat on protein profiles

When mycelia were stressed at 40 °C, more than 70 % of the control protein bands were observed in discontinuous SDS-PAGE gel electrophoresis (Fig 4: lane 3). In unstressed cells some proteins were present in low concentrations, whereas in stressed cells they accumulated at moderate levels, especially in the case of the band around 53 kDa (Fig 4A: lane 4; and B: lane 5). At 55 °C, most of the low molecular weight proteins disappeared (Fig 4B: lane 4). In cells exposed to 55 °C after prior heat shock at 40 °C, the 53 kDa protein was produced at elevated levels in both cultures tested (Fig 4A: lane 4; B: lane 5), whereas enhanced production of the 49 kDa protein was seen in UAMH 10764 (Fig 4A: lane 4). No protein synthesis occurred at 66 °C (Fig 4A: lane 5). Cells exposed to 7 % ethanol at 26 °C retained synthesis of almost all proteins produced in unstressed cells (Fig 4: lane 6). In ethanol-shocked cells transferred to 55 °C, the synthesis of the 53 and 49 kDa bands seen in the above heat-only studies was induced. In addition, a 40 kDa band was consistently induced in both isolates tested (Fig 4: lane 7).

Discussion

During the physiological study of some fungi, a very wide range of carbon sources is surveyed to determine whether they support growth (Barnett *et al.* 2000; de Hoog *et al.* 2000).

Table 4 – Effect of different amino acids as nitrogen sources on the mycelial growth of *B. compniacensis* in shaken flasks

Strain	Conc. (mM)	Average dry weight (mg) (±average deviation)					
		Alanine	Glutamate	Glutamine	Asparagine	Asparagine/ Phenylalanine	Casamino acids
UAMH 10839	2.5	4.4 (0.15)	1.25 (0.25)	4.28 (0.13)	3.23 (0.08)	2.98 (0.22)	4.28 (0.02)
	5	5.55 (0.35)	2.9 (0.25)	5.17 (0.27)	3.31 (0.19)	2.3 (0.2)	4.9 (0.3)
	10	4.3 (0.25)	1.23 (0.08)	4.2 (0.2)	2.69 (0.04)	2.18 (0.23)	8.18 (0.36)
UAMH 10762	2.5	5.1 (0.05)	–	4.49 (0.23)	4.28 (0.23)	3.68 (0.13)	5.21 (0.06)
	5	5.9 (0.4)	3.2 (0.2)	5.9 (0.2)	3.31 (0.19)	4.1 (0.2)	8.23 (0.13)
	10	5.5 (0.2)	2.11 (0.06)	4.6 (0.16)	2.69 (0.04)	3.41 (0.15)	17.9 (0.7)
UAMH 10764	2.5	2.3 (0.1)	2.2 (0.2)	4.3 (0.09)	1.69 (0.25)	–	6.06 (0.26)
	5	3.4 (0.3)	–	5.8 (0.2)	2.58 (0.08)	–	6.18 (0.18)
	10	2.7 (0.1)	2.29 (0.29)	5.08 (0.18)	2.18 (0.13)	–	7.95 (0.55)

Point two five percent (v/v) ethanol used as the sole carbon source. Initial pHs of the media were adjusted with 5 N HCl/NaOH as follows: alanine pH 6.4, glutamate pH 6.6, glutamine pH 6.9, asparagine pH 6.9, asparagine/phenylalanine pH 6.6, casamino acids pH 6.5. –, Not tested.

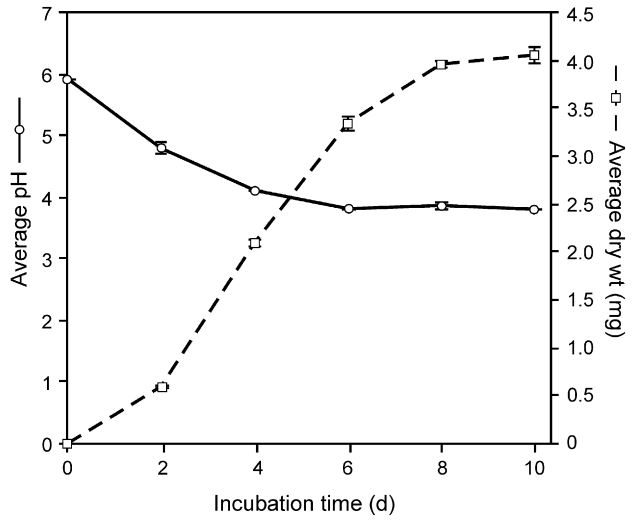

Fig 1 – Correlation between pH and ammonium in a medium supporting the growth of isolate UAMH 10762 (mg D.W. 50 ml^{-1}) in the presence of ammonium and pH of culture medium as a function of time. Values show mean of two to eight replicates. Bars show average deviation.

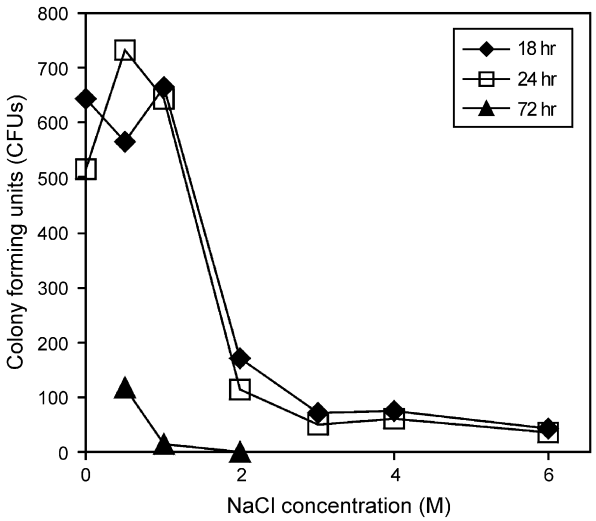

Fig 3 – Effect of different concentration of NaCl (0.5, 1, 2, 3, 4, and 6 M) on fungal growth of UAMH 10762. Growing mycelial fragments were shifted from the treated flasks after 18, 24, and 72 h. and inoculated on MLA. The plates were incubated at 26 °C and the number of the colonies was counted after two weeks. Values show mean of two to three replicates.

In the case of *Baudoinia compniacensis*, its specific ecological distribution in outdoor areas exposed to ethanol vapour gave an indication that its metabolism might be specialized for ethanol and related compounds. Moreover, preliminary studies showed that medium with 5 ppm ethanol greatly increased the success of isolation from environmental materials (Scott *et al.* 2007). Therefore the present study concentrated on substances related to ethanol, either simple alcohols, or, in the case of acetate, metabolites associated with normal ethanol breakdown pathways. The most commonly utilized simple sugar, glucose, was also tested. Simple alcohols other than ethanol were scarcely used, whereas ethanol, acetate, and glucose yielded optimal mycelial growth. Ethanol, normally metabolized in fungi by alcohol dehydrogenase enzyme ADH II, tends to integrate, *via* the intermediates acetate and acetaldehyde, into common metabolic pathways in a way that does not apply to other simple alcohols (Russell *et al.* 1983). Moreover, when acetaldehyde is produced in large quantities as a result of ethanol use as a primary carbon source, a key metabolic branch point exists allowing further metabolism either *via* the tricarboxylic acid (TCA) cycle or *via* the specialized fungal glyoxylate cycle (Kornberg & Madsen 1957) which efficiently processes the two-carbon units. This efficiency is not found with propanol, which may be converted to acetone (Dailly *et al.* 2001), a compound requiring additional specialized enzymes if it is to be utilized. Likewise, butanol is probably not efficiently handled by ADH (Green *et al.* 1993). Methanol requires specialized enzymes in order to be oxidized in ways that either do not generate potentially toxic formaldehyde or, if it is produced, allow it to be handled so that the cell is not damaged. Even though ethanol, as expected, strongly supported the growth of *B. compniacensis*, glucose also yielded good growth; this indicates that although ethanol may be important ecologically to the growth of this fungus, it is not physiologically essential.

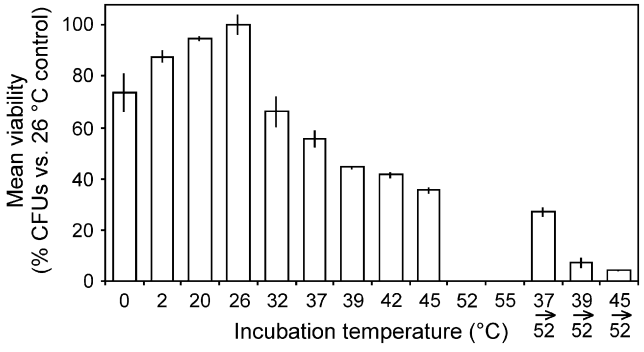

Fig 2 – Effect of different temperatures on the viability of cells of UAMH 10762 exponentially growing (6 d) mycelial fragments were shifted from 26 °C to fresh ML media with different temperatures (0, 2, 20, 26, 32, 37, 39, 45, 52, and 55 °C) for 30 min. The three histograms on the right represents spores incubated at 37, 39, and 45 °C for 30 min, then transferred to prewarmed fresh MLB, and incubated at 52 °C for another 15 min. Values show mean of two to eight replicates. Bars show average deviation.

As might be expected for a fungus often growing on bare inorganic surfaces, inorganic nitrogen sources were well utilized. In general, saprotrophic fungi grow better on ammonium or amino acids than on inorganic nitrate (Hacskaylo *et al.* 1954; Fries 1955; Niederpruem *et al.* 1964); however, in the case of *B. compniacensis* no distinct differences could be seen within the resolution of our data.

B. compniacensis did utilize all tested ammonium and amino acid substrates well as nitrogen sources, but among the amino

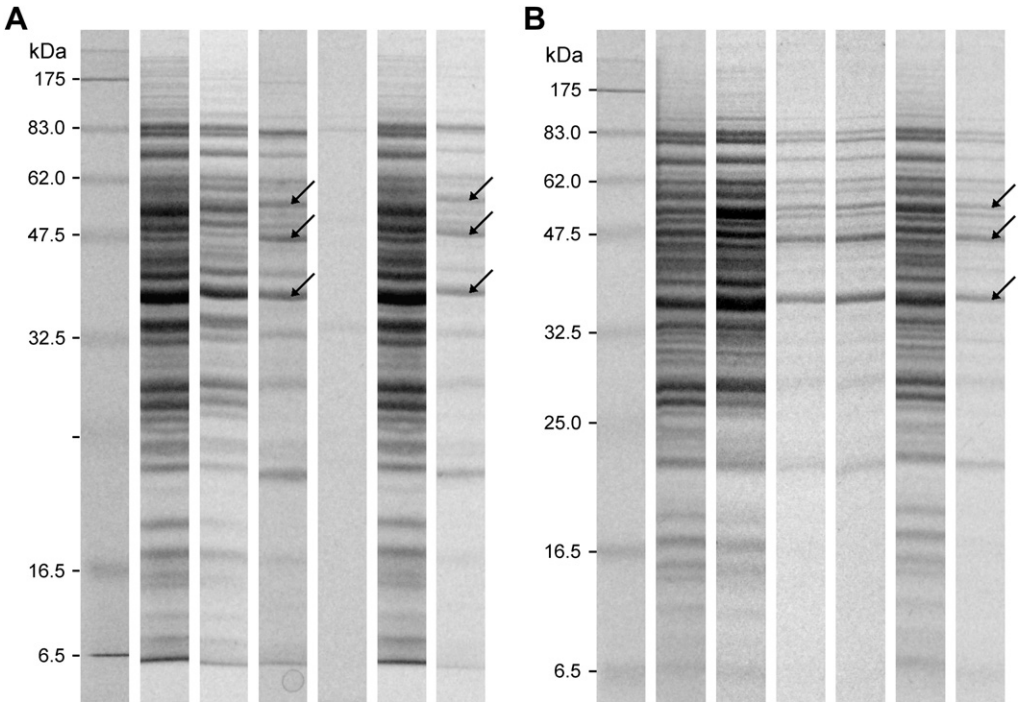

Fig 4 – Effect of heat and ethanol on *Baudoinia compniacensis*. Cellular proteins separated by discontinuous SDS-PAGE of isolates UAMH 10764 (A), and UAMH 10762 (B). In both gels, lane 1 contains marker proteins with the molecular masses indicated in kDa. Lane 2 [(A) 23 μg protein (B) 27.5 μg protein] control cells incubated at 26 °C. Lane 3 [(A) 19 μg protein, (B) 25 μg protein] control cells incubated at 40 °C for 30 min. Lane 4 [(A) 23.5 μg protein,(B) 27.5 μg protein], control cells incubated at 40 °C for 30 min then transferred to a prewarmed medium at 55 °C for 15 min (A); and heat-stressed at 55 °C for 15 min without pre-incubation at lower temperature (B). Lane 5 [(A) 25 μg protein, (B) 29 μg protein] control cells incubated at 40 °C for 30 min, then transferred to a prewarmed medium at 66 °C (A) or 55 °C (B) for 15 min. Lane 6 [(A) 25 μg protein, (B) 26 μg protein], control cells treated with 7 % ethanol incubated at 26 °C for 30 min. Lane 7 [(A) 27 μg protein, (B) 29.5 μg protein], control cells treated with 7 % ethanol incubated at 26 °C for 30 min then transferred to a prewarmed medium at 55 °C for 15 min. Arrows in both gels indicate putative stress protein bands at circa 53, 49 and 40 kDa.

acids tested, glutamine appeared to serve better than glutamate as a sole nitrogen source. This is not surprising given the presence and prominent metabolic role of its nitrogen-bearing amide side chain. Lundeberg (1970) pointed out in reference to asparagine that a preference for this amino acid as a nitrogen source might indicate that nitrogen in the amide position is more generally metabolically accessible for fungi than nitrogen bound as an amine. The assimilation of ammonium into glutamate to produce glutamine is believed to play a central role in the nitrogen metabolism of many fungi (Lara *et al.* 1982; Holmes *et al.* 1989).

The slow drop in pH seen when *B. compniacensis* utilized ammonium acetate as the sole carbon and nitrogen source is similar to that seen with diverse other fungi, e.g., the results seen by Rangel-Castro *et al.* (2002) with the symbiotic mushroom species *Cantharellus cibarius*.

The inability of *B. compniacensis* to utilize urea as the sole nitrogen source probably reflects lack of a specific transport system as was suggested for *Aspergillus nidulans* (Dunne & Pateman 1972) or else the absence of a urease enzyme. In many groups of fungi, such as members of the *Arthrodermataceae*, possession of a urease enzyme is indicative of a habitat association with soil (Summerbell 2000). No evidence to date supports an association of *B. compniacensis* with soil, though admittedly this small, non-descript, metabolically unusual organism would not be detected in most conventional soil mycology studies.

The lethal temperature for hydrated *B. compniacensis* cells was 52 °C, whereas for dried mycelium it was 75 °C for the same isolate (data not shown). Exposure of the cells to mild heat shock increased viability in later exposure to more extreme heat and also resulted in modifications to the protein expression profiles. When *B. compniacensis* was stressed by 7 % ethanol, the same profile of protein synthesis was displayed as in non-stressed cells. This type of thermotolerance acquired through production of specialized heat shock proteins in pre-incubation has been seen in various fungi since its original elucidation in *Saccharomyces cerevisiae* (Finton & Pringle 1980; Plesset *et al.* 1982). That chemical stressors, such as ethanol, can protect against heat shock has been taken to suggest that heat shock or ethanol-induced proteins function in a general cellular response mechanism to stress (Finton & Pringle 1980; Plesset *et al.* 1982; Watson & Cavicchioli 1983). Piper (1995) reported that the induction of heat shock proteins in *S. cerevisiae* was effected at temperatures above 35 °C or ethanol levels above 4–6 % (v/v). However, it is possible that other sorts of stresses may induce the expression of

these proteins on additional study of *B. compniacensis*. Further investigation is needed to determine whether the proteins found in heat and ethanol-treated cells of *B. compniacensis* represent genuine heat shock proteins.

The correlation seen between increased heat survival after preconditioning and changes in the protein expression profile does not necessarily imply these proteins were responsible for the protective effect. In *S. cerevisiae*, it has been suggested that heat-induced synthesis of the disaccharide trehalose is protective against thermal shock (D'Amore *et al.* 1991; Bell *et al.* 1992; van Dijck *et al.* 1995; Arguelles 2000; Elbein *et al.* 2003), whereas at least some heat shock proteins are involved more in repair (de Virgilio *et al.* 1991; Hottiger *et al.* 1992). The exact juxtaposition of the roles of trehalose and various stress response proteins is not entirely resolved (Gross & Watson 1998). Future experiments with *B. compniacensis* will study the relationship between trehalose accumulation and protein expression profiles after heat or ethanol preconditioning.

In overview, the physiological characters shown here for *B. compniacensis* appear to correspond well to an organism living in bare, inorganic habitats exposed to ethanol vapours, as well as extremes of heat and cold. The fungus is able to utilize ethanol particularly well but can also survive on simple sugars; it can utilize a wide variety of organic and inorganic nitrogen sources with good tolerance of any local pH changes that may result, and it can be pre-adapted to survive normally lethal heat levels by prior heat, ethanol exposure, or desiccation. It can also survive a wide variety of osmotic conditions, at least insofar as that is indicated by surviving a range of salt concentrations. The information gained helps in the design of optimal selective and maintenance media for this unusual and difficult to culture organism, and also facilitates the development and testing of control strategies in areas where widespread occurrence of dark fungal discolouration on outdoor residential and commercial structures is disfavoured.

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