MYCOLOGICAL RESEARCH 112 (2008) 1373–1380

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/mycres

Ethanol physiology in the warehouse-staining fungus, *Baudoinia compniacensis*

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ARTICLE INFO

Article history:
Received 31 December 2007
Received in revised form
22 April 2008
Accepted 1 May 2008
Corresponding Editor:
Nicholas P. Money

Keywords:
Alcohol dehydrogenase
Biodeterioration
Capnodiales
Glyoxylate cycle
Torula compniacensis

ABSTRACT

The fungus *Baudoinia compniacensis* colonizes the exterior surfaces of a range of materials, such as buildings, outdoor furnishings, fences, signs, and vegetation, in regions subject to periodic exposure to low levels of ethanol vapour, such as those in the vicinity of distillery aging warehouses and commercial bakeries. Here we investigated the basis of ethanol metabolism in *Baudoinia* and investigate the role of ethanol in cell germination and growth. Germination of mycelia of *Baudoinia* was enhanced by up to roughly 1 d exposure to low ethanol concentrations, optimally 10 ppm when delivered in vapour form and 5 mM in liquid form. However, growth was strongly inhibited following exposure to higher ethanol concentrations for shorter durations (e.g., 1.7 M for 6 h). We found that ethanol was catabolized into central metabolism *via* alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ACDH). Isocitrate dehydrogenases (IDHs) were active in cells grown on glucose, but these enzymes were not expressed when ethanol was provided as a sole or companion carbon source. The glyoxylate cycle enzymes isocitrate lyase (ICL) and malate synthase (MS) activities observed in cells grown on acetate were comparable to those reported for other microorganisms. By replenishing tricarboxylic acid (TCA) cycle intermediates, it is likely that the functionality of the glyoxylate cycle is important in the establishment of luxuriant growth of *Baudoinia compniacensis* on ethanol-exposed, nutrient-deprived, exposed surfaces. In other fungi, such as *Saccharomyces cerevisiae*, ADH II catalyses the conversion of ethanol to acetaldehyde, which then can be metabolized *via* the TCA cycle. ADH II is known to be strongly repressed in the presence of glucose.

Introduction

Tourists visiting distillery facilities in France where cognac and armagnac are held for ageing are likely to be told about ''la part des anges'' (''the angels' share''), the annual 2 % loss of ethanol by distilled spirits stored in traditional wooden casks. At the same time, they are shown a black fungus that forms a conspicuous, sooty covering wherever these escaping ethanol vapours contact nearby walls, fences, tree trunks, and other exterior surfaces. Despite the constant attention, the fungus involved in this growth has received almost no scientific study since it was described as *Torula compniacensis* (literally, ''the torula from Cognac'') in 1881 (Richon & Petit 1881). A significant obstacle has been that the microscopically nondescript and very slow growing fungus is difficult to obtain in pure culture, tending to be overgrown by competitors. Recently, the organism was isolated into pure culture from several locations around the world, and was investigated by nuclear ribosomal gene sequencing. It was revealed as belonging to an undescribed genus in the order *Capnodiales*

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0953-7562/$ – see front matter
doi:10.1016/j.mycres.2008.05.003

(Scott et al. 2007). This genus was named *Baudoinia*, and the fungus was redesignated *B. compniacensis*.

Investigation on the biosystematics of *B. compniacensis* was coupled with physiological studies, including ones on basic growth and specific responses to ethanol (Ewaze et al. 2007). *Baudoinia* was found to use ethanol, but not other simple alcohols, as a carbon source for growth; however, it proved to be equally capable of growing on ethanol-free medium using glucose as its carbon source. In addition, ethanol was shown to activate stress response proteins overlapping with those activated by heat shock (Ewaze et al. 2007). It was suggested that this activation of survival responses by ethanol might aid the establishment and growth of *Baudoinia* in the relatively extreme, bare, sun-exposed habitats where it is often seen around spirit maturation warehouses.

In the present study, further investigation was conducted on the metabolism of ethanol in *Baudoinia*, as well as on the positive and negative effects of various ethanol levels on growth and colony establishment. The ultimate objective was to study the basis of ethanol metabolism in *Baudoinia* and investigate the role of ethanol in cell germination and growth, with the goal of disclosing information that could be used to control the growth of this conspicuous organism around aging warehouses.

Materials and methods

Isolates used

Baudoinia compniacensis isolates UAMH 10762 (University of Alberta Microfungus Collection, Edmonton, AB, Canada) and UAMH 10839 were isolated from tree bark and a concrete wall, 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 of the fungi on which the paper is based have been permanently preserved in the University of Alberta Microfungus collection and Herbarium (UAMH).

Preparation of inoculum

Stock cultures of the isolates were grown on 90 mm Petri dishes containing modified Leonian's (ML) agar (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

ML agar was used as a general purpose cultivation medium. This medium was prepared by adding to 1 l distilled water the following ingredients: 6.25 g maltose, 6.25 g malt extract 0.63 g (BioShop, Burlington, ON), $MgSO_4 \cdot 7H_2O$, 1.25 g KH_2PO_4, 0.63 g Bacto peptone (Difco, Becton Dickinson, Sparks, MD), 1 g yeast extract (Difco), and 15 g agar (Sigma-Aldrich, St Louis, MO) for solid media.

A completely defined medium, *Baudoinia* physiological medium (BPM), was devised for use in 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 distilled deionized (DDI) water. It was added to all growth media at a rate of 10 $ml\,l^{-1}$ prior to autoclaving. Stock solution B (nitrogen source): nitrogen source stock solutions of NH_4Cl or NH_4NO_3 were added to media, as required, to a final concentration of 5 mM. Stock solution C (carbon source): a stock solution of 95 % (v/v) ethanol was added to cooled autoclaved culture medium under axenic conditions immediately prior to inoculation to a final concentration governed by the particular experiment. When acetate or glucose was used as a carbon source, it was added at the desired concentration to growth media prior to autoclaving. For carbon sources, either 5 mM acetate or 50 mM glucose were used. Acetate added in the form of ammonium acetate (CH_3COONH_4), served as sole carbon and nitrogen source. Media were dispensed 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, Jeio Tech Co., Ltd., Seoul, South Korea). At the end of the growth period, the contents of each flask were filtered through pre-weighed, 0.8 mm A/F glass fibre filter membranes (Gelman Sciences, Ann Arbor, MI). The filter membranes bearing mycelium were washed thoroughly with distilled water and dried at 60 °C to constant weight. Dry weight was measured using an electro balance (AD-6, Perkin Elmer, Waltham, MA) equipped with a Polonium-210 antistatic ionizing strip. Each nutrient variable was tested in duplicate, at minimum.

Growth in ethanol medium

Mycelial suspension (2 ml) of isolates UAMH 10762, UAMH 10839, and UAMH 10764 were prepared as described under 'preparation of inoculum' above, and grown in 50 ml defined medium with NH_4Cl or NH_4NO_3 as the sole nitrogen source and different concentrations of ethanol at 11, 22, 43, 87, and 170 mM [=0.063, 0.125, 0.25, 0.5 and 1 % (v/v), respectively] as

sole carbon source. At 7 d, the mycelia were harvested, filtered, washed, and weighed as detailed in the previous section.

Tolerance of elevated ethanol concentrations. Mycelial suspensions were prepared as described under 'preparation of inoculum', above, and incubated 6 d in ML broth at 26 °C on a rotary shaker to obtain exponential growth. The medium containing actively growing mycelium was filtered through sterile gauze in a thistle funnel to remove large pieces and the mycelial fragment suspension in the filtrate was collected in a small test tube. Inoculum was adjusted to 5×10^5 fragments ml^{-1} using a haemocytometer. Inoculum of UAMH 10762 was transferred to 50 ml ML broth plus different concentrations of ethanol: 0, 500, 900, 1600, 1700, and 2400 mM [=0, 3, 5, 9, 10 and 14 % (v/v), respectively]. Cultures were incubated on an orbital shaker at 26 °C and sampled at 0, 3, 6, and 8 h to assess survival on ML medium. At each sampling time, two flasks for each treatment were removed from the shaker and swirled to resuspend the material; a 200 µl aliquot, calculated to contain 1000 cfu, was removed and plated on an ML agar plate. The plates were incubated 14 d at 26 °C and the number of outgrowing colonies was counted. The very restricted, slow-growing, black-pigmented nature of the *Baudoinia* colonies facilitated accurate counting.

Stimulation of cells by pulse exposure to low ethanol concentrations. Inoculum of UAMH 10762 prepared as for the cell viability experiment was transferred to flasks containing 50 ml of different, relatively low concentrations of ethanol (0, 2.5, 5, 10, and 22.5 mM). The purpose was to determine whether short-term exposure to ethanol had a particularly strong potentiating effect on formation of new colonies in later incubation on ethanol-free medium. Aliquots of 200 µl, calculated to contain 1000 cfu, were transferred from each flask after 20 h and inoculated onto ML agar. The plates were incubated at 26 °C and the number of the colonies was counted after 14 d.

Effect of ethanol vapour on dormant cell germination. Five 2 l Erlenmeyer flasks each containing 1 l distilled water, were autoclaved, and different concentrations of ethanol were added to the cooled water under axenic conditions. Using the Henry's law constant for ethanol in aqueous solution (5×10^{-6} atm m^3 $mole^{-1}$ at 25 °C) (Gaffney *et al.* 1987), ethanol concentrations were calculated to obtain ethanol vapour levels in the head space ranging from 0.1–100 ppm; the actual volumes of 95 % ethanol added to the water were 1.23, 12.3, 123 and 1230 µL. Aliquots of 10 µl calculated to contain 300 cfu of UAMH 10762 were applied to the surface of mixed cellulose ester filter membranes (0.45 µm pore size, 25 mm; SKC, Eighty Four, PA). Each filter was affixed with a pin onto the lower surface of Neoprene stopper (Fisher Scientific, UE Tech Park, Singapore), these stoppers were then inserted firmly into the flasks, and covered with Parafilm to avoid ethanol loss. The flasks were incubated stationary for 14 d at 26 °C.

At the end of the incubation period, the filter membranes were removed from the stoppers and dried at room temperature. To facilitate counting the small colonies that had formed on the filters, optical clearing of the filters was performed as per the *NIOSH Manual of Analytical Methods* (NIOSH 1994), using an acetone vaporizing unit (Wonder Makers Enviromental, Kalamazoo, MI). Each filter was placed on a clean slide, which was placed on a cool polypropylene block positioned at the outlet of an aluminum retort (hot block) heated to 70 °C. A 1 ml aliquot of acetone was injected into the hot block with slow, steady pressure on the plunger button while holding the pipette firmly in place. Condensation of the acetone vapour on the membrane rendered the membranes transparent. A drop of clear lactic acid and a glass cover slip were applied to the surface of the cleared membrane. Microcolonies were counted using LM.

Protein electrophoresis studies

Preparation of cell-free extracts for protein studies. A weighed quantity of *Baudoinia* inoculum, mixed with a small quantity of perlite baghouse fines (Dicaperl Minerals, Thomason, MA), was ground with a pestle in an ice-cold mortar containing 100 mM Tris–HCl extraction buffer. The crude extract obtained was filtered through filter paper (Whatman No.1) and maintained at 4 °C prior to use.

Proteins elicited by growth on ethanol medium. Mycelia of UAMH 10762 and UAMH 10839 grown for 7 d at 26 °C in control ML broth and in 43 mM (0.25 %) ethanol plus 5 mM NH_4NO_3 and trace elements. Cell-free extracts were prepared as outlined above.

For electrophoresis, 60 µl of cell-free extract was loaded per well. Proteins were separated by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) using a 12 % resolving gel and 4 % stacking gel run in a Miniprotean® 3 Cell (BioRad, Hercules, CA). All steps were carried out at 4 °C at 200 V for 40 min. 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, Frederick, MD). Bands were scored as present/absent and strong/weak based on the graphical readouts produced by this reading method (not shown).

Alcohol dehydrogenase (ADH, NAD form, EC 1.1.1.1). All three study isolates were grown in a medium consisting of 43 mM ethanol, 5 mM NH_4NO_3 and trace elements at 26 °C for 7 d on a rotating shaker at 120 rev min^{-1}. As a positive control, *Saccharomyces cerevisiae*, in the form of commercial packaged yeast, was grown 7 d on Sabouraud glucose medium (in 1 l distilled water: 50 g glucose, 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, and 0.01 g $FeSO_4 \cdot 7H_2O$). A weighed quantity of yeast, mixed with a small quantity of fine perlite, was ground with a pestle in an ice-cold mortar containing 100 mM Tris–HCl extraction buffer, pH 7.5. The crude extract was filtered through filter paper (Whatman No.1) and maintained at 4 °C prior to use. The *Baudoinia* and *Saccharomyces cerevisiae* extracts were partially purified in an Amicon Ultra 4 centrifugal filter device (Millipore, Billerica, MA).

For electrophoresis, 60 µl of treated cell-free extract was loaded per well. ADH-NAD bands were determined using discontinuous native PAGE as described by Ornstein & Davis (1964). A 10 % resolving gel and 4 % stacking gel were run in a Miniprotean® 3 Cell. All steps were carried out at 4 °C at 200 V. Gels were run until the tracking dye was about 1 cm from the bottom. The gels were stained for ADH-NAD+ isozyme activity, according to Wendel & Weeden (1989). They were incubated in 100 ml of reaction mixture consisting of 50 mM Tris–HCl, pH 8, mixed with 1 mM ethanol (substrate),

1 mM NAD+, 0.7 mM phenazine methosulphate (PMS), and 0.37 mM nitroblue tetrazolium (NBT). Incubation was at 37 °C for 5 min in the dark with gentle rocking and held for and additional 5 min to develop the colour. The reaction was stopped by rinsing the gel with water.

Acetaldehyde dehydrogenase (ACDH, NAD form, EC 1.2.1.4). As above, the three *Baudoinia compniacensis* isolates were grown in a mixture consisting of 43 mM ethanol, 5 mM NH_4NO_3, and trace elements at 26 °C for 7 d on a rotating shaker at 120 rev min^{-1}. Mycelia were extracted in 100 mM potassium phosphate buffer at pH 7 containing 2 mM EDTA.

For electrophoresis, 60 µl of treated cell-free extract was loaded per well. ACDH-NAD bands were determined by discontinuous native PAGE as detailed in the previous section. All steps were carried out at 4 °C at 200 V for 50 min. The gels were stained for ACDH-NAD+ activity by incubating them in 100 ml of reaction mixture. The mixture consisted of 100 mM potassium phosphate buffer (pH 7.6) containing 70 mM acetaldehyde (substrate), 1 mM NAD+, 0.1 mM PMS, and 0.5 mM NBT. Staining was done overnight to develop the colour, as described by Jendrossek *et al.* (1987). The reaction was stopped by rinsing the gel with water.

Isocitrate dehydrogenase-NAD+ (IDH; EC.1.1.1.41) and IDH-NADP+ (EC.1.1.1.42)

The three *Baudoinia compniacensis* isolates were grown in two carbon source media, ML broth, as well as BPM with an ammonium acetate carbon and nitrogen source. Media were incubated at 26 °C for 7 d on a rotating shaker at 120 rev min^{-1}. The mycelium was extracted in a buffer consisting of 100 mM Tris–HCl and 2 mM EDTA buffer at pH 8.

For electrophoresis, 60 µl of treated cell-free extract was loaded per well. IDH-NAD and IDH-NADP bands were determined by using discontinuous native PAGE as described above. All steps were carried out at 4 °C at 200 V for 50 min. The gels were stained for IDH-NAD+ and IDH-NADP+ isozyme activities, according to Soltis *et al.* (1983), by incubating them in reaction mixture, consisting of: solution A: 50 mM Tris–HCl, pH 8 (15 ml) containing 200 mg agarose (used as an optical enhancer to increase the clarity of stained bands); solution B: 50 mM Tris–HCl, pH 8 (15 mL) containing, 35 mM $MgCl_2$, 1 mM NAD+ (alternatively, 0.4 mM NADP+ was used to test for the IDH-NADP+ isozyme), 25 mM Isocitrate-Na-salt, 0.8 mM NBT, and 0.4 mM PMS. Solution A was first boiled and cooled to 60 °C. Solution B was gently mixed in and the mixture was poured into a gel form so that it covered the existing gels. Once the agarose in the staining mixture had solidified, gels were incubated in dark at 30 °C until blue bands appeared.

Malate synthase (MS, EC 4.1.3.2) and Isocitrate lyase (ICL, EC 4.1.3.1). The three *Baudoinia compniacensis* isolates were grown on BPM with ammonium acetate as carbon and nitrogen source at 26 °C for 7 d on a rotating shaker at 120 rev min^{-1}. For MS enzyme assay, the mycelium was extracted in 50 mM Tris–HCl buffer, pH 7.5. For the ICL assay, the mycelium was extracted in 50 mM potassium phosphate buffer, pH 7. Both enzyme assays were conducted at 26–28 °C in a total volume of 2 ml.

MS activity was assayed by the method of Ono *et al.* (2003). The MS assay mixture contained 100 mM Tris–HCL (pH 8), 0.1 mM acetyl-CoA, 10 mM $MgCl_2$, 1 mM glyoxylate, and cell-free extract. The reaction was initiated by the addition of glyoxylate. The disappearance of acetyl-CoA was monitored with a Genova MK3 Life Science Analyser spectrophotometer (Jenway, Dunmow, Essex) at 232 nm.

ICL activity was again assayed by the method of Ono *et al.* (2003). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 30 mM D,L-isocitrate, 10 mM $MgCl_2$, 2 mM dithiothreitol, 4 mM phenylhydrazine and cell-free extract. The reaction was initiated by addition of isocitrate. The increase in absorbance resulting from the formation of the phenylhydrazone derivative of glyoxylate was assayed spectrophotometrically at 334 nm.

Protein determination

Cell-free extract was analysed for protein quantity, e.g. prior to gel loading, using the method described by Lowry *et al.* (1951).

Results

Ethanol growth

Different patterns of ethanol utilization were displayed by the three test *Baudoinia compniacensis* isolates (Fig 1). On one or more of the two ammonium-based nitrogen sources, isolates UAMH 10762, UAMH 10764, and UAMH 10839 demonstrated a growth peak with ethanol at a concentration of 43 mM. At that concentration, on medium with NH_4Cl, the total biomass yield per 50 ml medium was relatively high for UAMH 10762 and UAMH 10839 at 4 and 3.5 mg, respectively, and was distinctly lower for UAMH 10764 at 1.5 mg. Growth in all treatments diminished substantially above 170 mM ethanol (Fig 1). No growth was observed for all three isolates on 5 % ethanol with NH_4Cl.

Tolerance of elevated ethanol concentrations. UAMH 10762 demonstrated survival rates of 81, 65, and 36 %, respectively, after 6 h incubation at 520, 870, and 1700 mM ethanol (3, 5, and 10 %, respectively; Fig 2). Twenty percent ethanol was completely lethal at both time intervals. Six percent survival was observed at 2.4 M (14 %) ethanol for 3 h incubation, and no survival was observed after a 6 h incubation at 3.5 M (20 %) ethanol (data not shown).

Stimulation of cells by pulse exposure to low ethanol concentrations. Volatile organic compounds, such as ethanol, are known to act on fungi as triggers for germination (Fries 1973; Hase 1980; Zeuthen *et al.* 1988). Although this experiment was initiated using inoculum in exponential phase, the inoculum was mycelial in nature and in filamentous fungi, only apical cells of mycelium are actively growing, in the sense of extending to produce additional material. The scraping of colony surfaces to prepare inoculum is expected to have removed a mixture of apical and non-apical cells. The question addressed in the present experiment was whether relatively brief exposure to low ethanol concentrations might stimulate some otherwise partially or completely dormant cells, e.g. non-apical cells, to grow and produce colonies rapidly on subsequent incubation in ethanol-free medium. Isolate (UAMH 10762) was

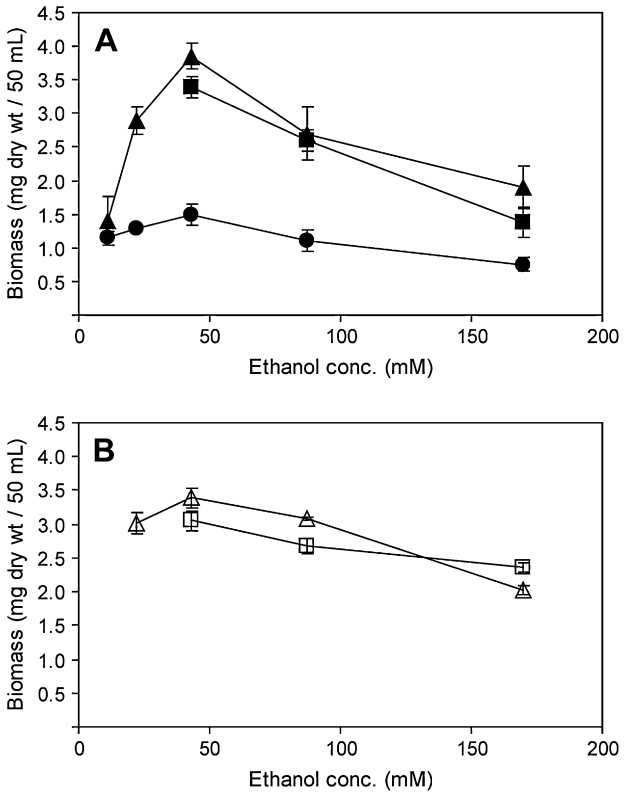

Fig 1 – Optimal concentration of ethanol in liquid growth medium at 7 d based on dry weight of isolates grown in different concentrations of ethanol (mM). (A) Treatments based on growth in medium with 5 mM NH_4Cl: UAMH 10762 (triangle), UAMH 10839 (square), and UAMH 10764 (circle). (B) Treatments based on growth in medium with 5 mM NH_4NO_3: UAMH 10762 (triangle), and UAMH 10839 (square). Values are mean of duplicates or triplicates. Error bars indicate average deviation.

used to determine the optimal ethanol concentration to induce outgrowth from mycelial fragments. Based on these assays we observed a trend towards increased colony formation following ethanol pretreatment at a concentration of 5 mM in aqueous solution for 18–24 h (Fig 3).

Effect of ethanol vapour on germination of dormant mycelial cells. Isolate UAMH 10762 was used to find the optimal ethanol vapour concentration triggering the germination of the typical dark, heavy-walled, chlamydospore-like cells composing mycelial fragments. Optimal germination was observed with 10 ppm of ethanol vapour (Fig 4). When 100 ppm of ethanol was used, germination was not stimulated but rather inhibited to 32 % lower than control levels (0-value on the ordinate of Fig 4).

Protein studies

Proteins elicited by growth on ethanol medium. In discontinuous SDS-PAGE gel electrophoresis, more than 28 protein bands were observed for untreated mycelium grown for 7 d on ML broth (Fig 5). Cells grown 7 d on 43 mM ethanol medium proved to have strongly different banding patterns for total protein than those seen for control cells.

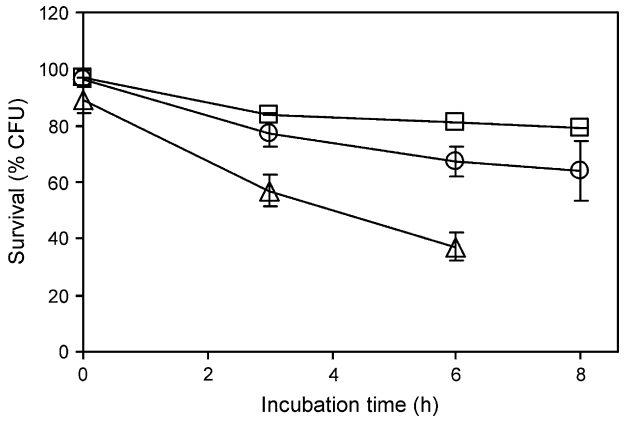

Fig 2 – Effect of ethanol on the viability of cells of isolate UAMH 10762 after transfer of exponentially growing cells to ML broth containing different concentrations of ethanol. Ethanol concentrations were 520, 870, and 1700 mM ethanol (3, 5, and 10 %, respectively). Values are mean of duplicates. Error bars indicate average deviation.

ADH. For each *Baudoinia compniacensis* isolate, one distinct band was identified on gel stained for ADH-NAD activity with ethanol used as substrate (Fig 6). The variation in ADH-NAD band intensity among the *B. compniacensis* isolates was influenced by the actual presence of ADH activity, as well as the concentration of cell-free extracts applied to the gel.

Various carbon sources were investigated for their influence on ADH activity (Fig 6). A distinct band for ADH-NAD dependent enzyme activity was observed for commercial yeast grown on glucose (in the constituent glucose of Sabouraud broth; data not shown), as well as for *Baudoinia* isolates grown on 43 mM ethanol with 5 mM NH_4NO_3. In contrast, no such band was detected for UAMH 10839 grown on glucose, in this case supplied as a component of ML broth (data not shown). Similarly, no corresponding band was seen for UAMH 10839 when grown in ML broth with 43 mM ethanol (data not shown).

ACDH. One band was identified for ACDH-NAD activity for the three test isolates grown on 43 mM ethanol with 5 mM NH_4NO_3 (Fig 6). Acetaldehyde-free control gels showed no bands.

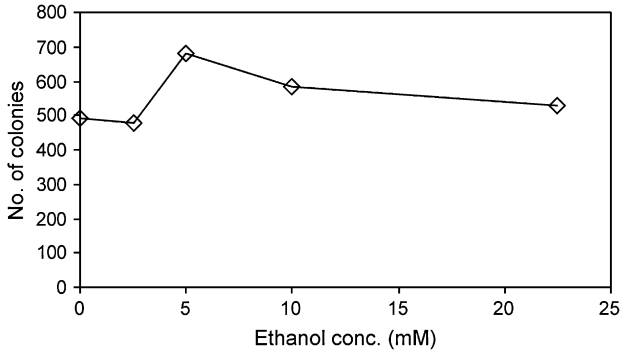

Fig 3 – The effects of pulse (20 h) exposure to ethanol on colony formation by UAMH 10762 on ethanol-free medium. Values are mean of duplicates.

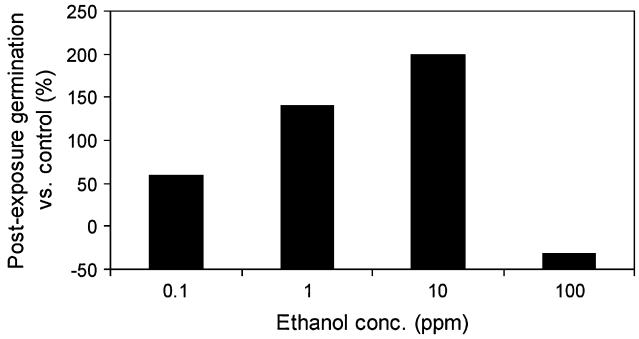

Fig 4 – Ethanol vapour stimulation or inhibition of colony formation by dormant inoculum of UAMH 10762. Zero on the ordinate was set by the germination level obtained under control conditions (no exposure to ethanol).

IDH, ICL, and MS. Isolates UAMH 10839 and UAMH 10762, showed an intense single band for both IDH-NAD (EC: 1.1.1.41) and IDH-NADP (EC: 1.1.1.42) when grown on ML broth inclusive of its glucose carbon source (Fig 6). Only a faint band was seen for both enzymes with UAMH 10764. The differences in band intensities seen in the figures reflect the amount of protein applied to the gel. Control gels in which isocitrate substrate was absent showed no activity bands. No isozyme activity corresponding to IDH-NAD or IDH-NADP was detected in the three *Baudoinia compniacensis* isolates grown in medium with acetate, a non-fermentable carbon source (Fig 6).

When ICL (EC: 4.1.3.1) and MS (EC: 4.1.3.2) activity levels were examined in the three *B. compniacensis* isolates grown on acetate medium as a carbon source (Table 1), moderately high activities were observed for both enzymes. ICL activity in UAMH 10839 was twice as high as in UAMH 10762 and four times as high as in UAMH 10764. Isolates UAMH 10839 and UAMH 10672 showed higher ICL activity than MS activity, but UAMH 10764 had ICL activity lower than its MS activity.

Discussion

Given the known association between *Baudoinia compniacensis* and habitats exposed to ethanol (Scott *et al.* 2007, Ewaze *et al.* 2007), it is perhaps surprising that 870 mM (5 %) ethanol completely suppressed the growth of this fungus. This tolerance level is well below that recorded for most alcohol-producing species of *Saccharomyces* (Casey & Ingledew 1986). We also observed that 2.4 M (14 %) ethanol reduced the viability of *Baudoinia* by 95 % in 3 h. This same reduction in viability of a strain *S. cerevisiae* required a significantly higher concentration of ethanol, 3.6 M (21 %) (Casey & Ingledew 1986). Therefore, we consider *Baudoinia* to be relatively intolerant of ethanol, in contrast to *S. cerevisiae*.

Isolate UAMH 10764 exhibited a lower growth rate than the other isolates when grown on ethanol. This observation may be related simply to a correspondingly lower rate of ADH II protein expression in this isolate, as the trend of the curve is the same for the three isolates. It is noteworthy that isolates UAMH 10762 and UAMH 10839 are genetically highly similar

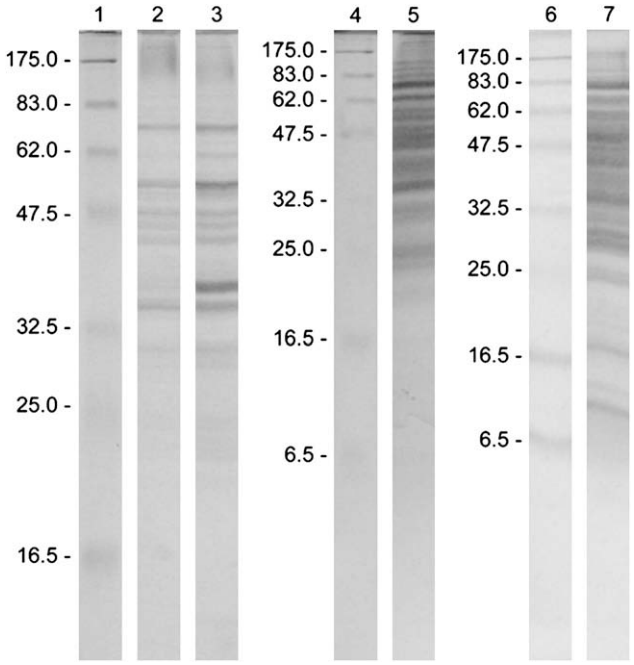

Fig 5 – Comparison between protein patterns of cell free extracts of UAMH 10762 and UAMH 10839 grown on two different media. Cellular proteins separated by discontinuous SDS-PAGE. Lanes 1, 4, and 6 are marker proteins with the molecular masses indicated in kDa. Lanes 2 (25 μg protein) and 3 (30 μg protein), mycelia of UAMH 10762 and UAMH 10839, respectively, grown for 7 d at 26 °C on 43 mM (0.25 %) ethanol plus 5 mM NH_4NO_3 and trace elements. Lanes 5 (22 μg protein) and 7 (30 μg protein) are total protein isolations from mycelia of UAMH 10762 and UAMH 10839, respectively, grown for 7 d at 26 °C on ML broth medium.

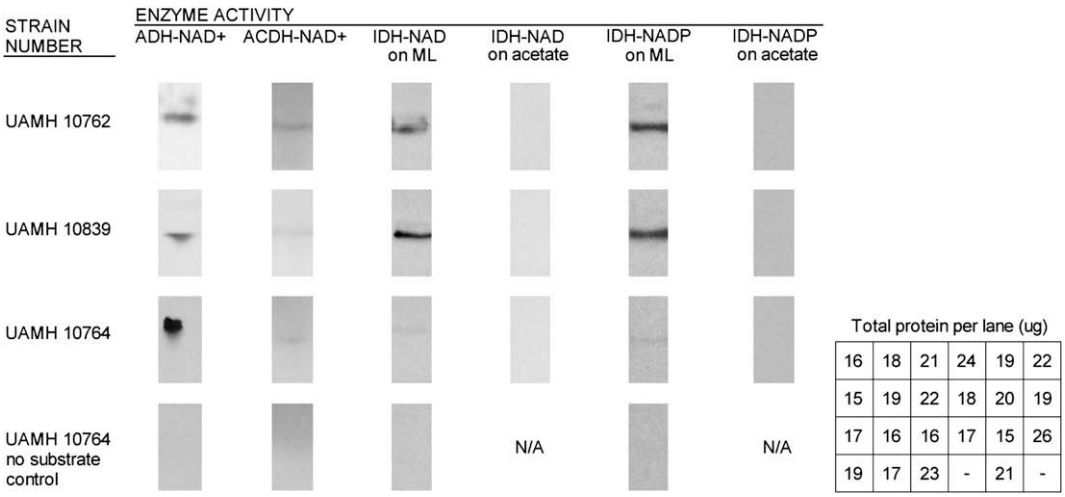

Fig 6 – Discontinuous native PAGE for the detection of ADH-NAD+, ACDH-NAD+, IDH-NAD, and IDH-NADP. Total protein loaded per lane is indicated in the corresponding cell of the legend at the lower right. Cells used to test ADH-NAD+, ACDH-NAD+ were cultivated on a medium containing 43 mM (0.25 %) ethanol and 5 mM NH_4NO_3. For ADH-NAD+, ACDH-NAD+ tests, a cell-free protein extract from *Saccharomyces cereviseae* grown on Sabouraud–glucose broth was used as a positive control (data not shown). Growth media used for the preparation of cell-free protein extracts for IDH-NAD and IDH-NADP testing are indicated above.

and were isolated from same area (Scott *et al.* 2007). Isolate UAMH 10764, although also highly similar, was obtained from a distant geographic locality.

The ethanol concentration most stimulatory to *Baudoinia* was 43 mM, while 20 h pulse exposure to a much lower concentration, 5 mM, proved optimal in inducing colony formation by mycelial fragments. When ethanol vapour was investigated, optimal germination of dormant *Baudoinia* mycelia was seen at a low level, 10 ppm. These results suggest that ethanol may play a stimulatory role in the growth of *Baudoinia* above and beyond its potential function as a source of metabolic carbon. Currently, it is unclear whether growth of *Baudoinia* in the vicinity of distillery warehouses could be diminished in direct proportion to the reduction of ethanol emission levels from the warehouses, or if even minute levels of ethanol emission would be sufficient to trigger the observed levels of growth of this fungus. If ethanol functioned mainly as a carbon source, any reduction in emission should lead to a corresponding reduction in the growth of *Baudoinia*. However, if low levels of ethanol triggered colony establishment even where ethanol levels were insufficient to provide all the carbon needed for growth, then only the reduction of ethanol to very low levels would be likely to reduce the environmental proliferation of *Baudoinia*.

Table 1 – Enzyme activities of isocitrate lyase and malate synthase in cell-free extracts of UAMH 10762, UAMH 10839, and UAMH 10764, cultivated in a medium consisting of 5 mM ammonium acetate plus trace elements

Isolate	Enzyme-specific activity (micromoles of product produced per min per milligram of protein)	
	Isocitrate lyase	Malate synthase
UAMH 10762	0.096	0.075
UAMH 10839	0.181	0.104
UAMH 10764	0.042	0.061

The results obtained on optimal ethanol levels for germination and growth of *Baudoinia* can be used in practical work. For example, preliminary studies suggest that the amendment of growth medium with 5 ppm ethanol greatly increases the success of isolation from environmental materials (Scott *et al.* 2007).

Although ethanol can function as a factor modifying the properties of the cell membrane and influence growth that way (Mishra & Prasad 1989; Ceccato-Antonini & Sudbery 2004), positive growth responses usually indicate usage as a respiratory substrate, perhaps *via* activation of ADH. The normal fungal pathway for ethanol metabolism is *via* the ADH enzyme ADH II. Ethanol tends to integrate into common metabolic pathways *via* the intermediates acetate and acetaldehyde (Russell *et al.* 1983). In *S. cerevisiae*, ADH II preferentially catalyses the conversion of ethanol to acetaldehyde, which then can be metabolized *via* the tricarboxylic acid (TCA) cycle and also serve as an intermediate for gluconeogenesis (Russell *et al.* 1983). Our finding that ethanol-grown *Baudoinia* forms an ADH II isozyme suggests that our fungus can also oxidize ethanol to acetate. The formation of ADH II by *Baudoinia* is not stimulated by growth on medium containing glucose, whether or not ethanol is added. In *S. cerevisiae* also, ADH II is known to be strongly repressed by the presence of simple, fermentable sugars such as glucose (Denis *et al.* 1981). Our data also showed the presence of ACDH in *Baudoinia*, suggesting that NAD-dependant ACDH reduces acetaldehyde to carboxylic acid when the fungus is growing on ethanol.

Kornberg & Krebs (1957) first recognized the ability of some microorganisms to grown efficiently on two-carbon

compounds using the glyoxylate pathway. The key enzymes in this pathway are ICL and MS (Kornberg & Elsden 1961). Our earlier work indicated that the normal TCA cycle functions in *Baudoinia* when glucose is metabolized (Ewaze *et al.* 2007). The present study showed that the TCA cycle enzyme IDH was active when *Baudoinia* was grown on glucose medium; it was detected in two forms, IDH-NAD+ and IDH-NADP+. Neither form of the enzyme, however, was found in extracts of acetate-grown mycelium, suggesting that the glyoxylate pathway was being used in conjunction with this two-carbon substrate. The switching on and off of IDH involves reversible phosphorylation mediated by a bifunctional enzyme, IDH kinase/phosphatase. When IDH is phosphorylated, isocitrate is diverted from the normal TCA cycle to the glyoxylate cycle.

The activities of the glyoxylate cycle enzymes ICL and MS in acetate-grown *Baudoinia* were comparable with those seen in various other microorganisms (McFadden & Howes 1962; Neumann *et al.* 1969). These results indicate that the glyoxylate cycle probably plays a crucial role in replenishing TCA intermediates, such as gluconeogenesis precursors, when *Baudoinia* is grown on acetate or ethanol.

The glyoxylate cycle enzymes may be important in functions other than basic metabolism on two-carbon substrates. They have been reported to contribute to protein synthesis in nitrogen-fixing bacteria (Green *et al.* 1998), riboflavin biosynthesis in *Ashbya gossypii* (Schmidt *et al.* 1996), and oxalate biosynthesis in a plant pathogen, *Athelia rolfsii* (Maxwell & Bateman 1968). *Baudoinia compniacensis* needs to be investigated to determine the overall contribution of glyoxylate cycle enzymes to its metabolism.

It is likely that the glyoxylate cycle of *Baudoinia*, conferring the ability to utilize ethanol, is a critical factor involved in the abundant growth of this organism in the nutrient-deprived conditions found on exposed surfaces around distillery warehouses and bakeries. Whether ethanol serves as a major carbon source under these conditions or only as a signal stimulating growth, it is likely that the glyoxylate cycle is involved: the level of simple sugars in the environment is unlikely to be high enough to switch it off.

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