

Osmotic shock tolerance and membrane fluidity of cold-adapted *Cryptococcus flavescens* OH 182.9, previously reported as *C. nodaensis*, a biocontrol agent of *Fusarium* head blight

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Received 10 August 2006; revised 3 November 2006; accepted 3 November 2006.
First published online 18 January 2007.

DOI:10.1111/j.1567-1364.2006.00193.x

Editor: Cletus Kurtzman

Keywords

Cryptococcus; osmotic; cold; membrane; drying.

Abstract

Cryptococcus flavescens (previously reported as *C. nodaensis*), a biological control agent of *Fusarium* head blight, has been previously shown to have improved desiccation tolerance after cold adaptation. The goal of the current study was to determine the effect of cold adaptation on the physicochemical properties of *C. flavescens* that may be responsible for its improved desiccation tolerance. The results show that cold adaptation improves liquid hyperosmotic shock tolerance and alters the temperature dependence of osmotic shock tolerance. Fluorescence anisotropy was used to characterize differences in the membrane fluidity of *C. flavescens* with and without cold adaptation. Force curves from atomic force microscopy showed a significant increase in the cell wall spring constant after cold adaptation. Cold adaptation of *C. flavescens* during culturing was shown to produce smaller cells and produced a trend towards higher CFU yields. These results suggest that cold adaptation significantly alters the membrane properties of *C. flavescens* and may be an effective method of improving the desiccation tolerance of microorganisms. In addition, we provide information on the correct naming of the isolate as *C. flavescens*.

Introduction

Cryptococcus flavescens OH 182.9 (NRRL Y-30216), which has been reported previously as *C. nodaensis*, has been shown to be effective in reducing *Fusarium* head blight (FHB) in greenhouse and field studies, as have *Cryptococcus* strains OH 71.4 (NRRL Y-30213) and OH 181.1 (NRRL Y-30215) (Schisler *et al.*, 2002; Khan *et al.*, 2004). FHB is a fungal disease that affects wheat, barley and other grain crops. The economic impact of FHB was estimated at \$2.7 billion for the USA during 1998–2000 (Nganje *et al.*, 2004). The potential to reduce the incidence of FHB has made *C. flavescens* an attractive candidate as a microbial biological control agent. In an effort to transform *C. flavescens* into a commercially viable FHB biological control option, studies in our laboratory have been performed to optimize its production and bioefficacy (Zhang *et al.*, 2005, 2006). Additional research is still needed to develop cost-effective methods of drying and stabilizing *C. flavescens* to produce a product with a suitable shelf-life.

Drying and stabilizing microbial biological control agents presents a challenging problem. Drying and subsequent rehydration subjects the microbe to high osmotic pressure gradients that can damage or kill the cells. Understanding how microbial cells withstand these pressures should lead to better methods for drying and rehydrating them. Recent studies with *Saccharomyces cerevisiae* have examined the effect of osmotic shocks on the viability of the cells (Beney *et al.*, 2001; Laroche *et al.*, 2001, 2005). These studies on microbial osmotic shock tolerance proposed membrane fluidity to be an important determinant in cell survival (Beney & Gervais, 2001; Laroche *et al.*, 2001; Beney *et al.*, 2004). Cell death has been attributed to membrane damage, as a result of the osmotic forces acting on the cell. Membrane phase transition temperature has also been proposed to relate changes in membrane fluidity with cell viability (Laroche & Gervais, 2003). Osmotic shocking of cells in the temperature region of the membrane phase transition resulted in greater cell death. These concepts have been recently extended to apply liquid osmotic shock data to the

prediction of cell survival in convective drying processes (Mille *et al.*, 2004, 2005). Although these methods appear to be useful tools with which to optimize microbial drying processes, additional research is needed to confirm their applicability to different types of microorganisms.

Our laboratory has recently shown that *C. flavescens* becomes more desiccation tolerant after cold adaptation at 15 °C for 20 h (Zhang *et al.*, 2006). The physiologic nature of this improved stress tolerance remains unclear. An objective of the current study was to evaluate the osmotic shock tolerance of *C. flavescens* with and without cold adaptation. An additional objective was to compare the membrane fluidity of *C. flavescens* cells with and without cold adaptation through fluorescence anisotropy experiments and to compare the rigidity of cell surfaces using atomic force microscopy (AFM) to detect changes in membrane properties. The objective was to characterize physiologic differences between the two cell preparations and to determine what implications any observed differences might have for known differences in desiccation tolerance. In addition, this biocontrol isolate and related *Cryptococcus* strains OH 71.4 and OH 181.1 were reidentified by sequence analysis of the D1/D2 domains of the 26S rRNA gene and the internal transcribed spacer (ITS) 1+2 spacer regions of the rRNA gene.

Materials and methods

Molecular identification of the isolates

The biocontrol isolates OH 182.9 (NRRL Y-30216), previously reported as *C. nodaensis*, and OH 71.4 (NRRL Y-30213) and OH 181.1 (NRRL Y-30215), previously identified as *Cryptococcus* spp., were reidentified by sequence analysis of the D1/D2 domains of the 26S rRNA gene and the ITS 1+2 regions of the rRNA gene using standard methods (Okoli *et al.*, 2006). The phylogenetic tree was constructed using neighbor joining with the uncorrected ('p') substitution model, alignment gaps were treated as missing characters, and all characters were unordered and of equal weight. Bootstrap values were based on 100 replicates.

Biomass production

Cryptococcus flavescens (= *C. nodaensis*, nomen nudem) OH 182.9 (NRRL Y-30216) was produced in a B Braun Biostat B fermenter (B. Braun Biotech Inc., Allentown, PA) charged with 1 L of semi-defined complete liquid (SDCL) medium (Slininger *et al.*, 2003). Antifoam 204 (Sigma, St Louis, MO) was added at 0.125 mL L⁻¹ before medium sterilization. No further antifoam was added, and cultures were not pH controlled after inoculation of the pH 7.0 medium. To initiate a production run, cells from a log-growth stage SDCL culture served as a 5% seed inoculum. Fermenters

were operated at 25 °C, with an air flow rate of 1500 mL min⁻¹ and a 200 r.p.m. agitation rate. Eighteen hours after inoculation, the temperature was reduced to 15 °C to cold adapt the cells for 24 h before harvest. No temperature adjustments were made to cultures that served as controls. After completion of biomass production at *c.* 42 h, a pH of 5.40 had been reached for cultures produced using either temperature condition, and dissolved oxygen had reached saturation. Harvested culture broths from either temperature production regime had ODs at 620 nm that ranged from 17 to 20. Colonized reactor broth was harvested and concentrated into a pellet through centrifugation at 8000 g for 10 min. The centrifugation was performed at the temperature at which the cells were grown (15 °C or 25 °C). The cell pellet was washed and resuspended twice with an aqueous solution containing 0.51% (w/w) glycerol. The washed cell pellet was resuspended in 0.51% (w/w) glycerol solution to yield a stock suspension containing ~10⁹ cells mL⁻¹. Each experiment represents a separate fermentation with replicates from the same production run.

Preparation of osmotic shock solutions

Aqueous solutions with varying amounts of glycerol were prepared to produce solutions with different water activities. The Norrish equation (Norrish, 1966) was used to estimate the amount of glycerol needed to produce a given water activity. The actual water activity of each solution was determined using an Aqualab water activity meter (Model Series 3, Pullman, WA). Glycerol solutions were produced with water activities of 0.993, 0.975, 0.881, 0.780, 0.648, 0.503, and 0.312. The solutions contained 0.51–90.0% (w/w) glycerol with a pH between 4.8 and 5.6.

Osmotic shock testing

Cryptococcus flavescens stock suspensions and osmotic shock solutions were equilibrated for 10 min in a water bath at the desired temperature. Osmotic shock was applied by rapidly injecting 50 µL of *C. flavescens* stock suspension into 950 µL of the osmotic shock solutions. Three minutes after the osmotic shock was applied, a 100-µL aliquot of the suspension was diluted into 900 µL of 0.51% glycerol solution. Viability of the cells was determined through serial dilutions in 0.03% phosphate buffer with plating on 1/5 tryptic soy agar (TSA) plates. Plates were incubated at 25 °C for 2 days until colony counting. The isothermal osmotic shock experiment was conducted at 25 °C. The temperature dependence experiments tested temperatures from 6 °C, increasing at intervals of 3.2 °C up to 34.8 °C. For each temperature, all concentrations of glycerol were tested. CFU mL⁻¹ data were converted to logarithmic values or viability percentages for error analysis. Each experiment was repeated a minimum of five times, with at least three

individual replicates per experiment. Each experiment represents a separate fermentation, with replicates from the same production run. The means were analyzed with a paired *t*-test ($P < 0.05$).

Scanning electron microscopy (SEM)

Stock suspensions of cells of *C. flaves-cens* (10^9 CFU mL⁻¹) that were prepared from cold-adapted or control cells were diluted to a concentration of 10^8 CFU mL⁻¹ with 0.51% glycerol solution. Five milliliters of this suspension was deposited by vacuum filtration on a 47-mm, 0.8- μ m, mixed cellulose esters filter membrane (Millipore Corp., Bedford, MA). Fixation and dehydration of the microbial lawn was performed sequentially as follows: submersion in 3% glutaraldehyde in 0.1 M sodium phosphate for 60 min, 0.1 M sodium phosphate for 5 min, 2% osmium tetroxide in 0.1 M sodium phosphate for 45 min, and 0.1 M sodium phosphate for 5 min. The sample was incubated with increasing concentrations of ethanol (15, 30, 50, 70, 90, and 100%) for 15 min each, and then subjected to critical point drying, sputter coated, and analyzed by SEM (JSM-6400 V, JEOL Inc. Japan).

Particle size experiments

Cold-adapted and control suspensions of cells of *C. flaves-cens* (10^9 CFU mL⁻¹) were diluted to a concentration of 10^6 CFU mL⁻¹ with distilled water. Particle size measurements were made at 23 °C with a dynamic light-scattering system, Nicomp 380 (Particle Sizing Systems, Santa Barbara, CA). The data were analyzed using the manufacturer's NICOMP algorithm to derive a volume-weighted average particle diameter (d_v). The measurements of cold-adapted and control cell suspensions were replicated using three separate cultures. The means were compared with a paired *t*-test ($P \leq 0.05$).

Fluorescence anisotropy experiments

Fluorescence monitoring of microbial membrane fluidity using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) has been described previously (Laroche *et al.*, 2001). For this work, a similar method was used. DPH was purchased from Molecular Probes Inc. (Eugene, OR) and kept protected from light in a desiccator until needed. A stock solution of DPH was made by adding the fluorescent probe to an amber vial and adding tetrahydrofuran (Sigma Chemicals, St Louis, MO) to produce a final concentration of 7.025 mg mL⁻¹. This stock DPH solution was kept frozen under argon until needed. Cells of *C. flaves-cens* were prepared for fluorescence measurements by diluting the cells to an OD of 1.5–2.5 in the 0.51% glycerol solution (absorbance was measured at 600 nm on a Himadzu spectrophotometer; Edison, NJ). DPH, after equilibrating to

ambient temperature, was diluted to a final concentration of 10 μ M in the diluted *C. flaves-cens* cells, protected from light, and vortexed periodically for 30 min. Incorporation into the membrane was confirmed by measuring the emission spectrum of DPH with an excitation wavelength of 340 nm (DPH free in solution has little or no fluorescence intensity). Aliquots of the sample were then placed into quartz cuvettes held in place by the four-position thermostatted holder of a Jobin Yvon-Horiba Fluorolog 3.21 fluorometer (Edison, NJ). The samples were allowed to equilibrate for 10 min per temperature over the range 6–35 °C. Temperature was maintained using a Thermo NESLAB RTE-7 refrigerated water bath/circulator (Portsmouth, NH). Dried N₂ was passed into the sample chamber to reduce any scattering due to condensation. Excitation and emission were measured at 340 and 432 nm, respectively, with slit widths of 5 nm and a KV-399 filter in the emission pathway. Fluorescence anisotropy was calculated according to Lakowicz (1999). All fluorescence measurements were background corrected by subtracting light intensities of unlabeled cells from that of the DPH-labeled cells. Each experiment was repeated a minimum of five times, with at least three individual replicates per experiment. Each experiment represents a separate fermentation, with replicates from the same production run.

Atomic Force Microscopy experiments

The use of AFM to explore the mechanical properties of microbial cells is well documented; for reviews, see Gad *et al.* (1997), Afrin *et al.* (2004), Kriznik *et al.* (2005), and Zhao *et al.* (2005). Samples were prepared using a method reported by Zhao *et al.* (2005). To prepare for AFM measurements, round glass coverslips 12 mm in diameter and 0.13–0.16 mm thick (Fisher Scientific, Pittsburgh, PA) were glued to metal pucks using double-sided sticking tape and allowed to set for at least 24 h. Just before use, the glass slips were put through a 10-min cycle in an UV – ozone cleaning system, rinsed with distilled water, and allowed to dry at ambient temperature. Poly-L-lysine 0.1% (w/v) solution (Sigma Chemicals, St Louis, MO) was then pipetted (40 L) onto the coverslip, which was allowed to dry in ambient conditions, rinsed with distilled water, and allowed to dry again. The yeast-prepared glass was then mounted in place, and an aliquot of the appropriate buffer was added. For cold-adapted cells, the cells and buffer were maintained on ice until used. The atomic force microscope was an MFP 3D stand-alone model from Asylum Research (Santa Barbara, CA) with a scan range ≥ 20 μ m. Cantilevers (calibrated by taking force curves on the bare glass coverslip and using the built-in thermal tuning) were of the V-shaped silicon nitride type with a measured spring constant of 0.227 ± 0.016 N m⁻¹ (Cleveland *et al.*, 1993). Scans were completed in contact

mode (using a minimum of force), where the cells were first located using a z -scan size of 20 μm . Force curves were then obtained by isolating a cell and approaching the cell surface after targeting it with the software. At least 10 replicate measurements were made for each sample.

Results

Molecular identification of the biocontrol isolates

Sequence analysis of the D1/D2 domains and the ITS 1+2 regions of the nuclear rRNA gene showed that the three biocontrol isolates belonged to at least two species. Isolate OH 182.9 (NRRL Y-30216) showed 99% and 100% sequence similarity with the D1/D2 and ITS 1+2 sequences of the type strain of *C. flavescens* CBS 942, respectively, but also showed similarity in these domains with the rRNA gene of an authentic isolate CBS 101036 of *C. nodaensis*. These relationships are indicated in Figs 1 and 2, and it can be seen that NRRL Y-30216 clusters in the *C. flavescens* lineage with high statistical support.

Isolates OH 71.4 (NRRL Y-30213) and OH 181.1 (NRRL Y-30215) belong to the *C. aureus* cluster (Figs 1 and 2).

Based on high sequence similarity values, the biocontrol strain NRRL Y-30213 may be regarded as conspecific with *C. aureus*, with 98.5% similarity for the D1/D2 and ITS sequences, respectively, of the type strain of that species, CBS 318. Strain NRRL-30215 is closely related to the type strain of *C. aureus*, with 98% sequence similarity in the D1/D2 domains, but the ITS 1+2 regions seem somewhat more divergent between these strains, with only 96.4% similarity. Both isolates NRRL Y-30213 and 30215 are also closely related to isolate SJ 196, which has been isolated recently from environmental sources at the San Juan Islands (WA) (Fraser *et al.*, 2006) with highly similar D1/D2 sequences.

Isothermal osmotic shock tolerance of *C. flavescens*

The remainder of this article is limited to the analysis of *C. flavescens* strain NRRL Y-30216. The viability of *C. flavescens* cells was determined after hyperosmotic shocks of varying strength. The results are shown in Fig. 3. Viability was shown to decrease with increasing severity of the osmotic shock for both preparations. The results show the cold-adapted preparation of *C. flavescens* to be more tolerant to hyperosmotic shock. The control sample showed a

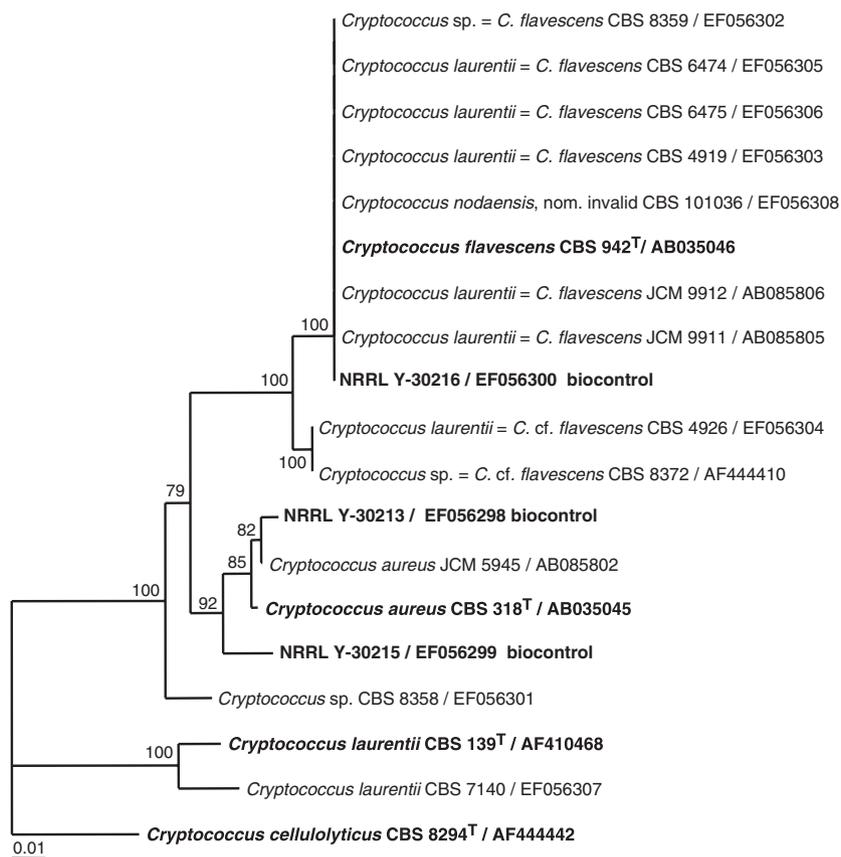
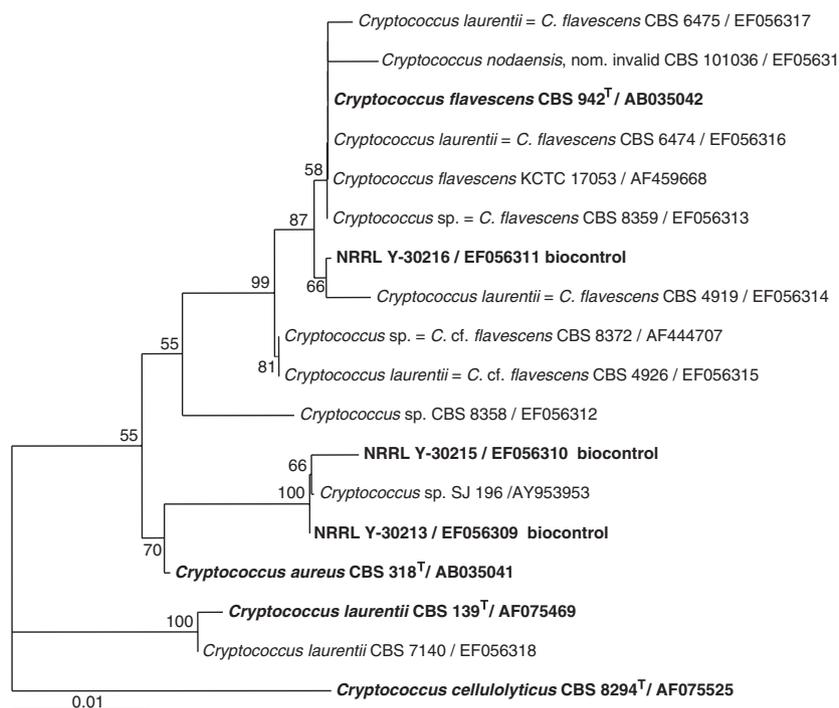


Fig. 1. Phylogenetic tree constructed with neighbor joining based on sequence analysis of the D1/D2 domains of the 26S rRNA gene, indicating the position of isolates OH 71.4 (NRRL Y-30213), OH 181.1 (NRRL Y-30215) and OH 182.9 (NRRL Y-30216) in relation to *Cryptococcus flavescens*, *Cryptococcus aureus* and *Cryptococcus laurentii* indicates that the identity of strain has to be changed; the respective type strains and the three biocontrol strains are indicated in bold; bootstrap values based on 100 replicates are indicated on the branches. *Cryptococcus cellulolyticus* is used as an outgroup. CBS, CBS Fungal Biodiversity Centre; NRRL, Agricultural Research Service Culture Collection; KCTC, Korean Collection for Type Cultures.

Fig. 2. Phylogenetic tree constructed with neighbor joining based on sequence analysis of the ITS 1+2 regions of the rRNA gene, indicating the position of isolates OH 71.4 (NRRL Y-30213), OH 181.1 (NRRL Y-30215) and OH 182.9 (NRRL Y-30216) in relation to *Cryptococcus flavescens*, *Cryptococcus aureus* and *Cryptococcus laurentii* indicates that the identity of strain has to be changed; the respective type strains and the three biocontrol strains are indicated in bold; bootstrap values based on 100 replicates are indicated on the branches. *Cryptococcus cellulolyticus* is used as an outgroup. Strain SJ 196 is a strain with no link to an official collection number; it is from Duke University: (-Fraser *et al.*, 2006). CBS, CBS Fungal Biodiversity Centre; NRRL, Agricultural Research Service Culture Collection; KCTC, Korean Collection for Type Cultures.



statistically significant loss in viability with an osmotic shock at a water activity of 0.65, compared to the same sample with no osmotic shock ($a_w=0.99$). The cold-adapted sample showed a statistically significant loss in viability with an osmotic shock at a water activity of 0.50, compared to the same sample with no osmotic shock ($a_w=0.99$).

Temperature dependence of osmotic shock tolerance

The temperature dependence of osmotic shock tolerance was determined at a constant water activity ($a_w=0.53$). The control preparation (Fig. 4a) showed considerable variation in its osmotic shock tolerance with respect to temperature. The cold-adapted preparation (Fig. 4b) showed less variation and a trend towards decreased osmotic tolerance with increasing temperature. These differences in the temperature dependence of osmotic shock tolerance suggest that cold adaptation induces physiologic changes in *C. flavescens* that alter the osmotic shock tolerance of the cells.

Fluorescence anisotropy experiments

To determine the effect of cold-shocking on the membrane properties of *C. flavescens*, fluorescence anisotropy was used to probe the membrane fluidity of control and cold-adapted cells. The fluorescent probe DPH was used to monitor membrane fluidity over the temperature range 6–35 °C and with a water activity of 0.993. The results are shown in Fig. 3. The control cells (Fig. 5a) exhibited a steady decrease in

anisotropy with increasing temperature. The change in anisotropy for the control cells was 0.17 over this temperature range. The cold-adapted cells (Fig. 5b) exhibited much less change over this temperature range, 0.05. The fluorescence anisotropy of the cold-adapted cells showed a variation in signal intensity that could be interpreted in terms of a lipid phase transition. This could represent a phase transition of the fluid–crystalline lamellar phase into the gel lamellar phase (Beney & Gervais, 2001). Additional data points are needed before the change in anisotropy at 15–21 °C can be definitively stated to represent a transition in membrane lipids. The control cells showed no evidence of a distinct phase transition over this temperature range.

Effect of cold adaptation on yield and cell volume

To determine the effect of cold adaptation on the yield of cells, the yields (CFU mL⁻¹) from replicate fermentations were examined for both control and cold-adapted cells. The results show a trend towards higher CFU mL⁻¹ for the cold-adapted cells. The average yield for control cells was $(5.2 \pm 3.4) \times 10^8$ CFU mL⁻¹. The cold-adapted cells had an average yield of $(1.1 \pm 0.9) \times 10^9$ CFU mL⁻¹. With this number of replicates ($n=6$), the means are not significantly different.

The effect of cold adaptation on the morphology of *C. flavescens* was qualitatively evaluated using SEM. Control and cold-adapted cells were collected on separate membrane filters and analyzed. Representative micrographs are

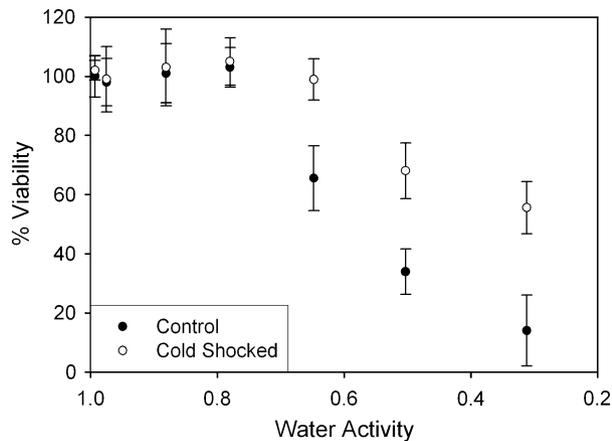


Fig. 3. Isothermal osmotic shock tolerance of *Cryptococcus flavescens* cell preparations at 25 °C. Error bars are reported at ± 1 SD.

presented in Fig. 6. Both samples showed a large distribution of cell sizes. The control samples appeared to have a greater preponderance of the larger cell sizes than the cold-adapted sample. All samples also showed signs of excreted mucilage, evident in the micrographs.

The size distribution of the *C. flavescens* preparations was further characterized with dynamic light scattering. The volume-weighted average diameter for control cells was 1540 ± 154 nm, whereas that for the cold-adapted cells was 1232 ± 125 nm. These values are based on the assumption that the cells have a spherical shape. The difference in the mean cell diameters was statistically significant ($P \leq 0.05$). The total cell volume yield was not significantly different between the two preparations. These results, in combination with the SEM and CFU results, suggest that cold-adapted cells are smaller and produce a higher CFU mL⁻¹ value without a change in biomass yield.

AFM experiments

The approach used here was to explore the mechanical properties of *C. flavescens* cells with and without cold adaptation. The goal was to ascertain any difference in cell stiffness that may result from cold adaptation. The slope of the AFM force curve, s , can be related to the cell's wall spring constant k_w with the equation $k_w = ks/(1-s)$, where k is the cantilever's spring constant (Boulbitch, 2000). A summary of cell wall spring constants is given in Table 1. The results show that the cold-adapted cells are almost five times stiffer than the control cells.

Discussion

Identification of biocontrol isolates

The biocontrol strain OH 182.9 (NRRL Y-30216) used in this study has been referred to as *C. nodaensis* in various

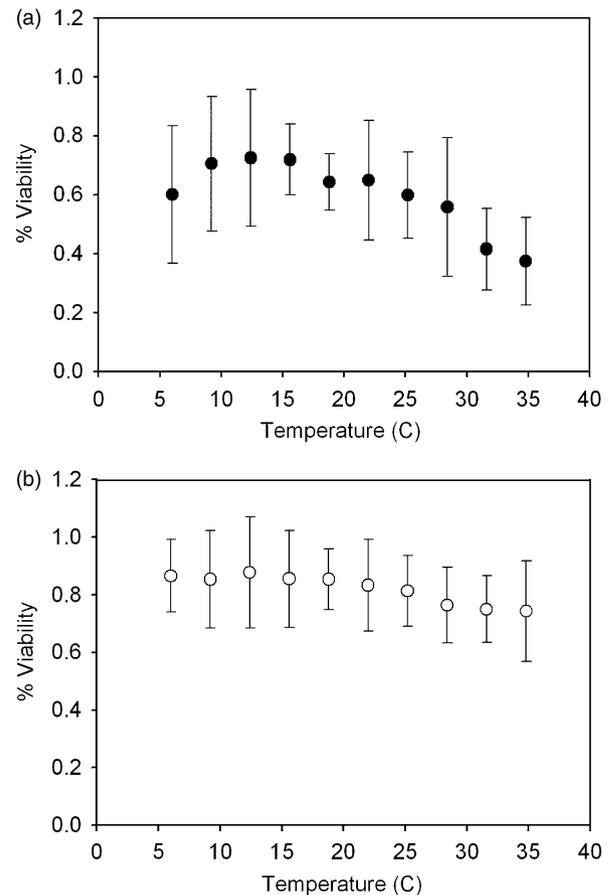


Fig. 4. Temperature dependence of *Cryptococcus flavescens* osmotic shock tolerance at constant water activity. (a) Control: *Cryptococcus flavescens* cultured at 25 °C for 48 h. (b) A cold-adapted sample cultured at 25 °C for 24 h and then at 15 °C for the final 24 h. The osmotic shock is from $a_w = 0.99$ to $a_w = 0.53$. Error bars are reported at ± 1 SD.

scientific reports (Schisler *et al.*, 2002; Khan *et al.*, 2004). It became clear, however, that *C. nodaensis* has never been validly described (Sato *et al.*, 1999), as a Latin diagnosis was missing and no type specimen was indicated. Hence, this species name has to be considered as a nomen nudum. This makes it important to determine the correct name for the biocontrol isolates OH 71.4 (NRRL Y-30213), OH 181.1 (Y-30215) and OH 182.9 (NRRL Y-30216), which have been investigated in this and other studies. Recent advances in the taxonomy of basidiomycetous yeasts have made it clear that comparative sequence analysis of parts of the rRNA gene is the most reliable tool for strain identification in this group of organisms (Fell *et al.*, 2000; Scorzetti *et al.*, 2002). In a recent re-evaluation of isolates previously considered to be synonyms of *C. laurentii*, Takashima *et al.* noted that *C. aureus*, *C. flavescens* and *C. penaeus* all have to be considered as individual species (Takashima *et al.*, 2003). Therefore, we analyzed the nucleotide sequences of the D1/D2 domain of the 26S rRNA gene and the ITS 1+2 sequences of the rRNA

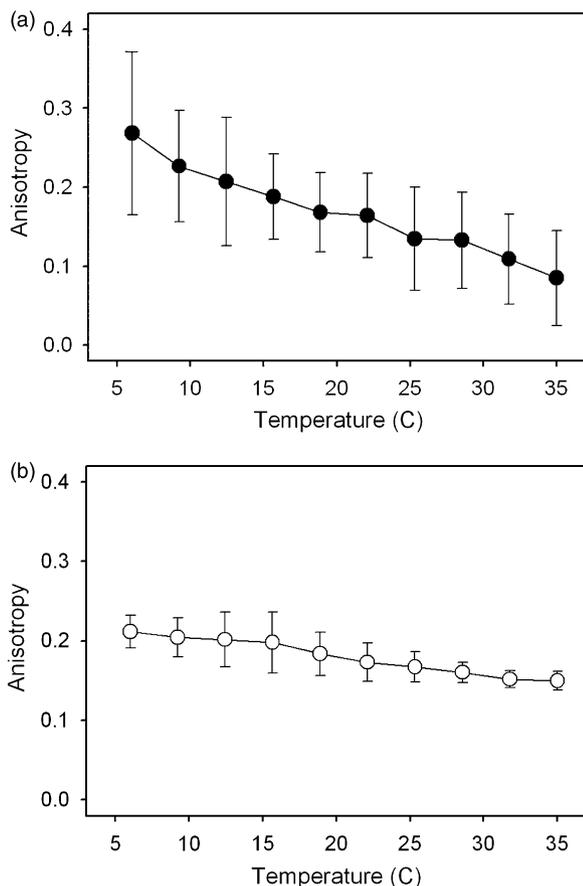


Fig. 5. Membrane fluidity of *Cryptococcus flavescens* preparations. The temperature dependence of fluorescence anisotropy as measured at constant water activity ($a_w = 0.99$). (a) Control: *Cryptococcus nodaensis* cultured at 25 °C for 48 h. (b) A cold-adapted sample cultured at 25 °C for 24 h and then at 15 °C for the final 24 h. Error bars are reported at ± 1 SD.

gene of strains NRRL Y-30213, Y-30215, and Y-30216. The results showed that strain NRRL Y-30216, which is further studied in this article, is identical to *C. flavescens*, as it showed high sequence identity with the type strain of that species. A number of other isolates, previously identified by physiologic and morphologic means as *C. laurentii*, were also found to be *C. flavescens*, e.g. CBS 4119, CBS 6474, CBS 6475, and CBS 6476. CBS 8359, isolated in Brazil, also belongs to this species. *Cryptococcus flavescens* is known from the surface of tropical plants in Indonesia, plant surfaces from the USA (i.e. wheat, and a maize plant from Illinois), and Brazil. Interestingly, sexually compatible isolates of *C. laurentii* (Kurtzman, 1973) also belong to *C. flavescens*. This most likely indicates that *C. flavescens* has a sexual stage; however, this needs further study.

The other two biocontrol strains, NRRL Y-30213 and Y-30215, do belong to the *C. aureus* complex, with strain NRRL Y-30213 being identical to this species. Strain NRRL Y-30215,

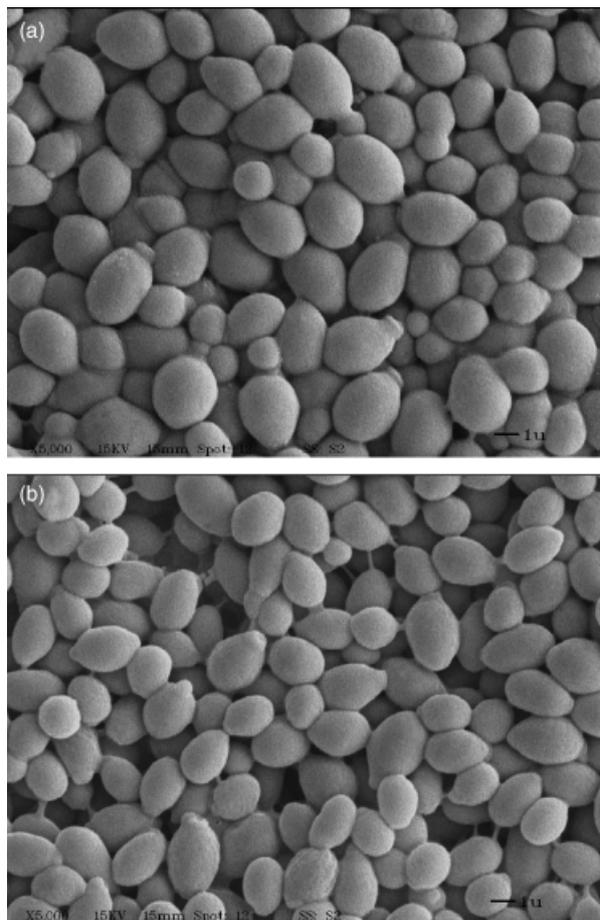


Fig. 6. Scanning electron micrographs of *Cryptococcus flavescens*. (a) A layer of control cells. (b) A layer of cold-adapted cells. Both micrographs were recorded with $\times 5000$ magnification.

Table 1. Summary of wall spring constants of *Cryptococcus flavescens*

Sample	Wall spring constant ^a (N/m)
Control	0.016 \pm 0.001
Cold-adapted	0.075 \pm 0.006

^aSpring constants are reported as the mean of individual measurements. Error reported at ± 1 SD. The means are statistically different ($P < 0.05$).

however, showed only 96.4% sequence similarity with the ITS sequences of the type strain CBS318 of *C. aureus*, indicating that *C. aureus* may contain infraspecific taxa or may represent a species complex comprising various subspecies. Interestingly, on the basis of D1/D2 analysis, an isolate obtained from environmental sources at the San Juan Islands (WA) (Fraser *et al.*, 2006) is closely related to isolate NRRL Y-30213 (Fig. 1).

In the current study, we assessed the effect of cold adaptation on the osmotic shock tolerance of *C. flavescens*, NRRL Y-30216. The results indicate that cold adaptation

increases the osmotic shock tolerance of *C. flavescentis* at 25 °C. In addition, the cold-adapted cells exhibited different temperature dependence of osmotic shock tolerance. The osmotic shock tolerance of cold-adapted cells decreased slightly with increasing temperature. The control cells showed a more dramatic decrease in osmotic shock tolerance with increasing temperature. These results confirm that cold adaptation induces physiologic changes in *C. flavescentis*. Our previous work with *C. flavescentis* (cited as *C. nodaensis*) demonstrated that cold-adapted cells were more desiccation tolerant (Zhang *et al.*, 2006). This study suggests that the increased desiccation tolerance may be correlated with the increased osmotic tolerance. It has been previously proposed (Mille *et al.*, 2004, 2005) that liquid dehydration behavior is predictive of drying tolerance. Our previous work with *C. flavescentis* showed that cold adaptation significantly improved storage stability, whereas with drying under mild conditions, both preparations maintained near 100% viability immediately following drying (Zhang *et al.*, 2006). Additional research is needed to provide a better understanding of the factors that affect initial drying viability and storage stability.

The membrane fluidity of microorganisms is known to change in response to environmental stresses (Beney & Gervais, 2001). In the current study, fluorescence anisotropy showed distinct differences in the membrane fluidity of cold-adapted cells of *C. flavescentis*, relative to the control cells. The cold-adapted cells showed much less change in fluidity over the temperature range tested and less variation in the data, relative to the control cells. These differences suggest that fundamental changes have occurred in the membrane and membrane regulation of the cold-adapted cells. Cold adaptation in an *Arthrobacter* species was shown to suppress the synthesis of charged lipids and reduce the membrane lipid composition to only lipids with glycerol head-groups (Mindock *et al.*, 2001). This suggests that cold adaptation may make the membrane more homogeneous and less responsive in changing its membrane fluidity. In *Saccharomyces* species, growth at 13 °C vs. 25 °C resulted in an increase in membrane unsaturated fatty acids for *S. cerevisiae*. In *S. bayanus*, no change in unsaturated fatty acids was observed, but the amount of medium-chain fatty acids increased (Torija *et al.*, 2003). These results suggest that yeasts use several methods to modulate membrane fluidity and adapt to growth at low temperatures. The theory of homeoviscous adaptation proposes that the viscosity of microbial membrane lipids will be constant at the temperature of growth (Sinensky, 1974). The fluorescence anisotropy results with *C. flavescentis* support this theory. When the fluidities of cold-adapted cells at 15 °C and control cells at 25 °C are compared, there is a small difference in fluidity, although it is not statistically significant. Whereas growth at low temperatures induces notable changes in membrane fluidity in *C. flavescentis*, these changes alone

appear insufficient to explain the difference osmotic shock and desiccation tolerance. Additional factors, such as membrane transport channels, may play an important role in these types of stress tolerance (Wei *et al.*, 2006).

In addition to changes in membrane fluidity, cold-adapted *C. flavescentis* cells were shown to be much stiffer with AFM force measurements. This technique determines the effective spring constant of the entire cell, which limits its interpretation to collective forces. Contributions from surface polymers, electrostatics and the elasticity of cellular components are embedded in these constants. It has been shown that peptidoglycan layer confers most of the rigidity to bacterial cells (Velegol & Logan, 2002). In this case, the interpretation of these results is further complicated by the cold-adapted cells being smaller than the control cells. The smaller cells would be expected to have a higher spring constant, even if the cell wall material for both samples had the same elastic modulus. The effect of size has been examined with AFM and Hertz's model using intact liposomes absorbed on mica (Liang *et al.*, 2004). This work showed a three-fold increase in bending modulus with a decrease in liposome size from 50 nm to 25 nm. However, this effect should be much less pronounced in our system, where the radii of curvature are much larger than the radius of the AFM tip. Although the size difference is likely to contribute to the increased spring constant of the smaller cold-adapted cells, we do not feel that this alone is sufficient to account for the five-fold increase in the cell spring constant. The greater rigidity of cold-adapted cells suggests that changes in the cell wall occurred in addition to changes in plasma membrane fluidity.

The effect of cold adaptation on the yield of cells (CFU mL⁻¹) of *C. flavescentis* was also examined. Cold-adapted cells showed a trend towards higher yields of CFUs under the defined fermenter conditions. Our previous work with cold-adapted *C. flavescentis* under shake flask conditions did not provide a significant yield enhancement (Zhang *et al.*, 2006). This may be a result of the increased efficiency of the fermenter or the higher-shear environment of the fermenter. The trend towards higher yields for the cold-adapted cells was coupled with a decrease in cell volume. Bacteria have also been shown to significantly decrease in cell volume when grown at low temperatures (Mindock *et al.*, 2001). This may represent a general adaptive strategy for microorganisms grown at low temperatures.

Recent proteomic studies have shown that the microbial response to cold temperatures occurs in two phases (Phadtare & Inouye, 2004; Schade *et al.*, 2004; Weinberg *et al.*, 2005). An immediate response to the cold shock and a latter acclimation response are observed after a period of time at the low temperature. This immediate response is believed to involve changes in membrane fluidity and RNA metabolism. Cold-shocking *Yersinia pestis* for 1 h induced transcription

changes in 28 membrane-related genes, which could alter the membrane composition in response to temperature shifts (Han *et al.*, 2005). A recent study with *Oenococcus oeni* reported a rapid decrease in membrane fluidity upon cold-shocking (Chu-Ky *et al.*, 2005). The acclimation phase involves the induction of proteins that protect the cell against a variety of environmental stresses. Schade *et al.* showed that exposure of *S. cerevisiae* to 10 °C for ≥ 12 h induced an environmental stress response, which did not occur with exposure for ≤ 2 h (Schade *et al.*, 2004). The environmental stress response or common environmental response (Causton *et al.*, 2001) is a genome-wide induction of common genes that occurs in response to a variety of stresses (Mindock *et al.*, 2001). This general stress response may help to protect the cells against other stresses after cold adaptation. Cold-shocking and cold adaptation of microorganisms has been shown to improve the microbial response to a variety of environmental stresses, such as freeze-thaw (Bryan *et al.*, 1999; Lee, 2004), heat resistance (Movahedi & Waites, 2002), and salt tolerance (Leblanc *et al.*, 2003). Our previous work with *C. flavescens* (cited as *C. nodaensis*) showed that short-term exposure (4 h) to low temperatures did not increase desiccation tolerance (Zhang *et al.*, 2006). These results, in combination with our current results and recent proteomic studies, suggest that a longer period (> 12 h) of cold acclimation is needed to induce the protective stress response.

Acknowledgements

The authors would like to thank J. Sloan and J. Nelson for excellent technical assistance. The authors also extend special thanks to the Center for Microanalysis of Materials at the University of Illinois at Urbana/Champaign for generous use of their Asylum Research 3D AFM.

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