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Macromolecular synthesis by yeasts under frozen conditions

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Summary

Although viable fungi have been recovered from a wide variety of icy environments, their metabolic capabilities under frozen conditions are still largely unknown. We investigated basidiomycetous yeasts isolated from an Antarctic ice core and showed that after freezing at a relatively slow rate (0.8°C min^{-1}), the cells are excluded into veins of liquid at the triple junctions of ice crystals. These strains were capable of reproductive growth at –5°C under liquid conditions. Under frozen conditions, metabolic activity was assessed by measuring rates of [^{3}H]leucine incorporation into the acid-insoluble macromolecular fraction, which decreased exponentially at temperatures between 15°C and –15°C and was inhibited by the protein synthesis inhibitor cycloheximide. Experiments at –5°C under frozen and liquid conditions revealed 2–3 orders of magnitude lower rates of endogenous metabolism in ice, likely due to the high salinity in the liquid fraction of the ice (equivalent of ≈ 1.4 mol l^{-1} of NaCl at –5°C). The mesophile *Saccharomyces cerevisae* also incorporated [^{3}H]leucine at –5°C and –15°C, indicating that this activity is not exclusive to cold-adapted microorganisms. The ability of yeast cells to incorporate amino acid substrates into macromolecules and remain metabolically active under these conditions has implications for understanding the survival of *Eukarya* in icy environments.

Introduction

Yeasts are present in extremely cold environments; they have been isolated from fresh snow and subglacial ice in the high Arctic (Starmer *et al*., 2005; Amato *et al*., 2007; Butinar *et al*., 2007), ancient glacier ice from worldwide locations (Christner *et al*., 2000) and a variety of terrestrial Antarctic environments (e.g. Vishniac, 2006). Cells entrapped in glacier ice remain preserved for extended periods (e.g. Abyzov *et al*., 1998; 2001; Christner *et al*., 2000; 2003; Miteva and Brenchley, 2005), and recent laboratory studies have shown that ice provides a habitat that can support microbial metabolism (e.g. Christner, 2002; Junge *et al*., 2006; Miteva *et al*., 2007). Liquid water is present in a network of veins at the grain boundaries between ice crystals, the diameter and chemistry of which is a function of the bulk salinity and temperature respectively (Price, 2000). Cryomicroscopic analysis has shown that the micrometer-sized veins in ice are sufficient to accommodate prokaryotic cells (Junge *et al*., 2004; Mader *et al*., 2006). The impurities excluded into the veins during freezing or during coarsening and re-crystallization (i.e. if introduced to the ice as insoluble particles; Price, 2000) can provide entrapped cells with the nutrients necessary to maintain a basal level of metabolic activity. The ability of cells in the ice to actively repair incurred macromolecular damage, especially of DNA, provides a plausible explanation for their capacity to remain viable for extended periods while frozen (Christner, 2002; Bidle *et al*., 2007; Johnson *et al*., 2007).

The anabolic activity of bacteria in ice has been measured in laboratory experiments down to temperatures of −20°C (Christner, 2002; Junge *et al*., 2006). In addition, *in situ* microbial respiration has been suggested as the cause of gas anomalies, which have been reported in some ice core horizons at temperatures of −10°C to −25°C (e.g. Campen *et al*., 2003; Ahn *et al*., 2004). Johnson and colleagues (2007) investigated permafrost samples up to 600 000 years old and concluded that bacteria actively repairing DNA were most likely to survive for extended periods while frozen. Extrapolation of various types of metabolic data available at subzero temperatures implies that rates of metabolism in ice are sufficient to offset macromolecular damage and the threshold for metabolism may be as low as −40°C (Price and Sowers, 2004). To our knowledge, the metabolic activity of eukaryal microorganisms under frozen conditions has only been experimentally investigated through CO_2 uptake and respiration measurements, which both exponentially declined with temperature down to −20°C (Panikov and Sizova, 2007).

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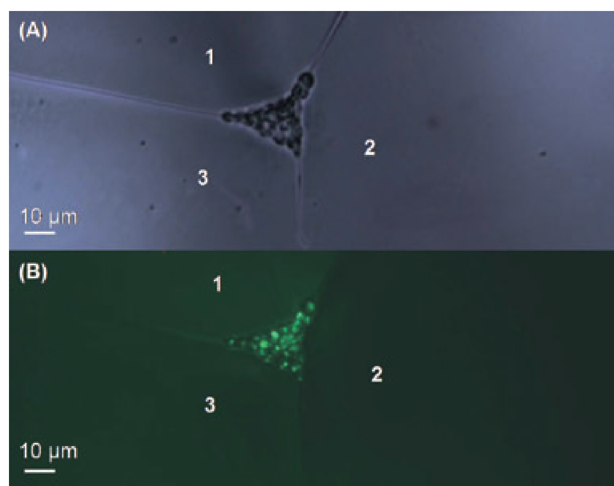

Fig. 1. (A) Bright field and (B) epifluorescent microscopic images showing the physical location of cells (*Cryptococcus* sp. 179-4) within a triple junction at −20°C. Numbers 1–3 indicate individual ice crystals.

Here we report on the metabolic activity of *Rhodotorula* and *Cryptococcus* yeast species isolated from the Vostok ice core, Antarctica and the mesophile *Saccharomyces cerevisae* under frozen conditions. Growth under liquid conditions and [^{3}H]leucine incorporation into macromolecules under frozen conditions were used to measure cellular activity over time. In addition, microscopic observations of thin sections of frozen cell suspensions were analysed to determine the physical location of cells in the ice. We discuss the ecological and basic scientific implications of these findings for the survival of microorganisms in permanently frozen environments.

Results and discussion

Physical location of yeast cells in frozen matrices

Thin ~250 μm ice sections containing fluorochrome-stained yeast cells were prepared from frozen cell suspensions and observed using epifluorescence cryomicroscopy. Figure 1 shows a triple junction, the point where three ice crystals interface, and the interstitial liquid veins that exist at the grain boundaries at −20°C. The crystals formed when cells suspensions were frozen in this way were ~0.5 mm in diameter. The cells imaged were clearly excluded into the veins during ice formation and appeared to be concentrated at triple junctions (Fig. 1B). Similar observations have been made with bacteria in sea ice and in freezing experiments done using 1.0–10 μM polystyrene beads (Junge *et al.*, 2004; Mader *et al.*, 2006). Mader and colleagues (2006) showed that particle size and freezing rate are important determinants in the partitioning of particles during freezing, with < ~30% of beads in the size range of eukaryotic cells (5–10 μm) excluded into the veins compared with values > ~80% for smaller beads (1–1.9 μm) in the size range of typical bacterial cells. Based on the data and freezing rates of Mader and colleagues (2006), we would expect that < ~20% of the ~6 μm yeast cells used in our experiments would have been excluded into the liquid fraction of the ice. Although quantitative counts of the cells embedded in ice crystals versus those excluded into the vein were not performed, the vast majority of cells observed were in the veins and appeared to be localized at triple junctions. It is possible that differences in freezing rates and the surface properties of cells and polystyrene beads affected the partitioning of these similar sized particles. It is important to note that ice formation under these laboratory conditions is distinctly different from that which occurs during the formation of glacier ice, so our data may not accurately reflect the cell partitioning processes that occur in natural ices.

Growth and metabolism at subzero temperatures

The growth rate of the Antarctic yeasts decreased exponentially with temperature between 15°C and −5°C (liquid conditions) (Table 1 and Fig. 2, opened symbols) and Q_{10} values ranged from 3.0 to 3.5. At 15°C, generation times ranged from 6.7 h (*Cryptococcus* sp. 179-4) to 11.6 h

Table 1. Rates of [^{3}H]leucine incorporation [(g leucine C) (g cell C)$^{-1}$ h^{-1}] at −15°C and −5°C (frozen and liquid conditions), and doubling times (generation h^{-1}) at −5°C (liquid conditions), 5°C and 15°C by the yeast strains in M9 mineral media supplemented with glucose, trace minerals and vitamins.

	Rate of leucine incorporation/doubling time				
Strain	−15°C	−5°C frozen	−5°C liquid	5°C	15°C
Rhodotorula sp. 179-2	–/–	1.59×10^{-9}/–	2.29×10^{-7}/0.009	–/0.056	–/0.111
Cryptococcus sp. 179-3	2.18×10^{-8}/–	6.54×10^{-7}/–	2.55×10^{-6}/0.017	–/0.047	–/0.145
Cryptococcus sp. 179-4	2.50×10^{-12}/–	2.53×10^{-10}/–	1.70×10^{-7}/0.015	–/0.049	–/0.148
Rhodotorula sp. 3605-11	–/–	4.76×10^{-9}/–	2.76×10^{-6}/0.009	–/0.013	–/0.086
S. cerevisae	7.52×10^{-10}/–	1.10×10^{-8}/–	–/–	–/–	–/–

The rate of leucine incorporation for each strain was calculated using the mean of triplicate experiments (coefficient of variation from 11.1% to 29.7% (average standard deviation was 17.2% of the mean value). '–' indicates that no data are available.

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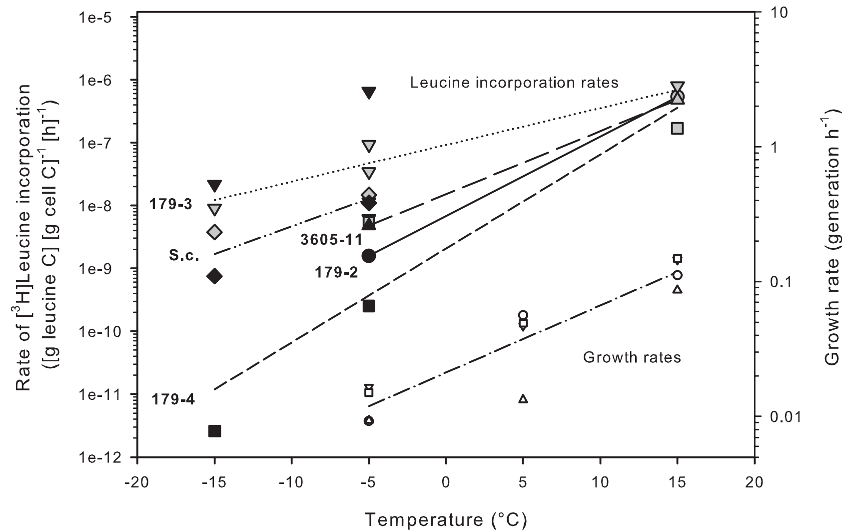

Fig. 2. Growth (opened symbols, right axis) and leucine incorporation (closed symbols, left axis) rates between –15°C and 15°C in *Rhodotorula* sp. 179-2 (circles), *Cryptococcus* sp. 179-3 (triangles down), *Cryptococcus* sp. 179-4 (squares), *Rhodotorula* sp. 3605-11 (triangles up) and *Saccharomyces cerevisae* (diamonds). Growth rates were measured under liquid conditions in M9 mineral media supplemented with trace minerals and vitamins. Rates of [^{3}H]leucine incorporation were measured under frozen conditions at –5°C and –15°C in either M9 mineral media supplemented with trace minerals and vitamins (black symbols) or deionized H_2O (grey symbols), and under liquid conditions at 15°C in 140 mM NaCl. Fit lines indicate the exponential decrease of activity for each strain with decreasing temperature in a particular incubation medium. Each data point represents the average of triplicate rate measurements. The coefficient of variation for rates of leucine incorporation averaged 17.2% for cells in M9 media and 29.9% for cells in dH_2O.

(*Rhodotorula* sp. 3605-11). All the Antarctic strains grew at –5°C with generation times of 61, 67, 107 and 108 h for *Cryptococcus* sp. 179-3, *Cryptococcus* sp. 179-4, *Rhodotorula* sp. 3605-11 and *Rhodotorula* sp. 179-2 respectively. According to the current terminology for describing the growth temperature range of a microorganism (e.g. Feller and Gerday, 2003), these strains are classified as eurythermal psychrophiles given a $T_{opt} > 15$°C and their ability to grow at $\leq$ –5°C.

The rate of leucine incorporation was used to compare macromolecular synthesis under liquid (–5°C and 15°C) and frozen conditions (–5°C and –15°C) (Table 1). All strains incorporated exogenous leucine into their cellular material in a time-dependant manner. At each time point, leucine incorporation by cells at –5°C or –15°C that were incubated in the presence of cycloheximide was not significantly different than the killed controls ($P < 0.05$) (Fig. 3), and after 30 days at –15°C, cells had incorporated significantly higher amounts of leucine than controls and cells treated with cycloheximide. This provides direct evidence that leucine was being incorporated into protein under frozen conditions, but some fraction of the leucine may also have been funneled into other pathways (e.g. lipid and nucleic acid synthesis), as others have reported (Christner, 2002; Junge *et al.*, 2006).

The inferred rate of heterotrophic production varied between strains, with larger differences observed at decreasing temperature. At –15°C, the activity was 2.50×10^{-12} to 2.18×10^{-8} (g leucine C) (g cell C)$^{-1}$ h^{-1} for *Cryptococcus* sp. 179-4 and *Cryptococcus* sp. 179-3 respectively. For comparison, a mesophilic type strain of *Saccharomyces cerevisae* was included in these experiments; this rate measured for the strain at –15°C was 7.52×10^{-10} (g leucine C) (g cell C)$^{-1}$ h^{-1} (Table 1). Christner (2002) measured leucine incorporation in *Arthrobacter* and *Psychrobacter* species at –15°C, and from his data we calculated corresponding rates of 7.1 and 11.1×10^{-9} (g leucine C) (g bacterial C)$^{-1}$ h^{-1} respectively. Junge and colleagues (2006) measured leucine incorporation rates at –10°C and –15°C of ~8.0 and 2.0×10^{-7} (g leucine C) (g bacterial C)$^{-1}$ h^{-1}, respectively, for the psychrophilic bacterium *Colwellia psychrerythraea* (from data visually deduced from figures). Linear interpolation of the data in Fig. 2 reveals that when normalized to cellular carbon, the rate of leucine incorporation in yeasts under frozen conditions is comparable to the lowest rates that have been measured in cold-active bacteria.

Compared with similar experiments carried out in deionized water (< 1 μmol l^{-1} total dissolved solute), incubations in M9 media counter intuitively resulted in lower rates of leucine incorporation under frozen conditions. The exception was *Cryptococcus* sp. 179-3, which had rates 7 and 2.4 times higher in M9 media at –5°C and –15°C, respectively, than in deionized water [65.4 and 2.18×10^{-8} (g leucine C) (g cell C)$^{-1}$ h^{-1} in M9 media; 9.25 and 0.91×10^{-8} (g leucine C) (g cell C)$^{-1}$ h^{-1} in deionized H_2O; at –5°C and –15°C respectively]. Due to the presence of higher solute concentrations, liquid veins were

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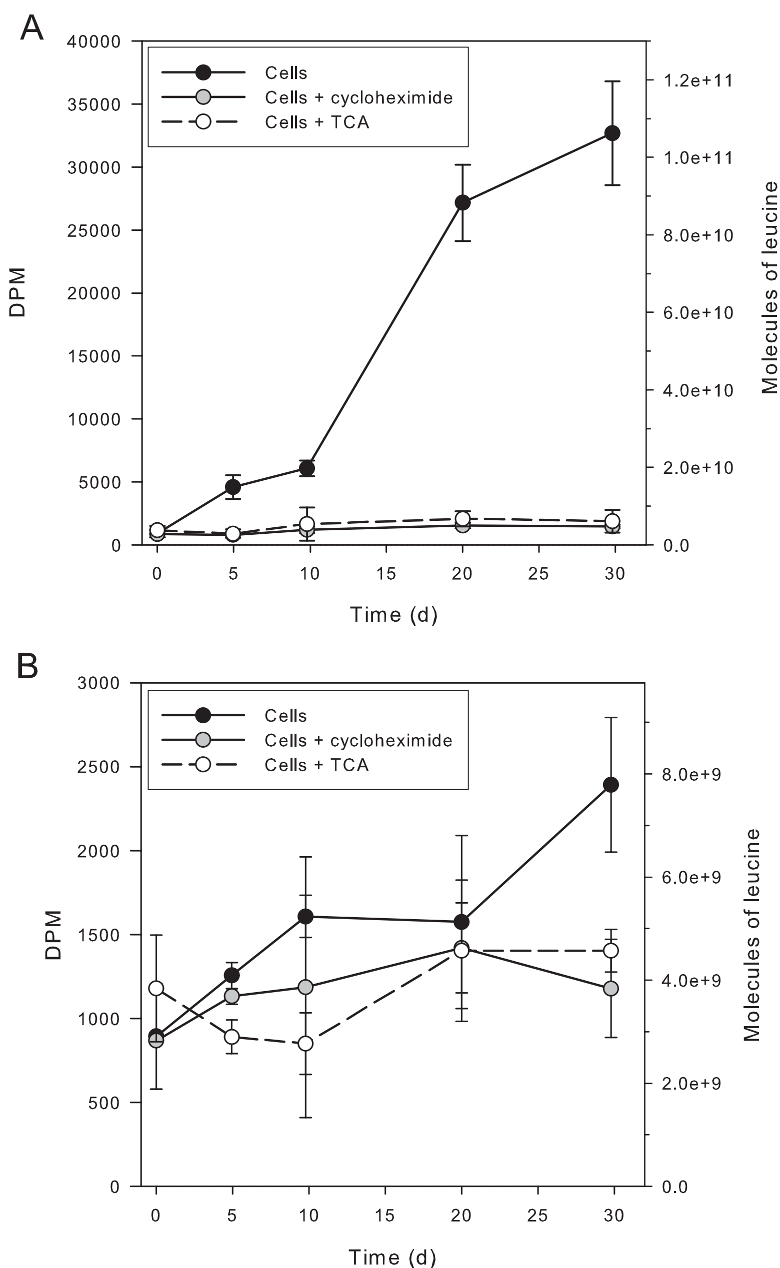

Fig. 3. Amount of [^{3}H]leucine incorporated into TCA precipitable material at –5°C (A) and –15°C (B) by frozen cell suspensions of *Cryptococcus* sp. 179-3 (closed symbols), cells incubated with cycloheximide (grey symbols) and cells killed by the addition of TCA (opened symbols). The initial cell concentration was $5.55 \pm 0.57 \times 10^4$ cells ml^{-1} and sample volumes were 0.7 ml. Errors bars are standard deviations from the mean of triplicate samples.

substantially larger in frozen M9 media than in frozen deionized H_2O, which would substantially influence the diffusion of substrate (i.e. leucine) or metabolic waste from the cell. Perhaps the *Cryptococcus* sp. 179-3 has a higher affinity leucine transport system, but we are unable to fully explain this result with the available data.

The rates of leucine incorporation under frozen and liquid conditions at –5°C in mineral medium are compared in Table 1. The Q_{10} value for the growth rate under liquid conditions between 15°C and –5°C was between 3.0 and 3.5. Under frozen conditions between –15°C and –5°C, the Q_{10} values for leucine incorporation ranged from 3.9 for *Cryptococcus* sp. 179-3 to 31.1 for *Cryptococcus* sp. 179-4, and was 7.8 for *S. cerevisae*. Although these are higher than values reported by others for communities of prokaryotes under cold conditions (Takacs and Priscu, 1998: deduced $Q_{10} = 2.3$; Lipson *et al.*, 2002: $Q_{10} = 3.6$ between 0 and 22°C), Q_{10} values for metabolism have been shown to increase at lower temperature (Bakermans and Nealson, 2004; Sand-Jensen *et al.*, 2007) and may be an indication that reaction rates are diffusion-limited (Van Hulzen *et al.*, 1999).

Based on models for freezing point depression for an ideal sodium chloride solution, the solute concentration in the liquid fraction of the ice is predicted to be equivalent to ~1.4 M ($A_w = 0.95$) and ~4.4 M ($A_w = 0.86$) NaCl at

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−5°C and −15°C respectively (Harris, 1981; Chen, 1987). Although the yeasts could not reproduce at these salt concentrations, they were capable of growth at −5°C under liquid conditions. This implies that temperature was not the growth-limiting constraint in the ice and that the low water potential of the solute-rich ice veins, along with the low diffusion of molecules, was probably the most important factor limiting metabolism under frozen conditions.

The detection of leucine incorporation at temperatures as low as −15°C in the mesophile *Saccharomyces cerevisae* was unexpected, but raises interesting questions regarding low temperature metabolism in non-psychrophilic organisms. Our experiments demonstrate that a basal level of cellular metabolism persists at temperatures far below an organism's lowest measurable growth temperature. The ability of a diverse variety of prokaryotic and eukaryotic microorganisms to conduct subzero metabolism implies that this phenotype may be an important survival mechanism and widely distributed. The high solute concentration coupled with the slow diffusion of metabolic substrates and wastes in ice veins (Wolff and Paren, 1984; Van Hulzen *et al*., 1999; Livingston *et al*., 2002) are likely factors that limit biological activity under these conditions. However, the ability to retain a low level of metabolic activity when frozen would provide a distinct advantage. Cells capable of repairing damage incurred during the freezing process and over extended periods of frozen incubation would be more likely to survive until conditions compatible with growth are reintroduced.

Although a number of recent reports have demonstrated bacterial activity under frozen conditions (Carpenter *et al*., 2000; Rivkina *et al*., 2000; Jakosky *et al*., 2003; Junge *et al*., 2004, 2006; Panikov *et al*., 2006; Miteva *et al*., 2007), few data are available on eukaryotic cells. Panikov and Sizova (2007) reported respiratory and biosynthetic activity of eukaryal strains. Under their experimental conditions, activity ceased after 2 weeks at temperatures below −20°C, suggesting that the cells entered a dormant state. Our rate measurements of [^{3}H]leucine incorporation by yeast cells in ice at −15°C and −5°C provide evidence for some level of basal metabolic activity and are consistent with the hypothesis of Price and Sowers (2004), who suggest that metabolism under frozen conditions is directed towards maintenance and survival, but not growth. The ability to remain metabolically active in ice may not be exclusive to cold-adapted species and may be more widely distributed in the tree of life than is currently appreciated. By definition, the terms 'psychrotolerant' and 'psychrophile' are applied to microorganisms that have the capacity to grow at low temperatures. Our results support the idea that enzymes and complex cellular pathways, such as protein synthesis, present in species not considered to be cold-adapted are functional at much lower temperatures and extremes of salinity than has been previously recognized.

These results are clearly relevant to discussions regarding the preservation of prokaryotic and eukaryotic cells in ancient frozen material such as glacier ice and permafrost (Abyzov *et al*., 1998; 2001; Christner *et al*., 2000; 2001; Miteva and Brenchley, 2005; Johnson *et al*., 2007). Cells may be capable of offsetting the macromolecular damage that inevitably occurs after prolonged entrapment in frozen materials through active metabolism and repair. If *in situ* microbial metabolism occurs in glacier ice, it could provide a plausible explanation for gas anomalies that have been documented in the basal portion of some ice cores (Flückiger *et al*., 1999; 2004; Campen *et al*., 2003).

Experimental procedures

Strain descriptions and culture conditions

The four cold-adapted yeast strains studied were isolated from previously analysed samples of the Vostok ice core, Antarctica (Christner *et al*., 2006). The isolates (*Rhodotorula* sp. 179-2, *Cryptococcus* sp. 179-3, *Cryptococcus* sp. 179-4 and *Rhodotorula* 3605-11) were subsequently enriched from melt water that was obtained using a quantitative decontamination protocol (Christner *et al*., 2005). The yeasts were initially cultured at 4°C from media inoculated with melt water using the procedures described by Christner and colleagues (2001). These isolates are designated by their respective genus (i.e. based on partial sequencing of the ITS/18S/26S rRNA genes; M. Blackwell, unpubl. data), the depth of the ice core sample, and the strain number (e.g. *Rhodotorula* sp. 179-2 is strain number 2 that was isolated from a depth of 179 m). Based on the molecular data, the nearest neighbours of strains 179-2, 179-3 and 179-4 are *R. glutinis*, *C. victoriae* and *C. adeliensis* respectively. Strain 3605-11 is likely a new species in the genus *Rhodotorula*, and its characterization is currently underway. The reference strain *Saccharomyces cerevisae* was obtained from the microbiology laboratory culture collection at Louisiana State University.

Cells were grown aerobically (200 r.p.m.) at 15°C, 5°C and −5°C in 5 ml of M9 mineral medium (Difco) that was supplemented with 22 mM of D-glucose (Fisher), vitamins (ATCC, ref MD-VS) and trace minerals (ATCC, ref MD-TMS). The solute concentration of the culture medium was sufficient (0.13 M) to remain liquid at −5°C. A growth curve for each yeast strain was conducted by measuring the optical density at 620 nm of cultures every 5–25 h for 14 days, and the growth rate and doubling time were calculated for cell populations in the exponential phase of growth.

Microscopic observations of yeast cells in ice

Cryptococcus sp. 179-4 was grown at 15°C until the stationary phase of growth. Then, 1 ml of the culture was stained with a 25× SYBR-Gold (Invitrogen) solution buffered with 89 mM tris-borate buffer containing 2 mM EDTA. The cells

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were harvested by centrifugation (17 000 *g*, 5 min) and rinsed twice in an equal volume of 0.8% NaCl (w/v). The washed cells were re-suspended in 0.8% NaCl (w/v) to achieve a concentration of 8.0×10^7 cells ml^{-1} and were frozen at –25°C (cooling rate of 0.8°C min^{-1}) in 1.5 ml microcentrifuge tubes. The ice sample was removed from the tubes, and thin sections (~250 μm) of the frozen cell suspension were made using a standard histological microtome that was housed within a –5°C environmental room. The frozen thin sections were mounted on glass microscope slides and observed with a Olympus BX51 epifluorescence microscope equipped with a cryostage (Linkam LTS350; ±0.1°C thermal stability) and a FITC filter cube (excitation from 455 to 500 nm and emission from 510 to 560 nm). Digital images were captured using a Qimaging Retiga 2000R camera.

Measurement of leucine incorporation

The incorporation of [^{3}H]leucine into the acid-insoluble macromolecular fraction was carried out following the procedure described by Christner (2002). Cells in the exponential phase of growth were harvested by centrifugation (3 min, 17 000 *g*), washed in deionized H_2O, and the cell suspension was re-suspended in experimental medium (either M9 supplemented with glucose, vitamins and trace minerals, or 0.14 M NaCl or deionized H_2O) to achieve $\sim 1 \times 10^5$ cells ml^{-1}. A 0.5 ml volume of the cell suspension was amended with 0.1 ml of 17 nM [^{3}H]leucine (L-leucine [4,5–^{3}H], 84 Ci $mmol^{-1}$ in ethanol : water 2:98; MP Biomedicals) after addition of 0.1 ml of 0.22-μm-filtered deionized H_2O or 50% trichloroacetic acid (TCA; final concentration of 7%); the TCA amended samples served as killed controls. In some experiments, prior to the addition of [^{3}H]leucine, the cell suspensions were amended with an inhibitor of eukaryal protein synthesis acting at the elongation step (cycloheximide, 50 μg ml^{-1}; MP Biomedicals).

For incubations under frozen conditions, ice-chilled samples were placed in an –80°C freezer < 30 s after the addition of [^{3}H]leucine. Rapid freezing minimized the uptake and incorporation of large amounts of leucine by cells before freezing occurred. The cooling rate at –80°C was measured using an electronic temperature probe (Hobo) placed into a microcentrifuge tube containing 70% ethanol. The cooling rate was 4.46°C min^{-1} (five times faster than that for microscopic observations) and linear down to –50°C, suggesting that samples froze < 5 min after being placed in the –80°C freezer (assuming that the suspensions were frozen when samples reached a temperature of –20°C). Time 'zero' in these experiments designates when samples were transferred from the –80°C freezer to the incubation temperature, which was 1 h after the addition of [^{3}H]leucine. Incubations at 15°C were done in a Gyromax 737R refrigerated incubator (Amerex Instruments). Frozen incubations were conducted in an environmental room (Environ CMP5090) at –5°C, or in a thermally stable freezer (Thermo Isotemp) at –15°C. Temperature was continually monitored with embedded loggers (Hobo) and thermal variation was ±1.5°C during all experiments.

At each experimental time point (4–6 time points, typically every 5 days for a total period of 30 days), triplicate frozen samples were removed from the freezers, amended immediately with 0.1 ml of cold 50% TCA (final concentration of 7%), and the frozen suspensions were completely melted within 5 min directly into the concentrated TCA solution. After melting, the samples were incubated for 30 min at 4°C and centrifuged for 15 min at 17 000 *g*. The pelleted acid-insoluble macromolecules were washed with 0.5 ml cold 5% TCA, centrifuged for 10 min (17 000 *g*), rinsed with ice-cold 70% ethanol followed by centrifugation, and the pelleted material was re-suspended in 1 ml of Cytoscint scintillation cocktail (Fisher, cat# BP458-4). Radioactivity was measured using a Beckman LS6000IC scintillation counter (10 min averaged counts per minute; CPM). The CPM were converted to disintegrations per minute (DPM) using quench curves constructed using [^{3}H]toluene (American Radiolabeled Chemicals, cat# ARC182) and acetone quenched standards in the Cytoscint cocktail. The level of background (i.e. due to traces of radiolabel that remained after washing) in the experiments was determined from killed controls and were measured in triplicate at each time point. Rates of leucine incorporation were calculated by subtracting background from data in which the level of radioactivity incorporated into the TCA-precipitable fraction was linear over time. In order to express the metabolic rates as (g leucine) (g cell $C)^{-1}$ h^{-1}, the cell diameter was measured microscopically in the initial suspension and biovolume was converted to cellular carbon assuming spherical cells and 0.22 (pg C) μm^{-3} (Bouvier *et al.*, 1998). Carbon contents for the strains analysed are estimated at 1.01×10^{-11} (g C) $cell^{-1}$ for 179-2, 1.05×10^{-12} (g C) $cell^{-1}$ for 179-3 and *S. cerevisae*, 2.88×10^{-11} (g C) $cell^{-1}$ for 179-4 and 2.86×10^{-12} (g C) $cell^{-1}$ for 3605-11.

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