RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2014) 17:131-139
doi:10.2436/20.1501.01.215. ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

Metabolomic charactetization of yeast cells after dehydration stress

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Received 28 January 2014 · Accepted 15 July 2014

Summary. In this study, we analyzed the metabolite features of the yeasts *Saccharomyces cerevisiae*, *Naumovia castellii*, and *Saccharomyces mikatae*. The three species are closely related genetically but differ in their tolerance of desiccation stress. Specifically, we determined whether certain metabolites correlated with cell viability after stress imposition. The metabolomic profiles of these strains were compared before cell desiccation and after cell rehydration. In *S. mikatae*, the presence of lysine or glutamine during rehydration led to a 20% increase in survival whereas during dehydration the levels of both amino acids in this yeast were drastically reduced. [**Int Microbiol** 2014; 17(3):131-139]

Keywords: *Saccaromyces mikatae* · *Saccharomyces cerevisiae* · *Naumovia castellii* · viability · dehydration stress · metabolite extraction · wild yeast

Introduction

Among the broad group of biological studies known as "omics," metabolomics focuses on the analysis of a large number of metabolites at an organism level [8]. In yeast populations, intracellular metabolome analysis has improved our understanding of cell metabolism and thus of cellular responses to external physiological conditions [27]. The metabolomic profile of a selected yeast strain with respect to its response to dehydration is of commercial interest. In the food industry, dried yeast (active dry yeast) formulations have gained widespread acceptance because of the greater genetic stability at room temperature, resulting in savings in transport and storage costs. However, the loss of cell viability during the industrial drying processes and the resulting lower activity have to some extent hindered the development of a high-quality inoculum [23]. Thus, identifying the changes in metabolites that occur in response to desiccation stress would facilitate efforts aimed at optimizing the drying process. During desiccation, the yeast's metabolic processes are in a suspended state [4,21] whereas during rehydration these organisms rapidly swell and return to active life.

The methodological steps required for metabolite detection and data analysis are well established [3,10,14]. Villas-Bôas et al. [29] described methods to improve yeast sample preparation, especially with respect to cold methanol quenching, metabolic extraction by chemical treatment, and sample concentration through solvent evaporation. The quenching step consists of a brief metabolic arrest, which is required to obtain a focused cell metabolome "snapshot" of the cellular response to environmental conditions. In microbial physiology studies, the most commonly applied cell quenching method involves immersion of the sample in a cold aqueous methanol solution [11]. An advantage of this method compared to environmental pH variations is that it allows the subsequent sepa-

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ration of extra- from intra-cellular fractions, which significantly reduces contamination between the two [9].

Other well-known and widely used extraction methods in yeast research include methanol-chloroform, pH-dependent, and boiling ethanol treatments; however, problems have been identified in each case, including the recovery of non-phosphorylated compounds, compound stability at extreme pH values, and compound thermolability, respectively [3]. In the case of metabolite extraction from *Escherichia coli* and *Saccharomyces cerevisiae*, these effects can be mitigated by extracting in 50% (v/v) methanol after a –80ºC cycle [13,29]. However, most of the methods for metabolite extraction have been optimized for laboratory strain batch cultures, which are much more easily manipulated than either industrial strains or cells from non-growing conditions, in which the cell wall becomes a problematic barrier that strongly impacts the repeatability of quantitative assays [25]. The majortiy of metabolite extraction protocols for microorganisms require a final sample concentration step, typically a lyophilization procedure, that prevents further modification of cell compounds by eliminating the water-miscible solvent mix from the sample [6].

In this study, we compared three species of yeast, *Saccharomyces cerevisiae*, *Naumovia castellii*, and *Saccharomyces mikatae*, that differ in their dehydration tolerance. The analysis described herein is a continuation of a previous study in which we explored the effect of changes in cell lipid composition on the viability of these yeast strains following rehydration [23]. Through metabolomic characterization, the aim of this study was to identify the putative cellular compounds involved in overcoming dehydration/rehydration stress. *S. mikatae* was from the *sensu stricto* group, which includes yeast species closely related to *S. cerevisiae*. *N. castellii* belongs to the *sensu lato* group, which includes heterogeneous yeast species that diverge more significantly from *S. cerevisiae* [16]. In tandem with these two strains, *S. cerevisiae* was analyzed. These three species differ from each other in a very limited number of physiological characteristics, some of which may be controlled by single-gene mutations [22]. The metabolite features of these yeasts during the dehydration/rehydration process were analyzed using an optimized gas chromatography-mass spectrometry (GC-MS) metabolomics method.

Materials and methods

Strains, growth conditions, and desiccation-rehydration. Overnight liquid cultures of the yeast species *Saccharomyces cerevisiae* (CECT-1477, from Burdeos sparkling wine), *Naumovia castellii* (CECT-11356, from Finland soil), and *Saccharomyces mikatae* (CECT-11823, Japan monosporic culture) at an initial OD_{600} of 0.5 were used as the inoculants. All three strains were grown in shake flasks (170 rpm) in YPD [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) glucose (Cultimed, Barcelona, Spain)] at 28ºC for 24 h. The effects of lysine (1, 2, 5, and 10%), Na_2HPO_4 (2, 5 and 10%), and glutamine (2, 5 and 10%) on cell viability during rehydration were studied by adding each compound individually to the pure water basal condition. Desiccation-rehydration, and cell viability were determined by flow cytometry, performed as previously described [22].

Metabolite extraction. Each step of the extraction was optimized based on the methods of Roessner et al. [24]. Five × 10^8 live cells were immediately frozen in cold 70% ethanol and stored at –20ºC until further analysis. After cell quenching, three methods for intracellular metabolite extraction were evaluated by assessing the percentage of unbroken cells. The mean number of unbroken cells per ml was calculated according to the CFU (colony-forming units) of treated cells after taking into account the CFU per ml before cell breakage. Frozen cells were pelleted and resuspended in methanol-water (1:1, v/v) to a volume of 400 µl, as described by Villas-Bôas et al. [29], with 10 µl of ribitol at 2 mg/ml (Sigma, Switzerland) added as an internal standard (IS). To optimize this step, samples were incubated at 90ºC either for 10 min, or for 5 min in the presence of 0.2-mm glass beads (BioSpec Products, USA) in a sonication bath (J.P. Selecta, Spain), or for 5 min in the presence of 0.5-mm acid-washed glass beads (~300 µl). The samples were then disrupted in a multitube bead-beater (BioSpec Products, USA) using five cycles of 1 min/beat, followed by 30-s rest for cooling. After centrifugation, the supernatant was dried in a SC110 speed vacuum system SC110 (Savant Instruments, USA) for 4 h. The dried residue was redissolved and derivatized for 1 h at 40ºC in 50 µl of 20 mg/ml methoxyamine hydrochloride in pyridine (Sigma, Japan; Fluka, India), followed by a 90-min treatment at 40ºC with 70 µl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (Sigma, USA).

Gas chromatography-mass spectrometry analysis. Gas chromatography (GC) was performed using an Agilent Technologies Network GC system 6890N connected to an HP computer with the ChemStation software (Agilent Technologies). Compounds were detected using an inert mass selective detector (MSD, model 5975, Agilent Technologies). Two µl of the cell extract was injected at a split ratio of 20:1 into a DB-5HT column (30 m × 0.25 mm × 0.1 µm; Agilent Technologies) with an automatic injector (7683B, Agilent Technologies). Helium was used as the carrier gas at a constant flow of 1.0 ml/min. The injector temperature was 200ºC. The column oven temperature was initially held at 80ºC for 4 min and then increased first to 200ºC at a rate of 5ºC/min and then to 300ºC at a rate of 25ºC/min, where it was held for 7 min. The MSD transfer temperature was 300ºC. The MSD quadrapole and source temperatures were maintained at 180ºC and 280ºC, respectively. The MSD data were acquired in electronic ionization scan mode at 70 eV within the range of 35–650 amu after a solvent delay of 4 min. Postrun analysis was performed with the Agilent MSD Chemstation. The relative abundance of each identified compound was calculated according to the respective chromatographic peak areas corrected with respect to the IS peak. These values were relativized to the 1 × 10^9 CFU, according to the cells plated and counted after 24 h of culture and desiccation/rehydration, and expressed as percentages.

Flow cytometry analysis. Flow cytometry was carried out using a CyFlow space instrument (PARTEC, Germany) fitted with a 22-mW ion laser for excitation (488 nm); a single emission channel (575-nm band-pass filter) was used for monitoring. Instrument control, data acquisition and data analysis were performed using FloMax software (Quantum Analysis, Germany). The two-color fluorescent probe from the LIVE/DEAD yeast viability kit was used to label the cells. In this system, plasma-membrane integrity and fungal metabolic function are required to convert the intracellular yellow-green fluorescence of FUN1 into red-orange intravacuolar structures, Calcofluor White

M2R labels cell-wall chitin with blue fluorescence regardless of metabolic state (Life Technologies). An overnight YPD culture of a *Saccharomyces* sp. strain was taken as a control of full viability (99% by FUN1 red-orange/Calcofluor white stain).

Microscopy. The cells were viewed with a Leica microscope (DM4000B, Germany) and a digital camera (Leica DFC300FX). The Leica IM50 software was used for image acquisition.

Statistical analysis. The data were analyzed by principal component analysis (PCA) using the SPSS 20.0 statistical software package. For further analysis, one-way ANOVA and the Tukey test were used with the same program. Statistical significance was set at $P \leq 0.05$.

Results and Discussion

Yeast cell viability after dehydration stress. Differences in the desiccation tolerance of the three closely related yeast species were assessed by flow cytometry. FUN1/Calcofluor co-staining was performed to quantify cell metabolic activity and cell wall integrity (Fig. 1A). This staining protocol allowed metabolically active cells (live cells) to be distinguished from damaged or non-active cells (dead cells). The mean number of viable cells after rehydration was calculated relative to cell viability before drying. After drying, the cells of each species were resuspended in either 10% trehalose or, as the reference condition, pure water to evaluate viability. The viability of cells in the deionized water condition was lower for *S. mikatae* and *N. castellii* than for *S. cerevisiae* (20%, 40% and 84%, respectively) whereas the viability of yeast cells dried in the presence of trehalose was increased by approximately 20% for all three species (Fig. 1A). Trehalose was previously shown to act as a membrane protector by reducing the membrane phase-transition temperature during the desiccation-rehydration process, without having a significant impact on cell metabolites [7,23].

Metabolite extraction. The metabolite fractions of the three strains were investigated to determine their dehydration-stress tolerance metabolite profiles of the yeasts. The extraction process was optimized for these strains based on the protocol of Roessner et al. [24]. After the cells were frozen to stop their metabolism, extracts were prepared using three different methods: thermal shock, sonication, and vortexing. Figure 1B shows the cells treated according to these methods and then evaluated by microscopy and flow cytometry to quantify the unbroken cell fraction, both before and after treatment. Resuspending the cells in the presence of 0.2-mm glass beads after shaking with a mini-bead beater for 5 × 1 min with a 1-min rest between treatments resulted in 98% broken cells whereas thermal shock and sonication resulted in 30% and 20% unbroken cells, respectively.

Metabolomic profiling of yeast cells during the desiccation process. To understand the metabolic differences between *S. mikatae, N. castellii*, and *S. cerevisiae* cells before the imposition of stress, their metabolomic profiles before dehydration (BD) and after rehydration (AR) were compared. The cellular metabolites were analyzed by GC-MS, with 44 peaks identified and quantified for each strain and condition (BD and AR). These metabolites included amino acids, glycolytic compounds, sugars, fatty acids, and organic acids. Multivariate data analysis was performed using PCA to examine the variations among metabolites between the BD and AR steps in *S. cerevisiae*, *N. castellii*, and *S. mikatae* (Fig. 2A, B, and C, respectively). Endogenous trehalose increased by 8.7-fold, 5.5-fold, and 3-fold during stress imposition in *S. mikatae*, *N. castellii* and *S. cerevisiae*, respectively, but for reasons of scale the data are not included in Fig. 2. However, intracellular trehalose did not correlate with the desiccation tolerance of the three species, as previously reported by Rodríguez-Porrata et al. [23]. PCA was performed after data processing, including normalization according to the IS and CFU corrections (see Materials and methods).

The relative amounts of fatty acids, organic acids, and compounds resulting from glycolysis differed significantly in the three species, both BD and AR. By contrast, there were no significant differences in the BD amounts of amino acids and sugars. The largest differences occurred in *N. castellii*, whose metabolite profile during dehydration was the opposite of that of *S. cerevisiae*, which also had a 40% greater viability AR (Fig. 2A and Fig. 1A). Although phosphoric acid is toxic to yeast cells, the BD concentration in *N. castellii* was 4-fold higher than in the two other yeast strains and increased 1.5-fold during stress, whereas in *S. mikatae* and *S. cerevisiae* the concentration decreased by 3-fold (Fig. 2C). These results suggested that the viability of *N. castellii* would be negatively affected during stress due to inefficient detoxification and/or a poor capacity for metabolic esterification of phosphoric acid into nucleic acids, proteins, lipids, and sugars [18]. Yeast cells store phosphate in vacuoles as polyphosphate, which normally increases during the stationary growth phase [18]. No significant differences among the yeast species were recorded BD in terms of phosphate content (Fig. 3). However, between the BD and AR steps, the phosphate content of *S. mikatae* and *S. cerevisiae*

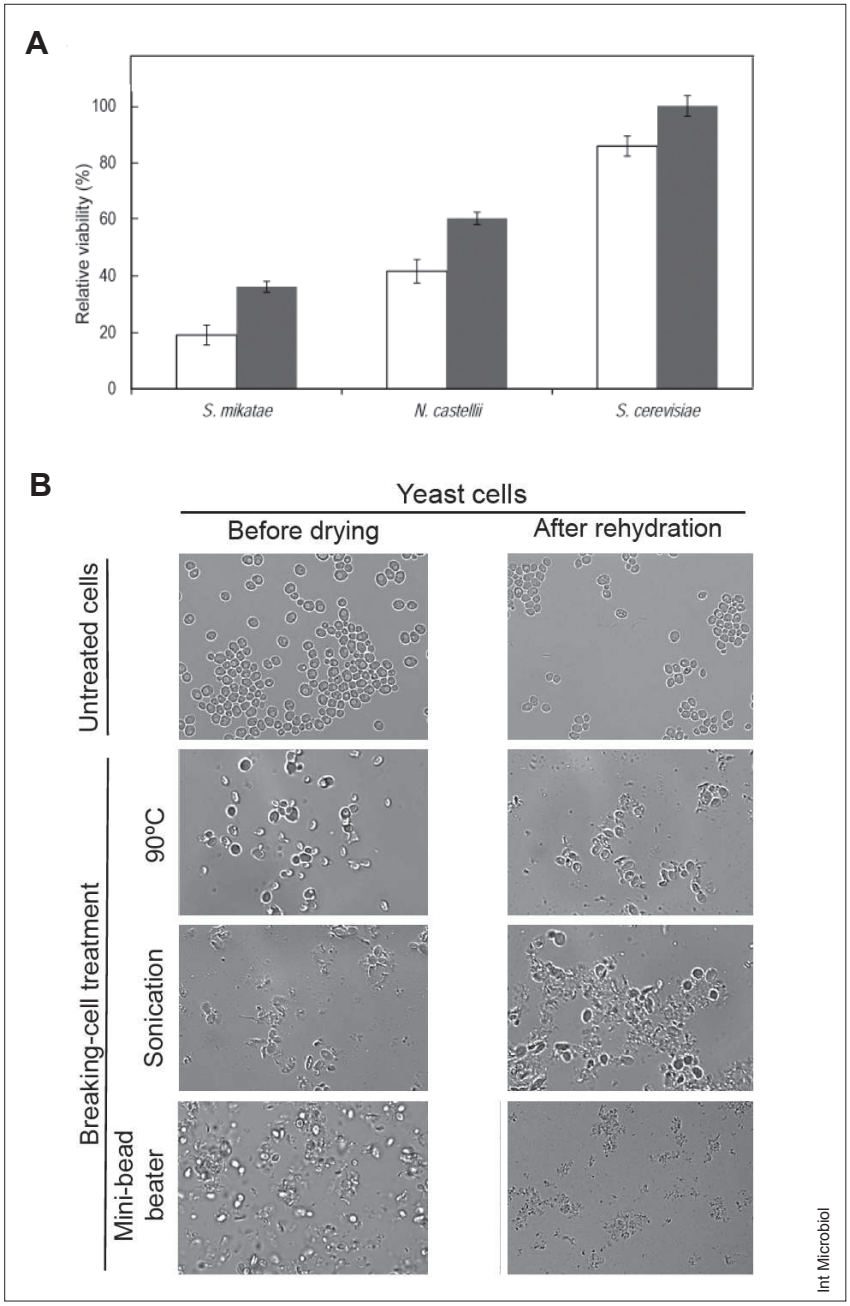

Fig. 1. Yeast cell viability following air drying and rehydration. (**A**) Cells were dried to a moisture content of 5% at 28ºC. The scale of relative viability (%) indicates the percentage of experimental values for the different yeasts relative to the highest viability for *Saccharomyces cerevisiae*. The values shown represent the means of five independent samples ± SD, as evaluated by flow cytometry. The viability of cells dried in the presence (gray bars) and absence (white bars) of trehalose was evaluated. (**B**) Microscopy images show the broken *S. cerevisiae* cells after treatment, and the predominance of large cell fragments. White spots are the cell walls of empty cells, and dark spots are intact cells.

decreased by 70% and 30%, respectively, while it doubled in *N. castellii* (Fig. 3). Nonetheless, this greater accumulation of phosphate by *N. castellii* than by *S. cerevisiae* during AR did not enhance its dehydration tolerance, as reflected in the differences in viability: 60% vs. 95%, respectively (Fig. 1A). *S. mikatae* had a significantly lower content of phosphate after drying and rehydration, consistent with its low viability (36%). The main metabolic response of *S. cerevisiae* to osmotic pressure,

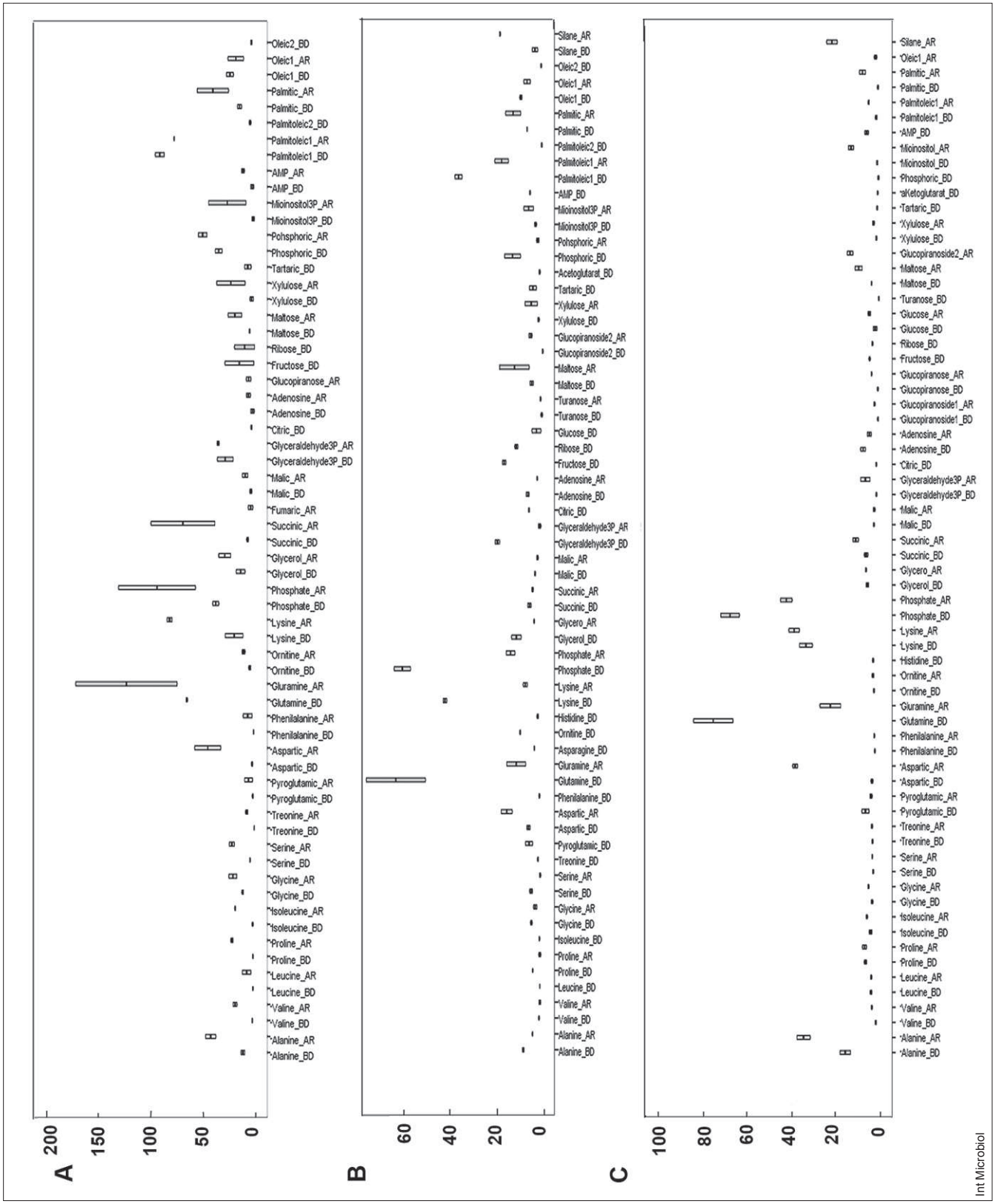

Fig. 2. Metabolic variations between before drying (BD) and after rehydration (AR), as examined by principal component analysis, during stress imposition in (**A**) *Saccharomyces cerevisiae*, (**B**) *Naumovia castellii*, and (**C**) *Saccharomyces mikatae*. Principal component analysis revealed the largest variance between the BD and AR steps, as shown by the changes in the intracellular alanine concentration (compare **A** and **C)** during stress imposition.

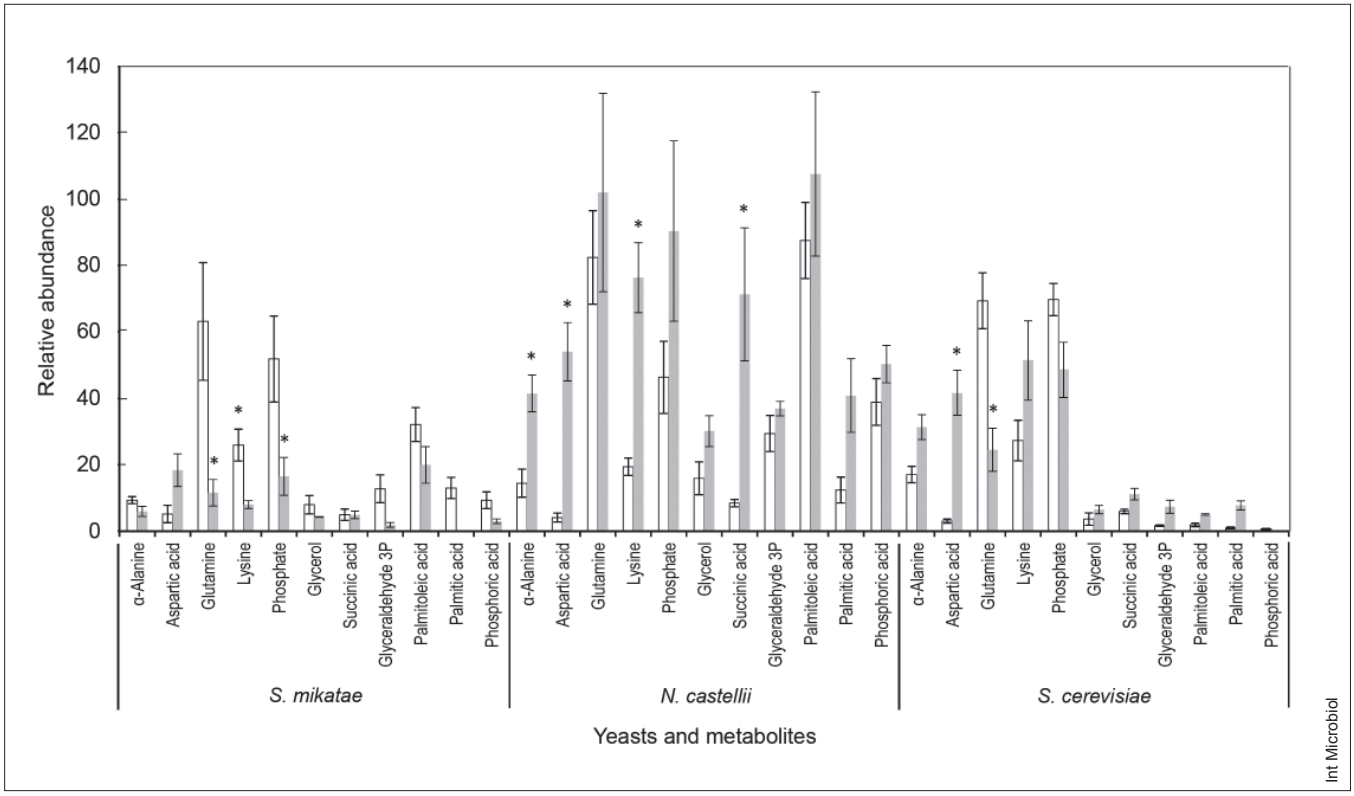

Fig. 3. Relative abundance of significant and stress-related compounds BD (white bars) and AR (grey bars) in *Saccharomyces cerevisiae, N. castellii* and *S. mikatae*. The values represent the means of three independent experiments. Results for each compound with statistically significant differences ($P < 0.05$) compared to the BD condition.

which fluctuates during dehydration and rehydration, is the cytoplasmic retention of glycerol [12,26]. In fact, in *N. castellii* and *S. cerevisiae* intracellular glycerol accumulation during stress imposition increased by 50%, whereas in *S. mikatae* it decreased by 50%, which further explains the low viability of these species (Fig. 3).

Our findings suggest a relationship between glycerol accumulation and desiccation tolerance, whereby a reduction in glycerol content during stress induction reduced cell viability. Glycerol biosynthesis is an important side-reaction of the glycolytic pathway, as it provides the NAD^+ necessary for cellular glycolysis activity [2]. Thus, according to our results, cells that synthesized glycerol during dehydration also had a backup supply of NAD^+ that allowed rapid glycolytic activation when cell activity was resumed. The NAD^+ generated is oxidized to maintain redox balance by the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate by the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In the GAPDH glycolytic pathway, under stress conditions, glycolytic flux is strongly directed to glycerol accumulation, depending on the kinetics of GAPDH [17]. *Saccharomyces*. *cerevisiae* is unusual in that three genes (*TDH1–3*) encode three different GAPDH isoenzymes, whereas other yeasts, such as *C. albicans* and *K. marxianus*, contain only one gene [1,15,28]. The *TDH1* gene encodes a less efficient GAPDH isoenzyme but it is the only gene that is expressed under conditions of cell stress [5,17]. Additional experiments are required to determine whether *TDH1* activation is responsible for glycerol accumulation during dehydration. Glycolysis is used by almost 800 yeast species in a sequence of enzymatic reactions that convert glucose to pyruvate, which, under aerobic conditions, is channelled into the mitochondria, where it enters the tricarboxylic acid cycle (TCA) and is eventually converted into acetyl-CoA.

Alternatively, pyruvate acts as a precursor for a side-reaction in α-alanine synthesis [19]. Intracellular α-alanine increased by 3-fold and 2-fold in N. castelli and *S. cerevisiae*, respectively, while in *S. mikatae* there was a 2-fold decrease between the BD and AR steps (Fig. 3). Acetyl-Co-A branchpoint metabolites can be converted into citric acid or lipids and

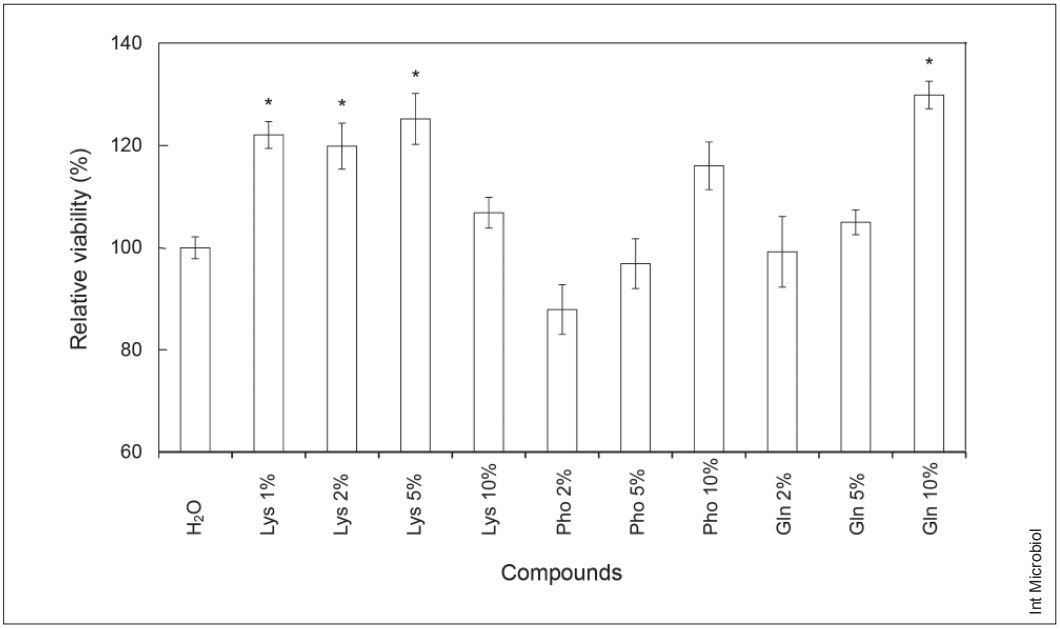

Fig. 4. Effect of rehydration on cell viability. *Saccharomyces mikatae* yeast cells were incubated at 37ºC for 30 min in pure water or in the presence of lysine (Lys), phosphate (Pho), or glutamine (Gln). The scale of relative viability (%) indicates the percentage of experimental values for the different yeasts relative to the non-complemented condition (H_2O). The values are the means of three independent experiments. Results with statistically significant differences ($P < 0.05$) compared to the H_2O condition are presented.

fatty acids, such as palmitoleic acid, which during dehydration increased in *N. castellii* and *S. cerevisiae* whereas in *S. mikatae* a 30% decrease was recorded. These results concerning palmitoleic acid and α-alanine indirectly suggest a greater reduction of pyruvate availability from the glycolytic pathway in *S. mikatae* than in the other two yeasts during stress induction. The only TCA cycle intermediate that showed significant variations during stress was succinic acid, whose levels increased by 9-fold and 2-fold in *N. castellii* and *S. cerevisiae*, respectively, while in *S. mikatae* the levels remained the same (Fig. 3). This accumulation of succinic acid in *S. cerevisiae* does not agree with previous results shown by Raab et al. [20]. The opposite is true in *S. mikatae* and could explain the insignificant differences between the BD and AR values while the high level of accumulation in *N. castellii* highlights its metabolic difference compared to the other two yeast species. In the TCA cycle, after three successive enzymatic steps, succinic acid is converted to oxaloacetate, another branch-point metabolite because it can be transaminated to form aspartate. The BD abundances of aspartic acid were similar in the three yeasts. However, in *N. castellii* and *S. cerevisiae* the AR levels of aspartic acid increased by 13-fold, but only by 3.5-fold in *S. mikatae* (Fig. 3). The changes in the abundances of aspartic acid and succinic acid suggested that *S. mikatae* cells should lack TCA activity during dehydration and rehydration, in contrast to *N. castellii* and *S. cerevisiae*. The TCA intermediate 2-oxoglutarate is also a branch-point metabolite; it is converted through transamination to lysine and glutamate, which can be used to synthesize other amino acids, such as glutamine, proline, and arginine. In our study, the BD contents of lysine and glutamine were similar in the three yeast species, whereas during stress imposition the changes in the abundances of these amino acids did not correlate with putative changes in yeast TCA activities. Thus, *S. mikatae* contained 3-fold less lysine after stress induction, while in *N. castellii* and *S. cerevisiae* lysine increased by 3-fold and 2-fold, respectively. Glutamine decreased significantly in *S. mikatae* and *S. cerevisiae*, by 6-fold and 3-fold, respectively. In fact, one of the two anaplerotic pathways responsible for replenishing the TCA cycle with intermediates, thereby maintaining its function, is the glyoxylate cycle, in which isocitrate dehydrogenase converts isocitrate into 2-oxoglutarate. The participation of both the TCA and glyoxylate cycles in providing 2-oxoglutarate to cells is not consistent which the changes in glutamine and lysine concentrations during stress induction. These variations also did not correlate with the dehydration tolerance profiles of any of the yeast species.

Effect of compound supplementation on yeast cell viability. We next sought to ascertain whether the low viability of *S. mikatae* after rehydration was due to low levels of lysine, Na_2HPO_4, or glutamine. Therefore, we tested several rehydration media in an attempt to overcome the drop in yeast viability during this process. In this experiment, all additives were resuspended in deionized water and pure water served as the reference condition for cell viability evaluation by flow cytometry. A statistically significant increase of 20% in cell survival driven by 1, 2, and 5% lysine or 10% glutamine supplementation was observed in *S. mikatae* (Fig. 4) but not in *S. cerevisiae*. Unexpectedly, phosphate supplementation was detrimental and increased cell death by 30% compared to the pure water reference condition (data not shown).

In conclusion, the changes that take place in cells that allowed them to overcome dehydration stress were investigated at the metabolomic level in *S. cerevisiae*, *N. castellii*, and *S. mikatae*. The extraction method for GC-MS analysis was optimized using a mini bead beater system, which enhanced cell breakage by ~20%. After data analysis and PCA, the observed variations in intracellular metabolites could be related to the form of central carbon metabolism, which offered a better understanding of the cellular metabolic differences between yeasts with respect to desiccation tolerance. Nevertheless, the poor survival of *S. mikatae* and *N. castellii* could not be ascribed to a single metabolite; rather, at least in *N. castellii*, the accumulation of both succinic acid and phosphoric acid by stress imposition might account for its low viability (60%). The metabolite profiles suggested the activity of the glycolytic pathway and TCA cycle in *N. castellii* and *S. cerevisiae*, but not in *S. mikatae*, which in the latter species could have led to a reduction in the levels of secondary metabolites required for viability following rehydration. Our results also demonstrated that the presence of lysine or glutamine during rehydration had a positive effect on the recovery of *S. mikatae* cell activity, although the benefits achieved by the supplementation of these compounds might be yeast dependent. Further investigations extending to other aspects such as global gene expression and proteomics will provide a better understanding of the cellular mechanisms involved in overcoming desiccation stress.

Acknowledgements. This work was financially supported by grants from the Spanish government (Projects AGL2010-22001-C02-02 and AGL2012-40018-C02-01, awarded to NR and RC, respectively). GL and AB also thank the Rovira i Virgili University for the doctoral fellowships FI-DGR 2012, AGAUR and 2009BRDI/12/12, respectively.

Competing interest: None declared.

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