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Keywords (separated by '-')	*D. bruxellensis* - Cold stress - Yeast - Ethanol production - Wine - Beer
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ORIGINAL PAPER

Cold exposure affects carbohydrates and lipid metabolism, and induces Hog1p phosphorylation in *Dekkera bruxellensis* strain CBS 2499

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Abstract *Dekkera bruxellensis* is a yeast known to affect the quality of wine and beer. This species, due to its high ethanol and acid tolerance, has been reported also to compete with *S. cerevisiae* in distilleries producing fuel ethanol. In order to understand how this species responds when exposed to low temperatures, some mechanisms like synthesis and accumulation of intracellular metabolites, changes in lipid composition and activation of HOG-MAPK pathway were investigated in the sequenced strain CBS 2499. We show that cold stress caused intracellular accumulation of glycogen, but did not induce accumulation of trehalose and glycerol. The cell fatty acids composition changed after the temperature downshift, and a significant increase of palmitoleic acid was observed. RT-PCR analysis revealed that *OLE1* encoding for Δ9-fatty acid desaturase was up-regulated, whereas *TPS1* and *INO1* didn't show changes in their expression. In *D. bruxellensis* Hog1p was activated by phosphorylation, as described in *S. cerevisiae*, highlighting a conserved role of the HOG-MAP kinase signaling pathway in cold stress response.

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Introduction

The ability of microorganisms to survive when exposed to environmental stress factors is regulated by different mechanisms, commonly referred as "stress response". Cold can represent such a factor for organisms living in temperate climate zones, where the temperature can fluctuate deeply during the day and all along the different seasons. Chilling operation is also widely used in food technology for the preservation and the shelf life extension of the products, since the temperature is one of the most important parameters that control the microbial growth. Particularly, in oenology cold treatments are generally carried out for technological purposes: firstly, a pre-fermentative cold maceration is a widespread technique used to extract higher anthocyanins and tannins contents with positive impact for the sensorial properties; secondly, the cold stabilization is a mandatory operation for white wines to keep tartaric acid crystals from forming after the wine has been bottled, avoiding the precipitation during the shelf life. *Saccharomyces cerevisiae* is known to accumulate molecules such as carbohydrates, glycogen and trehalose, as well as glycerol to face the environmental changes, ranging from nutrient limitations to

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temperature shift (Aguilera et al. 2007; Francois and Parrou 2001; Lillie and Pringle 1980). Consistent with this, an accumulation of glycogen and trehalose has been observed in baker's yeast after incubation at 10 and 0 °C, respectively (Kandror et al. 2004; Schade et al. 2004). High levels of trehalose have been shown to protect the cells also against freezing injuries; in fact, mutants unable to accumulate trehalose die more rapidly after freezing and thawing (Kandror et al. 2004).Cold-induced intracellular accumulation of glycerol has been proved to be activated upon a shift to low temperatures, with higher values reported at 4 °C than at 12 °C (Panadero et al. 2006). Another important and widespread response of living organisms to cold exposure is an increase in the proportion of unsaturated fatty acids in membrane phospholipids (Hazel and Williams 1990), and this is a clear example of homeostatic cellular control for structural and adaptive reasons.

In *S. cerevisiae* the adaptation to different stress conditions is controlled by the activation of a well characterised molecular signaling pathway, the HOG (High Osmolarity Glycerol) MAP Kinase pathway (Hohmann 2002), which is involved in several stress responses, like oxidative stress (Bisland et al. 2004), acetic acid stress (Mollapour and Piper 2006) and cold stress (Hayashi and Maeda 2006; Panadero et al. 2006).

D. bruxellensis is a frequent and increasing problem in wine industry as spoilage yeast, causing detrimental off-flavors but, on the other hand, its presence is desirable for the final aroma of Belgian lambic beer (Loureiro and Malfeito-Ferreira 2003; Schifferdecker et al. 2014). Interestingly, *D. bruxellensis* has been reported even to contaminate distilleries, especially in continuous fermentation systems, due to its ability to grow under anaerobic conditions and at high ethanol concentrations (Blomqvist et al. 2012; de Souza Liberal et al. 2007; Passoth et al. 2007). *D. bruxellensis* can use also nitrate as nitrogen source, and this characteristic can render this species able to overcome *S. cerevisiae* populations in fermentation processes (de Barros Pita et al. 2011; Galafassi et al. 2013b). Although *D. bruxellensis* and *S. cerevisiae* are considered two phylogenetically distant relatives, they share several peculiar traits, such as the ability to produce ethanol under aerobic conditions, high tolerance towards ethanol and acid, and ability to grow without oxygen (Rozpedowska et al. 2011). Apparently, these traits have evolved in parallel in both groups, but the molecular mechanisms involved may be different (Rozpedowska et al. 2011).

In order to elucidate the basis of the cold stress response in this industrially relevant yeast, mechanisms like synthesis and accumulation of intracellular metabolites, changes in lipid composition and activation of HOG-MAPK pathway upon the exposure to low temperatures (4 °C)were investigated in the sequenced strain CBS 2499 (Piškur et al. 2012).

Materials and methods

Media and growth conditions

CBS 2499 was the *D. bruxellensis* strain used in this work. Cellular stocks were stored at −80 °C in 15 % v/v glycerol and revitalized prior to each experiment in liquid mineral medium containing 20 g L^{-1} glucose and 6.7 g L^{-1} Yeast Nitrogen Base (YNB)without amino acids (Sigma-Aldrich, Saint Louis, MO, USA).

A first series of experiments was carried out (in triplicate) in liquid mineral medium (20 g $^{-1}$ glucose; 6.7 g L^{-1} YNB without amino acids), plus 0.1 M2-(*N*-morpholino) ethanesulfonic acid to maintain pH 5.5, by cultivating cells in agitated flasks at 200 rpm (aerobic conditions). Cell growth was monitored through the OD measurements at 600 nm by using a spectrophotometer (Jenway, 7315^{TM}Bibby Scientific Limited, Stone, United Kingdom), after appropriate dilution. For dry weight determinations, samples of cell cultures were collected, filtered through a glass microfiber GF/A filter (Whatman) and washed with three volumes of deionized water and dried at 105 °C for 24 h. To investigate the cold stress response, yeast cell suspensions were first inoculated at $OD_{600nm} = 0,1$ and cultivated at 30 °C and then, when the cultures reached the exponential phase ($OD_{600nm} = 1$), they were split in two aliquots: one (control culture) was maintained at 30 °C and the other one was chilled to 4 °C within 1 h and then incubated at the same temperature in an orbital shaker at 200 rpm.

A second series of experiments was performed in synthetic wine according to Vigentini et al. (2008), and without shaking (semi-anaerobic state) to simulate the oenological conditions. Briefly, *D. bruxellensis* was cultivated at 30 °C in static conditions in flasks

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using a synthetic medium similar in composition to wine (Vigentini et al. 2008). When the cultures reached the exponential phase ($OD_{600nm} = 1$), they were split in two aliquots: one (control) was maintained at 30 °C and the other was shifted to 4 °C, maintaining the static condition.

Viability and cultivability tests

The cell viability was assessed by staining aliquots from liquid cultures with a methylene blue solution (0.2 g L^{-1} methylene blue; 27.2 g L^{-1} KH_2PO_4; 0.071 g L^{-1} Na_2HPO_4; pH 4.6) and incubating for 15 min (Delfini and Formica 2001). Blue (dead) and white (live) cells were counted at the microscope by a Burker chamber, randomly selecting 10 sampling units. The viability was calculated as the percentage of viable cells (white) respect to the total counted cells (white plus blue).

The cell cultivability was determined by sampling aliquots from liquid cultures at 30 °C and after the shift to 4 °C they were decimally diluted in deionized water and plated onto YPD plates (glucose 20 g L^{-1}, yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, agar 20 g L^{-1}). After incubation at 30 °C for 7 days the colony forming units (CFU) were counted.

Quantification of reserve carbohydrates

Reserve carbohydrates were quantified, according to Parrou and Francois 1997, on cells corresponding to 10 mg of dry weight collected at different times from cultures growing on mineral medium at 30 °C (control cultures) and after the shift to 4 °C. Pellets were washed twice in deionized water and frozen at −20 °C. For glycogen and trehalose quantification pellets were lysed with 250 µL Na_2CO_3 0.25 M for 4 h at 95 °C; the pH was then decreased to 5.2 adding 150 µL of 1 M acetic acid and 600 µl 0.2 M sodium acetate, pH 5.2. Half of this suspension was then treated with 0.05 U mL^{-1} of trehalase (Sigma-Aldrich, Saint Louis, MO, USA) overnight at 37 °C, and the other half incubated overnight at 57 °C with 12 U mL^{-1} of amyloglucosidase. Clear supernatants of these suspensions were obtained by centrifugation at 5000×*g* for 3 min; glucose concentration was evaluated by a spectrophotometer using an enzymatic kit (Roche cat. num. 1 0716251 035, Hoffmann La Roche, Basel, Switzerland) prior and after treatment with trehalase and amyloglucosidase. Glycogen and trehalose concentration were expressed as the µg of glucose liberated by their hydrolysis per 10^7 cells ($\mu g_{eq\ glucose}\ 10^7\ cells^{-1}$).

For glycerol quantification, pellets were suspended in 1.5 mL of deionized water and boiled 10 min, and cell lysis was performed by mechanical disruption with acid-washed glass beads (425–600 µm, Sigma Aldrich, Saint Louis, MO, USA). Glycerol was determined with enzymatic kits (Roche, cat. 1 0148270 035) and expressed as $\mu g_{glycerol} mg^{-1}_{dry\ weight}$.

Hog1p phosphorylation

The phosphorylation of Hog1p protein was revealed by Western blotting analysis. Samples of cell suspensions from exponentially growing cultures ($OD_{600nm} = 1$) at 30 °C and at different times after the shift to 4 °C, were collected and frozen in liquid nitrogen. Protein extraction was performed in 5 % w/v SDS buffer containing proteases inhibitors (1mM phenylmethylsulfonyl fluoride, 1 µg/mL pepstatin and Protease Inhibitor Cocktail Complete (Roche) prepared as a 25× stock in dH_2O). Cells were mechanically disrupted with acid washed glass beads (425–600 µm, Sigma Aldrich) using the Precellys 24 tissue homogenizer (Advanced Biotech Italia Srl, Seveso, Italy), for 45 s alternating with 1 min incubations on ice. Unbroken cells and glass beads were removed by a 5 min centrifugation at 11,500×*g* at 4 °C. Proteins extracted were separated by SDS-PAGE on 8 % w/v polyacrylamide gel and immunoblotting was performed as previously reported (Galafassi et al. 2013a), with polyclonal anti-Hog1 (y-215) (sc-9079, Santa Cruz Biotechnology Inc., Dallas, TX, USA) and phospho-p38 MAPK (Thr180/Tyr182) (New England BioLabs Inc., Ipswich, MA, USA) respectively, diluted 1:1000 in TBS-BSA 0.5 % w/v and Tween 20 0.3 % v/v. Actin was used as reference to check the amount of protein loaded and it was detected with monoclonal anti-actin antibody (cat. no. MAB1501, Chemicon International Inc., MA, USA) 1000-fold diluted in TBS-BSA 0.5 % w/v and Tween 20 0.3 % v/v. Anti-rabbit and anti-mouse secondary antibodies were diluted 10,000 times. Bound antibodies were revealed using enhanced chemiluminescent substrate (LiteAblot Plus, EuroClone, Italy).

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Fatty acids extraction and analysis

Samples of cell suspensions, from exponentially growing cultures ($OD_{600nm} = 1$) at 30 °C and at different times after the shift to 4 °C, were extracted twice by the addition of 7.5 mL of a mixture of methanol/chloroform (1:2 v/v).The organic phase obtained after centrifugation 1200×*g* for 10 min, was evaporated with nitrogen and 2 ml of a toluene/methanol (1:4 v/v) mixture plus 200 µL of acetyl chloride were added. After 1 h of incubation at 100 °C, 5 mL of K_2CO_3 (6 % w/v in water) was added to the Pyrex glass tubes. After centrifugation at 1200×*g* for 10 min, the supernatant was transferred into amber glass vials and analyzed by gas chromatography.

The GC analysis was performed as described by Ackman (1986), partly modified. Separations were performed with a 30 m 0.32 mm i.d.Omegawax 320 capillary column (Supelco, Sigma Aldrich), under these conditions: initial isotherm, 140 °C for 5 min; temperature gradient, 2 °C min^{-1} to 210 °C; final isotherm, 210 °C for 20 min. The injector temperature was 250 °C. Injection volume was 1 µL with a split ratio of 1/100, and the FID temperature was 250 °C. Carrier and makeup gas were hydrogen and nitrogen, respectively. Fatty acid retention times were obtained by injecting the Omegawax test mix (Supelco) as standard. The obtained data were subjected to the Student's *t* test.

RT-qPCR analysis for transcription studies

Samples of cell suspensions, from exponentially growing cultures ($OD_{600nm} = 1$) at 30 °C and at different times after the shift to 4 °C, were collected for RNA extraction. To avoid RNA degradation, biomass was quickly recovered by centrifugation at 15,000×*g* for 1 min at 4 °C and immediately frozen with liquid nitrogen. Frozen pellets were mechanically disrupted with acid washed glass beads (425–600 µm, Sigma Aldrich) using the Precellys 24 tissue homogenizer and RNA was extracted with the RNeasy Plus MINI Kit (cat. num.74134, Qiagen,Velno, The Netherlands) following the supplier protocol. Concentration of the extracted RNA was determined measuring the absorbance at 260 nm and purity was checked with insuring that the absorbance ratios A_{260}/A_{230} and A_{260}/A_{280} were higher than 1.8. Integrity of RNA was controlled by agarose gel electrophoresis in denaturing conditions. 1 µg of RNA was used for the synthesis of cDNA using the QuantiTect Reverse Transcription Kit (cat. num. 205311, Qiagen).

Expression studies were carried out using the So Fast EvaGreen Supermix (cat. num. 172-5200, Bio-Rad, Richmond, CA, USA) in a BioRad C1000^{TM} Thermal Cycler. The mixtures for the amplification reaction were composed of 7.5 µL of EvaGreen master mix, 1 µL of each primer (333 nmol L^{-1}, final concentration), 2 µl of cDNA (5 times diluted cDNA synthesized from 1 µg of RNA) and 3.5 µL of deionized water. Cycling parameters were 98 °C for 30 s as hot-start, followed by 39 cycles at 95 °C for 3 s and at 60 °C for 5 s; a melting curve was included at end of each run, by increasing temperature from 65 to 95 °C. The sequences of target genes were obtained from the *D. bruxellensis* database (DOE Joint Genome institute database, JGI; http://genome.jgi-psf.org/Dekbr2/Dekbr2.home.html) after tBLASTn analysis by using their orthologous sequences in the *S. cerevisiae* genome from the SGD database (http://www.yeastgenome.org/) and the identity value here reported is the one automatically calculated by the database with default settings. The primers, listed in Table 1, were designed following the general rules suggested in the Real Time Application Guide supplied with the BioRad C1000^{TM} Thermal Cycler and each couple of primers was validated with the creation of standard curves by plotting Ct (cycle threshold) values obtained from real-time PCRs performed on dilution series of cDNA. From the standard curve, the amplification efficiency (E) was estimated by the software (BioRad CFX Manager). The *TUB1* gene was used as housekeeping gene (Rozpędowska et al. 2011), being also reported that the expression of the tubulin gene is not affected by cold stress in *S. cerevisiae* (Schade et al. 2004). Furthermore, the stability of the reference gene upon cold exposure was confirmed normalizing the amount of RNA used to prepare the cDNA and controlling that the resulting Ct of the reference gene didn't change between the condition tested. RNA was isolated from at least two independent growth experiments and each RNA batch was analyzed 3 times with an independent synthesis of cDNA. Each cDNA sample was run in technical triplicates during RT-qPCR assays, together with negative PCR control and negative RT control run in parallel as internal control. Results were analyzed with the $2^{-\Delta\Delta Ct}$ (Livak) method and statistical significances were calculated applying the *t*-test, setting the *p*-value at ≤ 0.05.

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Table 1 Target genes and relative primers sequences

Gene	Forward primer/reverse primer
Tubulin (*TUB1*)	5′-GTATCTGCTACCAGAAACCAACC-3′
	5′-CCCTCACTAACATACCAGTGGAC-3′
Trehalose-6-phosphate/phosphatase complex (*TPS1*)	5′-GCTGCCAGCGATATATGTCTAG-3′
	5′-CCAGCTCTTCCGTATTCCAC-3′
Inositol 3-phosphate synthase (*INO1*)	5′-CGGCTGTGGGTGATGATAAAG-3′
	5′-CCGCTCCCTCTTTCTTGTAGG-3′
Δ9-fatty acid desaturase (*OLE1*)	5′-CCGCTTTTACGTTGTCGTTGAC-3′
	5′-CAGAGCCAGACAAAACACCA-3′

Results

Effects of cold on growth, viability and cultivability in *D. bruxellensis*

To investigate the cold stress response in *D. bruxellensis*, we decided to focus our attention on the effects that a temperature downshift from 30 to 4 °C produces in *D. bruxellensis* CBS 2499, the genome of which was recently sequenced (Piškur et al. 2012). Experiments were carried out by cultivating the cells in aerobic conditions in shake flasks at 30 °C until the exponential phase was reached (1–2 OD_{600nm}), and by shifting the culture to 4 °C. After 6 h from the temperature downshift an abundant production of foam could be detected. This foam also adhered to the flask glass and, when examined under microscope, it contained a big amount of cells. This can explain the decrease of the biomass in the liquid medium detected after the temperature lowering to 4 °C (Fig. 1). On the other hand, both viability (Fig. 1) and cultivability (not shown) of the yeast cells remained higher than 90 %. During the cold incubation the cells consumed a small amount of glucose (approximately 2 g L^{-1}). As expected, in the control culture growing at 30 °C the biomass increased during the whole cultivation and glucose was exhausted (Fig. 2a). When cultures close to the end of their exponential growth phase were shifted to 4 °C a very similar behavior was also observed (data not shown).

D. bruxellensis is known to grow and spoil the wine (Boulton et al. 1996; Fugelsang 1996). To test the effect of cold when the cells were growing in such a challenging environment (presence of ethanol, low pH), *D. bruxellensis* was cultivated in static conditions on synthetic wine at 30 °C, as previously reported (Vigentini et al. 2008), and after reaching the exponential phase, the culture was shifted to 4 °C. Also in this case the cells maintained an high viability (85 %) (Fig. 1) and cultivability (not shown), despite the presence of ethanol 10 % v/v, pH 3.5 and low oxygen availability (due to the incubation in static conditions). This was observed also in the control culture that continued to be incubated at 30 °C (data not shown) and was in agreement with previously reported results (Vigentini et al. 2008).

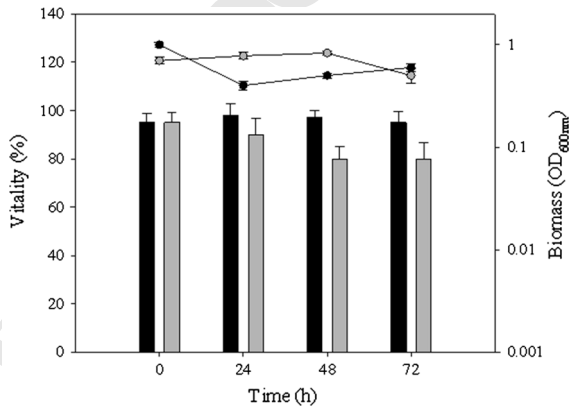

Fig. 1 Effect of the cold adaptation in *D. bruxellensis* CBS 2499 strain. Cells growing at 30 °C in exponential phase were shifted to 4 °C (time 0 corresponds to the downshift temperature). Biomass (*filled circle*, OD_{600nm}) of cells after the shift to 4 °C on mineral medium in aerobic conditions and on synthetic wine (biomass *open circle*, OD_{600nm}) in semi-anaerobic conditions are shown. *Bars* correspond to % viability (*black* on mineral medium, *grey* on synthetic wine)

Cold-induced accumulation of reserve carbohydrates

The synthesis and accumulation of reserve carbohydrates was analyzed in *D. bruxellensis* CBS 2499. Glycogen and trehalose were firstly accumulated up to

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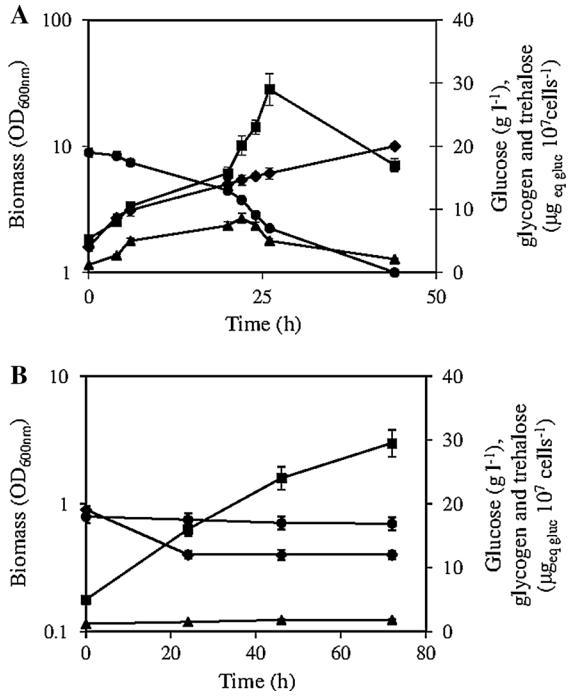

Fig. 2 Glycogen and trehalose accumulation **a** during the growth on glucose at 30 °C and **b** after the shift to 4 °C: *filled diamond* biomass (OD_{600nm}), *filled circle* glucose, *filled square* glycogen, *filled triangle* trehalose. Time 0 corresponds to the downshift temperature

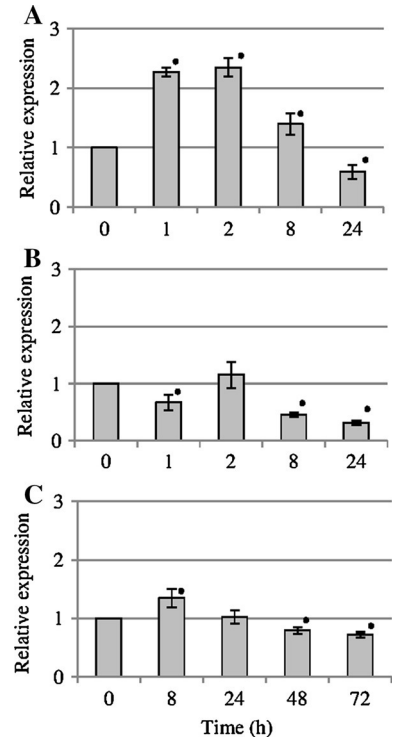

Fig. 3 Relative expression of *D. bruxellensis* genes involved in cold stress response during exponential growth on glucose at 30 °C (time 0) and at different times after the shift to 4 °C. **a** *OLE1*, **b** *INO1*, **c** *TPS1*; ● *p* value lower than 0.05

24 h at 30 °C and then were both degraded, in meanwhile glucose was exhausted within 48 h (Fig. 2a). However, when exponentially growing cultures were shifted to 4 °C, glycogen continued to be accumulated until to 72 h of incubation (Fig. 2b). On the contrary, the concentration of trehalose did not show any significant increase, in contrast to what observed at 30 °C (Fig. 2a, b). In order to verify the effect of the chilling at transcriptional level, the ortholog of *TPS1*, encoding in *S. cerevisiae* for trehalose synthetase, was identified in the CBS 2499 genome and showed a 67.8 % identity with the *S. cerevisiae* gene. The small increase of *DbTPS1* expression level observed during the incubation at 4 °C (Fig. 3) confirmed that in *D. bruxellensis* cold stress does not induce a specific accumulation of trehalose. Furthermore, the growth at 30 °C and the subsequent exposure to 4 °C did not induce the intracellular accumulation of glycerol, which maintained the same concentration measured at 30 °C (1 μg mg dry weight^{-1}).

Analysis of fatty acids composition upon exposure to cold stress

Among the genes that are mostly up-regulated in response to a temperature down-shift in *S. cerevisiae*, there are those encoding for enzymes involved in phospholipids synthesis and in fatty-acid desaturation (Murata et al. 2006; Nakagawa et al. 2002; Schade et al. 2004). We identified in the CBS 2499 genome the orthologs of *INO1* and *OLE1*, encoding respectively for inositol 3-phosphate synthetase and Δ9-fatty acid desaturase, that showed 70.6 and 65 % identity with the *S. cerevisiae* genes. The analysis of their expression patterns during exposure to low temperature indicated a two folds increased level only in the case of *DbOLE1* (Fig. 3), whereas *DbINO1* showed a down regulation (Fig. 3). This prompted us to examine the effect of cold on fatty acids composition in total lipids (Table 2). Noteworthy, the fatty acids profile of *D. bruxellensis* showed the presence of di- and polyunsaturated fatty acids, that are present in several yeast

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Table 2 Fatty acids composition (%) during the growth at 30 °C and after 24 h from the downshift to 4 °C

	30 °C	4 °C	*p*
14:0	1.3 ± 0.2	2.0 ± 0.1	0.002
16:0	25.0 ± 0.6	19.9 ± 0.3	<0.001
16:1n7	34.5 ± 1.8	41.6 ± 0.3	<0.001
18:0	5.7 ± 0.6	12.8 ± 0.3	<0.001
18:1n9	15.4 ± 0.9	8.5 ± 0.1	<0.001
18:1n7	5.7 ± 0.3	4.2 ± 0.1	<0.001
18:2n6	12.0 ± 0.5	9.9 ± 0.1	<0.001
18:3n6	Traces	Traces	
18:3n3	Traces	Traces	
20:0	Traces	1.2 ± 0.1	

species but not in *S. cerevisiae* (Ratledge and Evans 1989). Although the UFA/SFA (unsaturated fatty acids/saturated fatty acids) ratio was quite similar in the two conditions (2.1 at 30 °C and 1.8 at 4 °C), the level of C16:1n7 (palmitoleic acid) was significantly increased. The concomitant decrease of 16:0 could indicate that Δ9-desaturase, encoded by *DbOLE1*, could have a higher affinity for C16:0 than for C18:0. On the other hand, C18:1n9, C18:1n7 and C18:2n6 decreased, whereas C18:3n6 and C18:3n3 did not show significant variations.

Involvement of the HOG pathway in the cold stress response

S. cerevisiae mutants in the HOG-MAPK pathway are sensitive to osmotic stress and partially to cold (Albertyn et al. 1994; Panadero et al. 2006), demonstrating its involvement in the response to both stress conditions. In particular, the activation of Hog1p occurs through the dual phosphorylation of Thr-174 and Tyr-176 residues by Pbs2p, and this event determines its translocation to the nucleus where it participates to the transcriptional regulation of target genes (Alepuz et al. 2001; Ferrigno et al. 1998; Mas 2009; Rep et al. 2000). The immunoblotting analysis (Fig. 4) performed by using a commercial anti-phospho-p38 antibody, that specifically recognizes the dually phosphorylated form of Hog1p, showed the presence of a single band of the expected mobility in protein extracts obtained from cells collected after 30 min from the shift to 4 °C. This indicated that Hog1p was clearly phosphorylated, and its phosphorylation lasted for at least 6 h (Fig. 4).

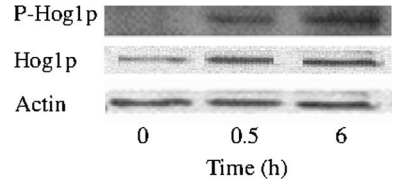

Fig. 4 Western blot image showing the phosphorylation of HOG1p (P-Hog1p) upon the exposure at 4 °C

Discussion

In this work mechanisms involved in the ability to survive upon the exposure to cold were analysed in the strain of *D. bruxellensis* recently sequenced (Piškur et al. 2012).

The ability to survive in mineral medium as well as in particularly "uneasy" environmental conditions, such as in the presence of high ethanol concentration, low pH, low oxygen (synthetic wine) even at cold temperature reinforces the idea that this species has evolved specific traits to occupy unfavorable niches. These phenotypic characteristics represent an interesting clue from a technological point of view, to develop appropriate strategies to counteract its presence in food processes as well as to use this yeast species for industrial processes.

Complex transcriptional and post-translational mechanisms regulate reserve carbohydrates synthesis and degradation, to integrate the control of their metabolism and cell growth in *S. cerevisiae* (Francois and Parrou 2001; Thevelein and Hohmann 1995). In *D. bruxellensis* few studies have been performed in different strains and conditions so far. Intracellular accumulation of glycogen has been shown in molasses fermentations, whereas the trehalose was found undetectable (Pereira et al. 2014). Increased expression levels of *D. bruxellensis* orthologs *TPS2* and *NTH1* during a model grape juice fermentation have been reported (Nardi et al. 2010). Our results pointed out that glycogen metabolism in CBS 2499 growing at 30 °C is very similar to the one reported in *S. cerevisiae* (Parrou et al. 1999), but the trehalose formation is very different. This carbohydrate was in fact accumulated and then degraded during the growth at 30 °C on glucose (Fig. 2a), in contrast to *S. cerevisiae* that starts to accumulate trehalose only after glucose depletion, at the onset of the diauxic shift (Parrou et al. 1999). In this regard, the differences in

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the kinetics of reserve carbohydrates metabolism between *D. bruxellensis* and *S. cerevisiae* could have important implications on the control of glycolytic flux, which deserve further investigations. The exposure to cold did not produce any rise of intracellular trehalose level in *D. bruxellensis*, but, on the contrary, glycogen was accumulated. Also in this case this behavior is very different to what occurs in *S. cerevisiae*, where the level of both carbohydrates has been shown to increase in response to cold (Kandror et al. 2004; Schade et al. 2004). In addition, intracellular accumulation of glycerol was not found in *D. bruxellensis*, again in contrast to what has been reported in *S.cerevisiae* (Panadero et al. 2006). Recently we showed that glycerol can accumulate upon exposure to osmotic stress in *D. bruxellensis* (Galafassi et al. 2013a), indicating that this compound plays a specific role in the osmotic response but it is not required to protect the cells in the cold response. Environmental temperature is known to affect the lipid composition in order to maintain an optimal membrane fluidity (Swan and Watson 1997). Phospholipids with unsaturated fatty acids have a lower melting point and more flexibility (Murata and Wada 1995), and such adaptation involves the induction of fatty acid desaturases (Nakagawa et al. 2002; Schade et al. 2004; Weber et al. 2001). In *S. cerevisiae* the increased expression of *OLE1*, which encodes the only desaturase found in this yeast (Stukey et al. 1989), results in an increased degree of unsaturation of total fatty acids (Nagawa et al. 2002). The analysis of cell fatty acids composition revealed that in *D. bruxellensis* CBS 2499 a significantly increased level of palmitoleic acid (16:1n7) resulted upon the downshift of temperature, in agreement with the induced expression of *DbOLE1* gene. This fatty acid has a lower melting point than C18:1n7, and its increase observed at 4 °C may help to maintain membrane fluidity. Interestingly, the presence of polyunsaturated fatty acids in this species can play a protective role in cold exposure.

Finally, the exposure to 4 °C triggered in *D. bruxellensis* the activation of HOG-MAPK pathway (Fig. 4). The activation of this pathway by cold stress in *S. cerevisiae* has been proposed to occur through the decrease of membrane fluidization (Hayashi and Maeda 2006), consistent with the observation that also hyper-osmotic stress as well as DMSO treatment, that produce the same stimulus, induce this pathway (Hayashi and Maeda 2006; Laroche et al. 2001). Our observations in *D. bruxellesis* are in agreement with the results obtained in *S. cerevisiae*, as recently we reported that the HOG-MAPK pathway is activated upon the exposure to osmotic stress (Galafassi et al. 2013a). The availability of the complete genome sequence and the development of molecular tools (transformation, gene deletion, RNA silencing, etc.) will allow in next future to identify the specific genes that are under the control of this regulatory pathway in stress response.

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