Ann Microbiol (2016) 66:351–359
DOI 10.1007/s13213-015-1115-0

ORIGINAL ARTICLE

Strain-dependent tolerance to acetic acid in *Dekkera bruxellensis*

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Received: 2 April 2015 / Accepted: 3 June 2015 / Published online: 26 June 2015
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Abstract *Dekkera bruxellensis*—a yeast species associated with wine and beer production—has recently received attention because of its ability to compete with *Saccharomyces cerevisiae* in distilleries producing fuel ethanol, and due to its resistance to high ethanol and acid levels. The tolerance to acetic acid in 29 strains of *D. bruxellensis* was investigated by screening growth at different concentrations up to 120 mM at pH 4.5. Different metabolic responses were exhibited in three strains (CBS 98, CBS 2499 and CBS 4482) that were analysed by their FTIR-metabolomic fingerprint. Physiological studies showed that the presence of acetic acid significantly affected their growth, causing a different reduction in growth rate, glucose consumption and ethanol production rates, as well as biomass and ethanol yields. The examined strains were unable to metabolise acetic acid in the presence of glucose, probably due to a glucose repression mechanism on the acetyl-CoA syntethase activity. Interestingly, the cells continued to produce acetic acid as byproduct of their fermentative metabolism. We also showed that the HOG MAP kinase pathway was not activated by phosphorylation upon exposure of the cells to acetic acid.

Electronic supplementary material The online version of this article (doi:10.1007/s13213-015-1115-0) contains supplementary material, which is available to authorized users.

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Keywords *Dekkera bruxellensis* · Acetic acid · Yeast metabolism · FTIR · Wine yeast · Ethanol production

Introduction

Several yeast species are able to grow at low pH and in environments containing weak acids. Weak acids like sorbic acid, benzoic acid, propionic acid and acetic acid have been approved by the EU Commission for use as food preservatives (Commission Regulation 2011). In particular, acetic acid, which is a byproduct of sugar fermentation by some yeasts belonging to genera *Saccharomyces*, *Brettanomyes/Dekkera*, and *Hanseniaspora/Kloeckera* (van Dijken and Scheffers 1986), is also one of the inhibitors commonly released during the hydrolysis of lignocellulosic materials used for second generation biofuel production. Therefore, elucidation of the mechanisms underlying the resistance of yeasts to acetic acid may have relevant impacts on the improvement of these biotechnological processes.

The effect of acetic acid has been investigated widely in *Saccharomyces cerevisiae* (Piper 2011) and in a few other non-conventional and more tolerant yeasts, like *Zygosaccharomyces bailii* (Guerreiro et al. 2012). The toxicity of acetic acid is strictly dependent on the pH of the medium: at low pH, in fact, since it is present mainly in undissociated form, it can diffuse into the cell in a passive way. In *S. cerevisiae*, the Fps1p aquaglyceroporin channel (Mollapour and Piper 2007) plays an important role in acetic acid uptake. Acetic acid dissociates within the cytosol, producing a decrease in the intracellular pH, which is detrimental to many cellular processes, including the activity of some glycolytic enzymes (Pearce et al. 2001). The protons generated are pumped out by the activity of the ATPase Pma1p and, at the same time, the anion is extruded by the activity of plasma

membrane multidrug resistance transporters (Holyoak et al. 1996; Carmelo et al. 1997; Mira et al. 2010a, 2010b; Stratford 2013a). The function of plasma membrane proteins has been shown to be linked to the lipid composition of the membranes (Wang and Chang 2002), and recent work reveals that the acetic acid stress response results in wide lipidome rearrangements, which could then represent one of the most important mechanisms of acetic acid resistance (Lindberg et al. 2013). In *S. cerevisiae*, the adaptation to acetic acid also requires the molecular signalling HOG (high osmolarity glycerol) MAP kinase pathway (Mollapour and Piper 2006; Hohmann 2009).

In the present study we investigated the response to acetic acid stress in *Dekkera bruxellensis* species. This yeast is associated with wine production and lambic beer fermentation, where it may contribute in a negative or positive manner to flavour development (Fleet 1992; Zuehlke et al. 2013; Schifferdecker et al. 2014). Indeed, this species is able to produce phenolic compounds, such as 4-ethylguaiacol and 4-ethylphenol that could spoil the wine, depending on their concentration (Vigentini et al. 2008; Malfeito-Ferreira 2011). Several strains have been isolated also from other sources, such as from apple cider, as well as from other sweet drinks (Morrissey et al. 2004; Teoh et al. 2004; Gamero et al. 2014). *D. bruxellensis* has also been reported to contaminate distilleries producing fuel ethanol, especially in continuous fermentation systems, where this yeast can compete with *S. cerevisiae* (de Souza Liberal et al. 2007; Passoth et al. 2007). Recently, it has been shown that *D. bruxellensis* can also use nitrate as nitrogen source—a characteristic well suited to industrial fermentation (de Barros Pita et al. 2011; Galafassi et al. 2013a). All these metabolic features have led to the idea that *D. bruxellensis* could be used for industrial ethanol production (Pereira et al. 2012). Although *D. bruxellensis* and *S. cerevisiae* are considered as two phylogenetically very distant relatives, they share several peculiar traits, such as the ability to produce ethanol under sugar excess and aerobic conditions, a high tolerance towards ethanol and acid, and the ability to grow without oxygen (Galafassi et al. 2011; Rozpędowska et al. 2011) and they cohabitate in several niches. In this work, we analysed how the presence of acetic acid affects its growth and sugar metabolism. In recent years Fourier transform infrared spectroscopy (FTIR) has been applied to whole cell analysis in various microbiological studies (Zhao et al. 2004; Szeghalmi et al. 2007; Roscini et al. 2010). FTIR analysis can discriminate the physiological states of microbial cells throughout growth and differentiation, irrespective of the type of cells considered (Adt et al. 2006; Cavagna et al. 2010; Corte et al. 2011). A FTIR-based bioassay was developed recently to determine the presence and extent of cellular stress, with the rationale that stressing conditions can alter the cell metabolome before and after cell death (Corte et al. 2010). Using this tool, we obtained information about the effects on *D. bruxellensis* caused by exposure to acetic acid.

Materials and methods

Strain collection and screening for acetic acid tolerance

The following *D. bruxellensis* strains were used to screen for acetic acid tolerance: CBS 72, CBS 73, CBS 74, CBS 75, CBS 78, CBS 96, CBS 97, CBS 98, CBS 1940, CBS 1941, CBS 1942, CBS 1943, CBS 2336, CBS 2499, CBS 2547, CBS 2796, CBS 2797, CBS 3025, CBS 4459, CBS 4480, CBS 4481, CBS 4482, CBS 4601, CBS 4602, CBS 4914, CBS 5206, CBS 5512, CBS 6055, CBS 8027 (CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands).

For long-term storage, yeast strains were maintained at −80 °C on 15 % (v/v) glycerol and 85 % (v/v) YPD broth (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose).

Overnight cultures were grown in YPD broth at 30 °C under orbital shaking. Cells at exponential growth phase were collected by centrifugation (4000*g*), washed twice in distilled water and re-suspended in 0.1 M phosphate buffer (pH 4.5). A 10-fold dilution series was obtained in order to spot 100.000 to 100 cells onto YPD agar (15 g/L) plates supplemented with 0 , 40, 80, 100 and 120 mM acetic acid and at pH adjusted to 4.5 (in triplicate). Growth at 30 °C was monitored from 7 to 11 days.

Growth conditions and metabolite assays

In order to investigate tolerance to acetic acid, liquid cultures of selected strains were grown in YPD broth at 30 °C under shaking and monitored through the increase in OD at 600_{nm} using a spectrophotometer (Jenway, 7315^{TM} Bibby Scientific Limited, Stone, UK). Cells from pre-cultures grown in YPD broth were harvested during the exponential phase by centrifugation and, after washing, inoculated at OD_{600nm} 0.1 into YPD broth at pH 4.5 supplemented with acetic acid (120 mM) or not supplemented (control cultures). The ability to use the acetic acid as the only carbon source was also tested in YP (10 g/L yeast extract, 20 g/L peptone) supplemented with acetic acid (80, 120 mM) and pH adjusted to 4.5. The media were sterilised by filtration (Millipore, 0.22 μm). All the experiments were replicated twice.

For dry weight measurements (DW), samples from the shake flask cultures were collected at different times (in triplicate at each point), the cells were filtered through a glass microfiber GF/A filter (Whatman) and washed with three volumes of de-ionised water and dried at 105 °C for 24 h. Glucose, ethanol and acetic acid concentrations in the supernatants were assayed (in triplicate) by using commercial enzymatic kits (Roche, cat. no. 1 0716251 035, 1 0176290 035

and 1 0148261 035 respectively, Hoffmann La Roche, Basel, Switzerland). The growth rates (μ_{max}), the specific glucose consumption rates and the specific ethanol/acetic acid production rates (Table 1) were calculated. Biomass yield was determined as the amount of biomass obtained divided by the amount of glucose consumed. Product yields were calculated as the total amount of products divided by the total amount of glucose utilised.

Cell viability and cultivability

To test the effect of acetic acid on cell viability, aliquots from liquid cultures growing exponentially were collected, centrifuged, washed and incubated with a methylene blue solution (0.2 g/L methylene blue; 27.2 g/L KH_2PO_4; 0.071 g/L Na_2HPO_4, pH 4.6) for 15 min. Blue (dead) and white (live) cells were counted under a microscope. The percentage of viable cells with respect to the total counted cells is given as viability.

To assess the cultivability in the presence of acetic acid, aliquots from liquid cultures growing in YPD broth and in YPD broth supplemented with acetic acid were collected after defined growth times, serially diluted (10-fold dilution series) and plated (in triplicate) on YPD agar at pH 4.5 (control) and YPD agar supplemented with acetic acid 120 mM at pH 4.5. After incubation at 30 °C, the cultivability was calculated as the percent of colony forming units (CFU) observed in YPD plus acetic acid with respect to those detected in the control YPD plates.

Determination of acetyl-CoA synthetase activity

The activity of acetyl-CoA synthetase (ACS) was determined in cells collected during the exponential growth phase from cultures growing on YPD broth at pH 4.5 supplemented with acetic acid or not supplemented (control). Cell extracts were prepared by disruption with acid-washed glass beads (425–600 μm, Sigma, St. Louis, MO) using the Precellys 24 tissue homogenizer (Advanced Biotech Italia, Seveso, Italy), as previously described (Pereira et al. 2012), and total protein concentrations were determined using a Bio-Rad kit no. 500-002 (Bio-Rad, Hercules, CA) using bovine serum albumin as standard. The specific activity of ACS in cell extracts was determined at room temperature using a spectrophotometer at 340 nm as described previously (Postma et al. 1989), with the only exception that the enzyme reaction started with 100 mM potassium acetate instead of 10 mM.

FTIR-metabolomic fingerprint analysis

Three strains (CBS 98, CBS 2499 and CBS 4482) were selected to investigate the metabolomic changes induced by the presence of acetic acid stress during the growth. A 10 mL sample of cell suspension growing in exponential phase at 30 °C under orbital shaking in YPD broth at pH 4.5 without (control) and with 120 mM acetic acid, was centrifuged, washed twice with distilled sterile water and re-suspended in 1.5 mL HPLC grade water in polypropylene tubes, in order to have a standardized OD_{600nm} around 5.0. An aliquot of 105 μL was sampled for three independent FTIR readings [35 μL each, according to the technique suggested by Essendoubi et al. (2005)].

FTIR analysis was carried out with a TENSOR 27 FTIR spectrometer, equipped with HTS-XT accessory for rapid automation of the analysis (Bruker Optics, Ettlingen, Germany). FTIR measurements were performed in transmission mode. All spectra were recorded in the range between 4000 and 400 cm^{-1}. Spectral resolution was set at 4 cm^{-1}, sampling 256 scans per sample. The software OPUS version 6.5 (Bruker) was used to carry out the quality test, baseline correction, vector normalization and the calculation of the first and second derivatives of spectral values. The script MSA (metabolomic spectral analysis) employed in this study was developed in "R" language to carry out the following operations on the matrices of spectral data exported as ASCII text from OPUS 6.5. The analytical procedure consisted of calculating the distance between the spectrum of the cells under test and that of the cells without the stressing agent; this procedure

Table 1 Growth parameters of *Dekkera bruxellensis* strains cultured in YPD (control) and YPD supplemented with 120 mM acetic acid (YPDAA). Experiments were performed in duplicate (the range of values is reported)

Strain	Growth conditions	Growth rate μ_{max} (h^{-1})	Specific consumption rate (mmol gDW^{-1} h^{-1})	Specific production rate (mmol gDW^{-1} h^{-1})		Yield (g g^{-1})			Viability
			Glucose	Ethanol	Acetic acid	Biomass	Ethanol	Acetic acid	
CBS 4482	YPD	0.16–0.18	3.85–3.80	3.25–3.05	1.47–1.32	0.27–0.25	0.24–0.22	0.15–0.13	99 %
	YPDAA	0.06–0.07	1.62–1.53	1.32–1.27	0.94–0.087	0.24–0.20	0.23–0.21	0.20–0.18	93 %
CBS 2499	YPD	0.18–0.20	4.63–4.55	6.36–6.01	1.15–0.95	0.24–0.22	0.36–0.31	0.08–0.07	99 %
	YPDAA	0.05–0.06	1.89–1.76	1.40–1.35	0.96–0.91	0.19–0.17	0.24–0.22	0.024–0.20	86 %
CBS 98	YPD	0.11–0.13	1.94–1.89	2.51–2.47	0.76–0.70	0.24–0.21	0.29–0.26	0.14–0.12	99 %
	YPDAA	0.017–0.019	0.81–0.76	1.14–0.98	0.27–0.22	0.15–0.13	0.33–0.30	0.18–0.15	73 %

was extended to five different spectral regions in order to differentiate the stress response among the various classes of molecules. Each single spectrum was normalized in order to have the range spanning from 0 to 1 in the manner suggested by Huang et al. (2006). Average spectra from the three repetitions were calculated. Response spectra (hereinafter reported as RS) were calculated as the difference between each average spectrum and the average spectrum of the same cells maintained in YPD broth without acetic acid, defined as control spectrum (Corte et al. 2010).

Western blotting analysis

For Western blotting analysis, exponentially growing cells in YPD pH 4.5 (around 1–2 OD600nm) were harvested, suspended in fresh medium containing 120 mM acetic acid and incubated at 30 °C. After different times of incubation, cells were collected and frozen in liquid nitrogen. Protein extraction was performed in 5 % (w/v) SDS and cells were mechanically disrupted by glass beads using the Precellys 24 tissue homogenizer (Advanced Biotech Italia). Proteins extracted were separated by SDS-PAGE on 8 % (w/v) polyacrylamide gel and immunoblotting was performed as previously described (Gatti et al. 1994). Hog1p and phospho-Hog1p were analyzed as reported (Hernandez-Lopez et al. 2006) with rabbit polyclonal anti-Hog1 (y-215) (sc-9079) raised against amino acids 221–435 of Hog1p of *S. cerevisiae* (Santa Cruz Biotechnology, Dallas, TX) and anti-phospho-p38 MAPK (Thr180/Tyr182) respectively, diluted 1:1000 in TBS-BSA 0.5 % (w/v) and Tween 20 0.3 % (v/v). Actin was used to check the amount of protein loaded and it was detected with monoclonal anti-actin antibody (cat. no. MAB1501, Chemicon International, Billerica, MA) 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. Bounded antibodies were revealed using enhanced chemiluminescent substrate (Lite Ablot Plus, EuroClone, Pero, Italy).

Results and discussion

Strains screening for acetic acid tolerance

The tolerance of *D. bruxellensis* species to the acetic acid was investigated through screening of 29 strains, cultivated on plates of rich glucose medium (YPD) adjusted to pH 4.5 and containing acetic acid at concentrations ranging from 0 to 120 mM. After 7 days of incubation, all the strains were able to grow in the presence of 40 mM acetic acid, while several strains failed to grow on plates containing higher concentrations of acetic acid. Prolonging the incubation to 11 days, all the strains formed colonies at 120 mM acetic acid at the highest spotted cell concentration (corresponding to 10^5 cells in the spot), whereas only two strains, CBS 2499 and CBS 4482, grew even at the lowest cell concentration (corresponding to 100 cells spotted) (Online Resource 1). This prompted us to determine the proportion of cells that were able to form a colony under these conditions (cultivability, see Materials and methods). A decrease of 10 % and 55 % in colony forming units (CFU) due to the presence of 120 mM acetic acid resulted for CBS 4482 and CBS 2499, respectively. In the case of CBS 98—one of the less resistant strains—the cultivability fell by more than 99 %: in fact approximately 200 CFU were obtained on plates containing acetic acid that were spread with 10^5 cells. In conclusion, all the tested *D. bruxellensis* strains proved able to face increasing concentrations of acetic acid on glucose-containing media (YPD) at pH 4.5, but the results of the screening revealed that only a part of population was able to proliferate under these conditions, with the size of this fraction being strain dependent.

The presence of acetic acid affects growth and glucose metabolism

A more detailed analysis of acetic acid tolerance in *D. bruxellensis* was performed on three strains: CBS 2499 and CBS 4482 (the most resistant strains from the screening trial), and CBS 98 (one of the less resistant strains). The genome of one of these strains (CBS 2499) has been sequenced recently (Piškur et al. 2012). At first, we tested the ability of the strains to use acetic acid as carbon source. Liquid cultures were grown at 30 °C under aerobic conditions on YP containing acetic acid and adjusted to pH 4.5. CBS 2499 and CBS 4482 strains were able to grow in the presence of 120 mM acetic acid and to consume it, while CBS 98 strain grew at acetic acid concentrations not greater than 80 mM.

Saccharomyces cerevisiae has been reported to be unable to co-metabolize glucose and acetic acid simultaneously (Fernandes et al. 1997), but co-consumption has been described recently (Lindberg et al. 2013). In contrast, the high acetic acid resistance in *Z. bailii* species has been associated with its ability to metabolise this acid also in presence of glucose (Rodrigues et al. 2012), due to a specific and not glucose repressed transport system and a higher metabolic flux through acetyl-CoA synthetase (Sousa et al. 1996; Rodrigues et al. 2012). In order to investigate the influence of acetic acid on glucose metabolism, the selected strains were cultivated in YPD medium at pH 4.5 supplemented with acetic acid and compared to the control (without acetic acid). In general, the three strains showed a typical respire-fermentative metabolism, producing biomass and ethanol (Fig. 1), but differences in all the investigated growth parameters (growth rates, specific glucose consumption rates, ethanol and acetic acid production rates, and yields) with respect

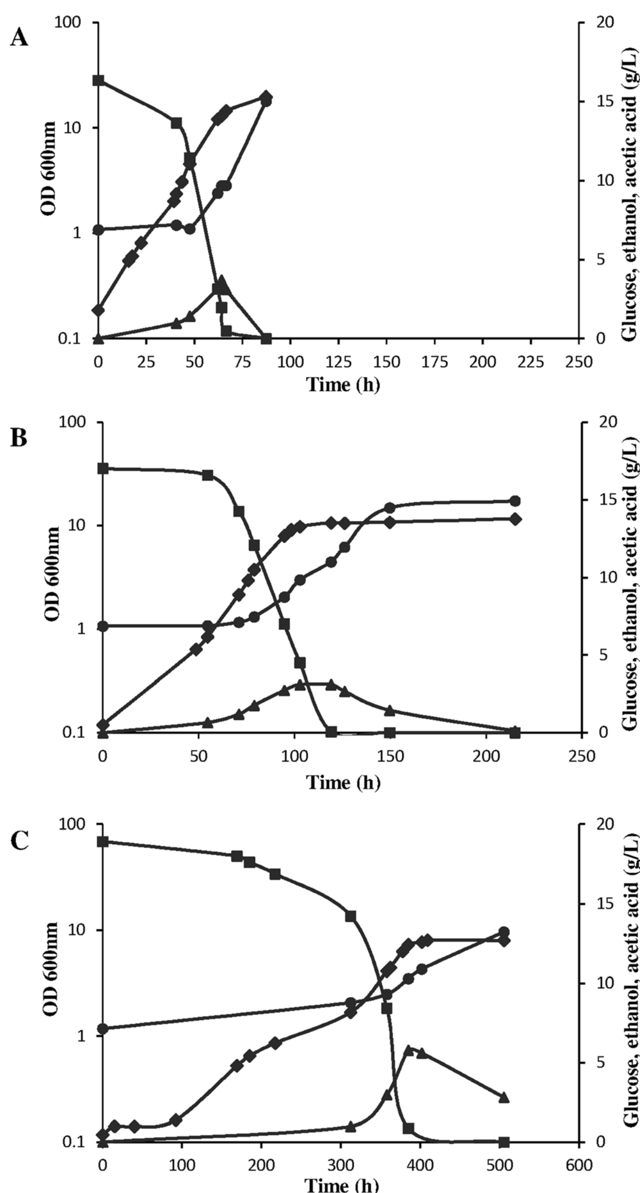

Fig. 1 Growth of *Dekkera bruxellensis* strains in YPD supplemented with 120 mM acetic acid at pH 4.5. **a** Strain CBS 4482, **b** Strain CBS 2499, **c** Strain CBS 98. ■ Glucose, ♦ OD_{600nm}, ● acetic acid, ▲ ethanol

to control conditions were observed (Table 1). Notably, the control culture of CBS 4482 strain showed the highest biomass yield and the lowest ethanol yield (Table 1), which could reflect a greater contribution of respiratory metabolism when this strain utilises glucose as carbon source.

The maximum specific growth rates (μ_{max}) were affected greatly by the presence of acetic acid for all three strains, with reductions in values ranging from 56 % to 84 % in comparison to control cultures (Table 1). From a metabolic point of view, *D. bruxellensis* behaved similarly to *S. cerevisiae*, with all three strains unable to co-metabolize glucose and acetic acid simultaneously (Fig. 1). This is probably due to a mechanism of glucose repression on acetyl-CoA synthetase activity (Table 2) that was below the limit of detection in cell extracts from cultures growing on glucose medium supplemented with acetic acid compared to those grown with acetic acid only. Glucose repression of acetyl-CoA synthetase has been reported also in *Dekkera anomala* (Geros et al. 2000). The biomass yields were lower in all cultures exposed to acetic acid (Table 1), but the reduction was greater in CBS 98 (46 %) than in strains CBS 4482 and CBS 2499 (10 % and 20 % respectively). In the former strain, this phenomenon could indicate that a higher amount of energy (ATP) had to be redirected from biomass synthesis toward the extrusion of protons out of cells.

The presence of acetic acid also reduced the specific glucose consumption rates (Table 1), with the lowest value of glucose consumption rate being observed in the CBS 98 strain. Ethanol was synthesised as in the control cultures, but the specific ethanol production rates decreased as well as the yields (Table 1). Surprisingly, we found that the tested *D. bruxellensis* strains continued to produce acetic acid as a byproduct of fermentative metabolism, even when this compound was present as a component of the medium (Fig. 1, Table 1), thus resulting in a further rise in its final concentration. All these facts could indicate that the acetaldehyde dehydrogenase activity was not inhibited by the increasing concentration of acetic acid, and we speculate that a very efficient export system is operating. In *S. cerevisiae*, expression of *TPO2*, *TPO3* and *AQR1*, encoding transporters of the major facilitator superfamily thought to mediate the active expulsion of acetate, is activated in presence of acetic acid (Fernandes et al. 2005; Mira et al. 2010a). Furthermore, the presence of acetic acid causes oxidative stress in cells (Giannattasio et al. 2005; Martani et al. 2013), which increases the demand for NADPH to counteract this. This fact might play an important role in maintaining active acetic acid production, as a way to generate this reduced cofactor. A similar situation has been reported recently in *D. bruxellensis*, due to a specifically increased NADPH demand for nitrate utilisation as a nitrogen source (Galafassi et al. 2013a). When the glucose was exhausted, the cells started to consume ethanol, converting it to acetic acid, and this resulted in a further increase in the acetic acid concentration, up to 250 mM (Fig. 1), and a

Table 2 Acetyl-CoA synthetase activity of *D. bruxellensis* strains cultivated under different growth conditions

Strain	Growth conditions	Acetyl-CoA synthetase activity (U/mg)
CBS 4482	YP+120 mM acetic acid	0.056±0.004
	YPD+120 mM acetic acid	Not detectable
CBS 2499	YP+120 mM acetic acid	0.052±0.004
	YPD+120 mM acetic acid	Not detectable
CBS 98	YP+80 mM acetic acid	0.187±0.02
	YPD+120 mM acetic acid	Not detectable

Fig. 2 Response spectra (RS) of the three *D. bruxellensis* strains grown in YPD and in YPD supplemented with 120 mM acetic acid at pH 4.5. *Solid line* Strain CBS 98, *dotted line* strain CBS 2499, *dashed line* strain CBS 4482

decrease of the pH to 4.0, but the cells maintained a high viability (never below 60 %).

It was noteworthy that, in the case of strain CBS 98, the presence of 120 mM acetic acid in the medium caused an adaptation phase that lasted for several days (Fig. 1c). To check the ability to proliferate, aliquots of cultures were spread on solid medium after 16 h growth. Indeed, the results indicated that only part of the cell population was able to form colonies in the presence of acetic acid (10^6 CFU/mL obtained on YPD and 10^4 CFU/mL on YPD supplemented with 120 mM acetic acid, respectively). On the other hand, cell viability, assayed by the methylene blue test, was approximately 90 % for both cultures. After 300 h growth, an increase of CFU on YPD supplemented with acetic acid plates was observed (3 x 10^5 CFU/mL), which corresponded to the cell population adapted to the presence of acetic acid in the liquid medium. In the case of CBS 4482, after 16 h growth we obtained the same level of CFU/mL on both YPD and YPD supplemented with acetic acid. This phenomenon has been well described recently in *S. cerevisiae* by Swinnen et al. (2014). They demonstrated that only a part of the population grew after exposure to acetic acid, whereas all the other cells persist in a non-proliferating state or die after exposure; the size of this fraction is strain-specific and determines the duration of the latency phase in the different strains (Swinnen et al. 2014). The results obtained suggest that a similar behavior can occur also in *D. bruxellensis* species.

FTIR metabolomic fingerprint

The metabolomic response indicates a variation in intracellular metabolites in response to specific conditions. Exponential phase cells, growing in YPD (control) and in YPD supplemented with acetic acid (120 mM), were analyzed by FTIR spectroscopy. The metabolomic response of CBS 4482 was very weak as shown by its RS close to 0 (Fig. 2). Since the RS is the difference in spectra obtained from challenged and unchallenged cells, RS close to zero means that the condition tested is not very stressing. The RSs obtained for the three cultures differed in the amides (W2), carbohydrates (W4) and mixed region (W3) (Fig. 2), whereas negligible effects were observed for fatty acids (W1) and in the typing (W5) regions (data not shown). Strain CBS 2499 showed a positive response from 1800 to 900 cm^{-1}, whereas the other two strains had a slightly positive (CBS 98) and faintly negative (CBS 4482) response. An intense response was observed for both CBS 2499 and CBS 98 at ca 1040 cm^{-1} (carbohydrate region) and a weaker response at ca. 1570 cm^{-1} (amide region); the latter can be probably ascribed to the GMP and AMP (Harz et al. 2009). An increased level of the AMP/ATP rate has in fact been reported to be essential for the activation of the Snf1p pathway, which is known to occur in response to acetic acid stress (Wilson et al. 1996). Moreover, acetic acid induces a depletion of ATP, thus raising the AMP/ATP ratio (Pampulha and Loureiro-Dias 2000; Mira et al. 2010a). This could be linked also to a difference in

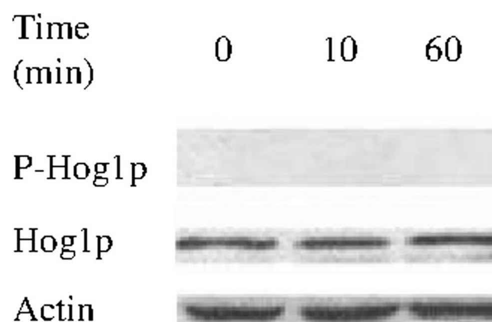

Fig. 3 Western blot showing that Hog1p is not involved in the acetic acid response in *D. bruxellensis* CBS 2499. Cells collected during the exponential phase of growth on YPD pH 4.5 were washed and incubated in the same medium containing 120 mM acetic acid for 10 min and for 60 min. Time 0 represents the point just before starting acetic acid exposure

ATP availability in the three strains, possibly due to glucose respiration, as suggested by the lower ethanol yield observed when this strain was cultivated in the absence of acetic acid (Table 1). The significance and position of these response peaks was confirmed by second derivative analysis of the spectrum (data not shown). A relatively intense peak was detected in CBS 98 and CBS 2499 around 1620 cm^{-1}—a region attributed to acetyl C=O (Mäntele et al. 1988), and therefore likely to witness the presence of acetic acid within the cell.

The different metabolomic reactions displayed by the three strains, and their relationship with the other physiological results, indicate that strain-specific mechanisms are involved in the response to acetic acid, which warrants further study. Interestingly, the strain with the lowest metabolomic response was also that with the highest viability (CBS 4482), suggesting that cells tried somehow to contrast the devitalization (Table 1). The fact that cell viability and metabolomic responses are not tightly correlated, produces a complex and complete description of strains—a powerful tool for screening industrial fitness in yeasts.

DbHog1p is not phosphorylated upon exposure to acetic acid

We explored whether the presence of acetic acid induced in *D. bruxellensis* one of the main stress responses observed in *S. cerevisiae*, i.e. the dual phosphorylation of Hog1p (Mollapour and Piper 2007). CBS 2499 cells cultivated on YPD and collected at the exponential phase of growth were exposed to the presence of acetic acid (120 mM). Proteins were examined after different exposure times by Western blot analysis, and phosphorylation of Hog1p was tested using a commercial anti-phospho-p38 antibody, which specifically recognizes the dual-phosphorylated form of Hog1p. As shown in Fig. 3, incubation of cells in the presence of 120 mM acetic acid did not result in any band corresponding to the phosphorylated form of Hog1p, indicating that this regulatory mechanism is not operating as a consequence of exposure to acid stress. This is in contrast to what was observed recently when this strain was exposed to other stresses like osmotic and cold stress, which both resulted in Hog1p phosphorylation (Galafassi et al. 2013b, 2015).

Conclusions

Our investigation on acetic acid tolerance showed that *D. bruxellensis* species exhibited strain-dependent behavior. Other traits, including nitrate utilisation as well as sulphite tolerance, have also shown strain-dependent occurrence (Curtin et al. 2012; Vigentini et al. 2013; Borneman et al. 2014), which can be linked easily to high genetic strain divergence (Vigentini et al. 2012).

Furthermore, the tested *D. bruxellensis* strains proved to unable to metabolize acetic acid in the presence of glucose, probably due to a mechanism of glucose repression of ACS activity. Acetic acid affected cell growth, causing a reduction in growth rate, glucose consumption rate and ethanol production rate, as well as biomass and ethanol yield. Interestingly, all three strains tested continued to produce acetic acid even when this compound is present at high concentration, suggesting an efficient export system. Work is in progress to characterize at the single cell level the influence of intracellular pH, which has been demonstrated to play an important role in acetic acid resistance in yeast (Stratford et al 2013b). Exposure to acetic acid did not cause activation of the HOG MAP kinase pathway, which is conversely known to be involved in other stress responses in this species (Galafassi et al. 2013b, 2015). Although the HOG pathway appears to be conserved even among distantly related species, this pathway may also be adapted to the specific niche requirements of each species.

Acknowledgements Md Moktaduzzaman has a fellowship from Marie-Curie FP7-PEOPLE-2010-ITN "CORNUCOPIA" project to C.C. This work is dedicated to the memory of Jure Piškur.

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