

Received Date : 08-Aug-2013

Revised Date : 10-Oct-2013

Accepted Date : 16-Oct-2013

Article type : Research Paper

Editor: Jens Nielsen

## Improved sake metabolic profile during fermentation due to increased mitochondrial pyruvate dissimilation

**Running title:** *Mitochondrial activity in sake yeast*

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1567-1364.12120

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**Keywords:** Yeast, Metabolic Flux Analysis, cell physiology, brewer's yeast.

### **Abstract**

Although the decrease of pyruvate secretion by brewer's yeasts during fermentation has long been desired in the alcohol beverage industry, rather little is known about the regulation of pyruvate accumulation. In former studies, we developed a pyruvate undersecreting sake yeast by isolating a strain (TCR7) tolerant to ethyl  $\alpha$ -transcyanocinnamate, an inhibitor of pyruvate transport into mitochondria. To obtain insights into pyruvate metabolism, in this study, we investigated the mitochondrial activity of TCR7 by oxigraphy and  $^{13}\text{C}$ -metabolic flux analysis during aerobic growth. While mitochondrial pyruvate oxidation was higher, glycerol production was decreased in TCR7 compared to the reference. These results indicate that mitochondrial activity is elevated in the TCR7 strain with the consequence of decreased pyruvate accumulation. Surprisingly mitochondrial activity is much higher in the sake yeast compared to CEN.PK 113-7D, the reference strain in metabolic engineering. When shifted from aerobic to anaerobic conditions, sake yeast retains a branched mitochondrial structure for a longer time than laboratory strains. The regulation of mitochondrial activity can become a completely novel approach to manipulate metabolic profile during fermentation of brewer's yeasts.

### **INTRODUCTION**

Pyruvate is located at the intersection between two of the main catabolic pathways of the cell: glycolysis and the Krebs cycle. Pyruvate is either transported into the mitochondrial matrix where it is converted to acetyl-CoA and  $\text{CO}_2$  and then fueled into the Krebs cycle or alternatively is dissimilated to  $\text{CO}_2$  and ethanol or acetate in the cytosol. In addition, pyruvate is one of the key intermediates for many anabolic reactions (38 reactions in (Kuepfer *et al.*, 2005)) including amino acids and lipid de novo synthesis.

The regulation of fluxes at the pyruvate branch-point by mitochondrial pyruvate transport is by far the least known step of central carbon metabolism in yeast and higher eukaryotes. In *S. cerevisiae* the kinetics of pyruvate transport into the mitochondria are likely impacting the competition of mitochondrial pyruvate oxidation with cytosolic dissimilation, and might consequently be involved in triggering the Crabtree effect (Pronk *et al.*, 1996). In applied microbiology the Crabtree effect has important consequences on the extent of fermentation (production of ethanol) versus respiration (production of biomass and recombinant proteins). Mitochondrial pyruvate uptake and oxidation has important consequences for mammals too; for example the Warburg effect present in cancer cells can be partly explained by a decrease of pyruvate uptake by mitochondria, followed by an increase in the glycolytic rate (Schell & Rutter, 2013).

How pyruvate enters mitochondria has remained a mystery until recently, when the proteins responsible for the uptake of this organic acid have been identified (Herzig *et al.*, 2012). These are encoded by the MPC1, 2 and 3 genes, whose products are believed to act as dimers catalyzing the uptake of pyruvate into mitochondria. The transporters can be inhibited by cinnamate derivatives like UK-5099 (Bricker *et al.*, 2012, Herzig *et al.*, 2012).

Low pyruvate and  $\alpha$ -acetolactate secretion are important characteristics of brewer's yeasts, since residual pyruvate and  $\alpha$ -acetolactate during alcoholic fermentation lead to production of off-flavours in alcoholic beverages. Notably, although industrially relevant, the parameters influencing the accumulation of these substances in brewer's yeast have never been studied. Knowledge on these parameters could enable rational strain design and subsequent improved breeding of non-off-flavor producing brewer's yeasts.

Sake yeast is used for brewing of sake, a Japanese traditional alcoholic beverage (Kitagaki & Kitamoto, 2013). The widely used sake yeast strain Kyokai no. 7 (K7) has been employed and maintained for more than 70 years (Tsukahara *et al.*, 1947) and produces the highest ethanol concentration among brewery yeasts worldwide (Watanabe *et al.*, 2011, Watanabe *et al.*, 2012). During sake brewing, a high concentration of glucose (10-15%) is successively provided by saccharifying enzymes produced by *Aspergillus oryzae*.

The sake yeast strain TCR7 has been developed by isolating a mutant of Kyokai no.7 tolerant to ethyl  $\alpha$ -transcyanocinnamate, an inhibitor of mitochondrial pyruvate transport. Differently from its WT, TCR7 secretes less pyruvate during sake mash fermentation both on laboratory and factory scales, and is now utilized in the actual sake industry to prevent secretion of off-flavors (Horie *et al.*, 2010). However, the mechanism of its lower pyruvate secretion was not known, which has hindered its further development and improvement. In order to obtain insights into the mechanisms underlying this phenotype, we have carried out a bioenergetic and quantitative physiological characterization of TCR7 and Kyokai no.7.

## MATERIALS AND METHODS

### Strains and growth conditions.

The *S. cerevisiae* sake strains Kyokai no.7 (Tsukahara *et al.*, 1947, Akao *et al.*, 2011), its derivative TCR7 (Horie *et al.*, 2010) and the reference strain CEN.PK 113-7D, were used throughout this study. Batch cultures of 25 ml were performed in 500 ml Erlenmeyer flasks on a rotary shaker at 30 °C and 250 r.p.m / 25 mm amplitude ensuring fully aerated conditions, in yeast minimal medium containing (per litre) 5 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3 gKH<sub>2</sub>PO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 4.5 mg ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.3 mg CoCl<sub>2</sub>·6H<sub>2</sub>O, 1.0 mg MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.3 mg CuSO<sub>4</sub>·5H<sub>2</sub>O, 4.5 mg CaCl<sub>2</sub>·2H<sub>2</sub>O, 3.0 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.4 mg NaMoO<sub>4</sub>·2H<sub>2</sub>O, 1.0 mg H<sub>3</sub>BO<sub>3</sub>, 0.1 mg KI, 15 mg EDTA, 0.05 mg biotin, 1.0 mg calcium pantothenate, 1.0 mg nicotinic acid, 25 mg inositol, 1.0 mg pyridoxine, 0.2 mg p-aminobenzoic acid and 1.0 mg thiamin. To avoid pH changes due to ammonia uptake and acetate production the medium was supplemented with 50 mM potassium hydrogen phthalate. The reference pH value was set to 5.0. Filter-sterilized glucose was added prior to the experiment at a final concentration of 5 g l<sup>-1</sup>. For mitochondria isolation yeast cells were grown on YP supplemented with 2% lactate at pH 4.8; cells were harvested during the exponential phase (O.D.<sub>600</sub> 0.8-1).

### Isolation of mitochondria and measurement of oxygen consumption and pyruvate dehydrogenase activity.

The isolation of mitochondria has been described in detail (Daum *et al.*, 1982) and proceeded essentially as follows. Cell walls were enzymatically degraded with Zymolyase. The resulting spheroplasts were disrupted by 13 strokes in a cooled Potter-Elvehjem homogenizer in hypotonic medium. Cytosolic and mitochondrial fractions were separated by differential centrifugation. Mitochondria were spun down gently for 10 min at 10,000 x g and were resuspended in a buffer containing 0.6 M mannitol, 20 mM Hepes/KOH pH 7.4, 1 mM EGTA, 0.2% bovine serum albumin (BSA) at approximately 10 mg of yeast protein/ml. Oxygen consumption by isolated mitochondria was measured with a Clark-type oxygen electrode using a Hansatech oxygraph (Hansatech Instruments Ltd, UK) in a buffer containing 0.6 M mannitol, 20 mM Hepes/KOH pH 6.8, 10 mM potassium phosphate pH 6.8, 2 mM MgCl<sub>2</sub>, 1 mM EGTA, and 0.1% BSA. Measurements were started by adding pyruvate plus L-malate at concentrations of 5 mM and 0.5 mM, respectively, followed by the addition of 0.5 mM ADP. Oxygen uptake rates were calculated based on a dissolved oxygen concentration of 236 µM in air-saturated buffer at 30°C. Pyruvate dehydrogenase was assayed as described in (Flikweert *et al.*, 1997) on mitochondria lysed with 0.05% Triton X-100.

## Analytical procedures.

Cell growth was monitored by measuring the optical density of cultures at a wavelength of 600 nm ( $OD_{600}$ ). Glucose, glycerol, acetate and ethanol were analysed by isocratic UV-RI-HPLC (Dionex Ultimate 3000. VWD-3100 UV-Detector, RI-101 RI-Detector). Analytes were separated on an Organic Acid Column, dimensions 300 x 8 mm (CS-Chromatographie) with 5 mM  $H_2SO_4$  as eluant ( $0.7 \text{ ml min}^{-1}$ ) at  $40^\circ\text{C}$ . The physiological parameters maximum specific growth rate, biomass yield on glucose, and specific glucose consumption rate were calculated during the exponential growth phase (Sauer *et al.*, 1999). Data consistency was investigated using a simple black box model of respiro-fermentative growing yeast (Stephanopoulos, 1998).

## $^{13}\text{C}$ -labelling experiments

All labeling experiments were performed in batch cultures assuming pseudo-steady-state conditions during the exponential growth phase in respiro-fermentative conditions (Fischer & Sauer, 2003, Blank & Sauer, 2004).  $^{13}\text{C}$ -labelling of proteinogenic amino acids was achieved by growth on 5 g glucose  $\text{l}^{-1}$  as a mixture of 80% (w/w) unlabelled and 20% (w/w) uniformly labeled [ $^{13}\text{C}$ ]glucose ( $^{13}\text{C}$ , 99 %; Cambridge Isotope Laboratories, Inc). Cells from an overnight minimal medium culture were washed and used for inoculation below an  $OD_{600}$  of 0.03.  $^{13}\text{C}$ -labelled biomass aliquots were harvested by centrifugation during the mid-exponential growth phase at an  $OD_{600}$  of  $<1$ . The cells (about 0.3 mg of dry biomass) were washed once with sterile water, and hydrolysed in 150  $\mu\text{l}$  6 M HCl at  $105^\circ\text{C}$  for 6 h. The hydrolysate was dried in a heating block at  $80^\circ\text{C}$  under a constant airflow. The free amino acids were derivatized at  $85^\circ\text{C}$  for 1 h using 30  $\mu\text{l}$  acetonitril and 30  $\mu\text{l}$  N-methyl-N-tert-butyldimethylsilyl-trifluoroacetamide (MBDSTFA). Gas chromatography and mass spectrometry (GC-MS) analysis were carried out using a Thermo Scientific Trace GC Ultra, combined with an Thermo Scientific ISQ quadrupole MS. Fifteen detectable amino acids were separated on a Restek Rxi®-5Sil MS column (Length: 15 m; inner diameter: 0.25 mm; film: 0.25  $\mu\text{m}$ ) at a constant flow rate of 1 ml helium  $\text{min}^{-1}$ . The split ratio was 1:15, the injection volume was 1  $\mu\text{l}$ , and the injector temperature was  $230^\circ\text{C}$ . The temperature of the GC oven was kept constant for 1 min at  $140^\circ\text{C}$  and afterwards increased to  $310^\circ\text{C}$  for another minute with a gradient of  $10^\circ\text{C min}^{-1}$ . The temperatures of the transfer line and the ion source were  $280^\circ\text{C}$  and  $230^\circ\text{C}$ , respectively. Ionization was performed by electron impact (EI) ionization at 70 eV. For detection, a Full Scan Method from 180 – 550  $m/z$  was used. GC-MS raw data were analyzed using the Xcalibur software (Thermo Scientific).

## $^{13}\text{C}$ -constrained metabolic flux analysis.

The stoichiometric model for  $^{13}\text{C}$ -constrained metabolic flux analysis comprises the major pathways of yeast central carbon metabolism (Blank *et al.*, 2005). The model used contains 30

fluxes and 27 metabolites. To calculate intracellular fluxes, the stoichiometric model was constrained with five extracellular fluxes (growth rate, evolution rates of ethanol, glycerol and acetate, and glucose uptake rate) and six intracellular flux ratios (fraction of cytosolic oxaloacetate originating from cytosolic pyruvate, fraction of mitochondrial oxaloacetate derived through anaplerosis, fraction of phosphoenol-pyruvate originating from cytosolic oxaloacetate, fraction of serine derived through glycolysis, upper and lower bounds of mitochondrial pyruvate derived through malic enzyme). Only NADH-dependent isocitrate dehydrogenase activity (Idh1 and Idh2) was considered in the model; NADPH-dependent activity of the IDP isozymes was neglected. Mass balances of O<sub>2</sub>, CO<sub>2</sub>, ATP and NADPH were excluded from the analyses. The overly constrained system was solved by a least square optimization as described in (Fischer *et al.*, 2004).

### **Determination of yeast mitochondrial morphology**

Two yeast expressing mito-GFP were used: a sake yeast (Kyokai no. 7 *his3/his3* + pRS413GPD-mitochondrial GFP)(Motomura *et al.*, 2012)) and the laboratory yeast BY4743 (MAT a/*his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0*) + pRS413GPD-mitochondrial GFP ). Cells were grown in a synthetic complete medium (SCM) supplemented with 2% (w/v) glucose, 790 mg/l of a complete supplement mixture - histidine (CSM-His) medium (Formedium), and 0.67% (w/v) yeast nitrogen base without amino acids and ammonium sulphate (Becton Dickinson and Company). Yeast cells were first incubated aerobically (culture shake at 200 rpm) at 30 °C to saturation (OD<sub>600</sub> 4-6) and then inoculated to a static culture at OD<sub>600</sub> of 0.1 in SCM + 2% glucose. Yeast cells were incubated at 30 °C for 4, 12 and 36 h without shaking, and mitochondrial morphologies were observed under a fluorescence microscope. Before observation, yeast cells were fixed with 3.7% formaldehyde for 30 min and washed twice with distilled water.

## **RESULTS**

### **TCR7 mitochondria display a higher rate of pyruvate oxidation**

TCR7 shows a low pyruvate-secreting characteristic during anaerobic growth in sake brewing medium (Horie *et al.*, 2010) and in YP medium supplemented with 10% glucose (Fig. 1A), and during aerobic growth in YP medium supplemented with 2% lactate (Fig. 1B) relative to its parent strain Kyokai no. 7. In order to elucidate the cause of this phenotype, Kyokai no.7 and TCR7 were grown in YP medium supplemented with 2% lactate. Mitochondria were isolated and their efficiency to oxidize pyruvate + malate was tested. The malate concentration was kept at 0.5 mM whereas the pyruvate concentration was varied between 0 and 5 mM (**Figure 2**).

Pyruvate oxidation followed a Michaelis-Menten kinetics. TCR7 displayed a  $V_{\max}$  of 305 nmol  $O_2$ /(min x mg prot) which was 58% higher than that of Kyokai no.7. Surprisingly the  $K_m$  of the mutant was also higher (0.38 mM vs. 0.15 mM for Kyokai no.7). Based on the determined kinetic parameters at a pyruvate concentration higher than 0.08 mM, TCR7 has a pyruvate oxidation rate in vitro higher than that of Kyokai no.7. The calculated affinity constants are similar to that measured by (van Urk *et al.*, 1989)(0.3 mM), which is near the  $K_m$  value that has been reported for the pyruvate dehydrogenase complex (0.2-0.6 mM) (Kresze & Ronft, 1981). For comparison, the  $K_m$  value of pyruvate transport by mitochondria rat liver mitochondria was 0.15 mM (Halestrap, 1975).

To further investigate the high pyruvate oxidation activity of TCR7, pyruvate dehydrogenase activity of TCR7 was investigated. TCR7 displayed a significantly lower in vitro pyruvate dehydrogenase activity (**Figure 3**), which was about half that of Kyokai no.7. In contrast no difference in the activity of the cytosolic pyruvate decarboxylase was observed (not shown).

In order to demonstrate that the two strains contained the same amount of mitochondria we determined by Western Blot the level of mitochondrial proteins (Aac2, Cyb2, Atp2) on cellular extracts (Figure S1). No significant differences were found between the strains Kyokai no.7 and TCR7.

These results indicate that mitochondrial pyruvate oxidation in TCR7 differs from the parental strain Kyokai no. 7. This was elucidated further by metabolic flux analysis.

### **Quantitative physiology and $^{13}C$ MFA of sake yeasts and CEN.PK 113-7d**

Since in vitro activities of enzymes can differ from in vivo activities, a detailed physiological characterization of the sake strains Kyokai no.7 and TCR7 was performed. In order to benchmark the physiological properties of these strains, the most widely used yeast strain in cellular physiology, CEN.PK 113-7D, was used as reference. The three strains were grown in synthetic medium in the presence of 5 g/l of glucose in respiro-fermentative conditions; 20% of the glucose provided, was uniformly labeled with  $^{13}C$ . To better highlight differences in mitochondrial metabolism, in a separate set of experiments, we added 0.5 M NaCl to slow down the rate of growth. It has been demonstrated that glucose repression irrespective of the glucose concentration present is partly lifted at lower growth rates, with the result that in the presence of NaCl a higher fraction of carbon is diverted towards mitochondria (Blank & Sauer, 2004, Heyland *et al.*, 2009).

The physiological parameters of the three studied strains are shown in **Table 1**. Both, in the presence and in absence of NaCl no significant differences in carbon uptake and product formation



rates were observed for the two sake strains, while the phenotype of the reference strain CEN.PK 113-7D differed strongly.

In the absence of NaCl CEN.PK 113-7D displays a mainly fermentative metabolism with a glucose uptake rate and an ethanol production rate much higher than Kyokai no.7 and TCR7. Accordingly the biomass yield for Kyokai no.7 and TCR7 was significantly higher than that of CEN.PK 113-7D ( $0.21 \pm 0.01$  vs.  $0.14 \pm 0.05$  g biomass x g<sup>-1</sup> glucose;  $P < 0.01$ ).

In the presence of NaCl as already reported (Heyland *et al.*, 2009), CEN.PK 113-7D showed lower glucose uptake and ethanol production rates. Importantly the effect of 0.5 M NaCl on Kyokai no.7 and TCR7 followed the same trend but not to the same extent. All the strains responded to NaCl by producing more glycerol probably as a response to the increase in osmolarity (Hohmann, 2002); CEN.PK 113-7D produced more glycerol than Kyokai no.7 and especially TCR7, which showed the lowest production rate. This indicates that the mutant has higher cytoplasmic NADH oxidation, since this feature has an inverse correlation with the glycerol formation (Vemuri *et al.*, 2007). It is noteworthy that in the presence of NaCl, the sake strains produced significantly less acetate than CEN.PK 113-7D.

The use of <sup>13</sup>C-glucose allowed us to perform a METAFOR (metabolic flux ratio) analysis of these yeasts (Blank & Sauer, 2004): the mass isotopomer distribution of proteinogenic amino acids was used to calculate the split ratios of key branching points of yeast central metabolism using the software FIAT FLUX (Zamboni *et al.*, 2005). In **Figure 4A and B** the relative rate of the oxaloacetate (OAA) generated from mitochondrial malate (corresponding to the oxidative TCA cycle activity) and of the pentose phosphate pathway (PPP) - glycolysis split ratio are shown. Both sake strains displayed a fivefold higher TCA cycle activity than the CEN.PK 113-7D strain; TCR7 showed the highest TCA cycle activity. In the presence of NaCl an increase of the oxidative TCA cycle activity was observed for all strains. The relative increase (minus vs. plus NaCl) was however much higher for the CEN.PK 113-7D. Also the PPP was higher for the sake strains than CEN.PK 113-7D. This flux ratio did not change significantly in the presence of 0.5 M NaCl.

Intracellular flux ratios and consumption and secretion rates (**Table 1**) were integrated in a model of yeast central metabolism using the NETTO subprogram of the Fiat Flux software, to obtain network-wide absolute fluxes (Blank & Sauer, 2004, Zamboni *et al.*, 2005). The normalized rates of carbon fluxes in the presence and absence of 0.5 M NaCl are shown in **Figure 5** and **Figure 6** respectively.



Metabolic flux analysis indicated that both in the presence and in the absence of NaCl TCR7 displayed a higher flux of carbon towards mitochondria compared to Kyokai no.7. In the mutant more pyruvate was transported and oxidized within mitochondria (**Figure 5**). In both sake strains the TCA cycle was fully operative in its cyclic mode; it is noteworthy that this is not the case of many *S. cerevisiae* strains under these experimental conditions (Blank *et al.*, 2005, Christen & Sauer, 2011) for which the TCA cycle is almost completely a bifurcated pathway whose function is to sustain biomass precursor synthesis. In TCR7 glycerol production was lower than in the other strains indicating a more sustained mitochondrial oxidation of cytosolic NADH. In all the tested strains NaCl treatment increased the C flux towards mitochondria and glycerol production (**Figure 6**). Also in this case the effect of NaCl on the sake strains was weaker than that on CEN.PK 113-7D.

The sake strains displayed a metabolism very different from the reference laboratory strain CEN.PK 113-7D. The main differences were: a) a sustained mitochondrial metabolism with a completely cyclic TCA cycle. A slightly higher oxidative TCA cycle activity was measured in the presence of 0.5 M NaCl; b) the sake strains showed a much higher PPP and a lower glycolytic flux; c) under these experimental conditions we detected a lower ethanol production for the sake strains; d) NaCl had a weaker effect on sake yeast metabolism compared with the changes measured for CEN.PK 113-7D (increase of mitochondrial metabolism, decrease of the growth rate and of the glucose uptake rate, increased glycerol production) (**Figure 6**).

### **Differences in mitochondrial morphologies of sake and laboratory yeasts**

The above results indicated a higher mitochondrial activity and flux of carbon in the sake yeasts compared to the laboratory strain under aerobic conditions. While sake yeasts are exposed to gaseous oxygen before the main fermentation, sake production proceeds under anaerobic conditions. Therefore, the mitochondrial morphologies of these strains, reflecting their activity, were investigated during the shift from aerobic to anaerobic cultivation conditions. The auxotrophic laboratory strain BY4743 was used in these experiments as reference. BY4743 has comparable growth characteristics to CEN.PK113-7D under aerobic culture conditions and is genetically similar to CEN.PK113-7D, although not strictly isogenic (van Dijken JP *et al.*, 2000). Both strains exhibited filamentous and branched mitochondrial structures after 4 h of static cultivation (Figure 7A and B). After 12 h of static cultivation, in contrast, the mitochondria of the laboratory strain were fragmented and very few exhibited filamentous and branched structures (Figure 7C) while the mitochondria of sake yeast remained filamentous and branched (Figure 7D). After 36 h both strains showed fragmented mitochondria (data not shown). Since filamentous and branched structure

reflects elevated activity of mitochondria (Skulachev, 2001), the morphological transformation observed, indicates a decrease of the mitochondrial activity of these strains upon transfer from aerobic to anaerobic state. In addition, the results indicate that sake yeast maintain a higher mitochondrial activity upon transfer from aerobic to anaerobic state for a longer time period, a finding consistent with the elevated mitochondrial function of sake yeast observed in this study.

## DISCUSSION

The aim of this work was to understand the physiological and molecular mechanisms leading to the lower pyruvate secretion of the sake yeast strain TCR7. Since this strain was isolated for being resistant to alpha-transcyanocinnamate (inhibitor of mitochondrial pyruvate transport), we hypothesized an increased transport of pyruvate into mitochondria required to overcome the inhibitory effect and subsequently a higher mitochondrial pyruvate dissimilation.

Indeed, mitochondria isolated from TCR7 showed a much higher  $V_{\max}$  and  $K_m$  for pyruvate oxidation than Kyokai no.7. The higher  $V_{\max}$  is in agreement with more pyruvate being transported and subsequently oxidized, with the consequence that less pyruvate is secreted into the medium. The increase in  $K_m$  might seem contradictory; however when intracellular pyruvate concentration is higher than 0.08 mM, TCR7 mitochondria can oxidize pyruvate more efficiently than its WT. In *S. cerevisiae* the influence of the extracellular glucose concentration on intracellular pyruvate concentration was reported. van Urk et al. measured a concentration of 1-2 mM under glucose-limited conditions (van Urk *et al.*, 1989). Pyruvate levels are much higher (about 40 mM) in the presence of excess glucose (Postma *et al.*, 1989). These data suggest that TCR7 mitochondria, despite their higher  $K_m$  have a potentially higher pyruvate dissimilation activity in many growth conditions.

Pyruvate oxidation in isolated mitochondria can be dependent on pyruvate uptake, dehydrogenation, TCA cycle activity and the rate of oxidative phosphorylation. Depending on the growth and assay conditions it is likely that all of these steps can contribute to the overall rate of pyruvate oxidation; mitochondrial uptake was proposed as the rate limiting step (Schell & Rutter, 2013). Very recently three isoforms of the mitochondrial pyruvate carrier (*MPC1-3*) have been identified (Bricker *et al.*, 2012, Herzig *et al.*, 2012). They are inhibited by an analogue of alpha-transcyanocinnamate, the molecule used to breed TCR7. Our real-time PCR analysis of *MPC1-3* expression level in Kyokai no.7 and TCR7 did not reveal any significant difference (Figure S2). In accordance with the altered pyruvate metabolism of TCR7, the in vitro activity of the pyruvate dehydrogenase was strongly decreased. PDH is a highly regulated complex whose activity is

controlled for example by phosphorylation and dephosphorylation during the diauxic shift (Ohlmeier *et al.*, 2010). It would be interesting to find out the molecular mechanisms of the lower in vitro PDH activity in TCR7.

The increased oxidative efficiency of TCR7 mitochondria was confirmed in vivo by detailed quantitative physiology including  $^{13}\text{C}$ -metabolic flux analysis. TCR7 displayed a slightly but significantly higher flux of pyruvate towards mitochondria. This was indicated by the increased flux ratio of oxaloacetate produced by mitochondria vs. oxaloacetate produced by anaplerosis (indicating the cyclic or oxidative activity of the TCA) (**Figure 4**) and by the distribution of the absolute in vivo fluxes (**Figure 5-6**).

$^{13}\text{C}$ -MFA also highlighted profound and interesting differences between the sake yeast strains (Kyokai no.7 and TCR7) and the common laboratory strain CEN.PK 113-7D. It is known that sake yeasts are characterized by a high fermentative capability producing high concentration of ethanol (Watanabe *et al.*, 2011). In the conditions used in this work (synthetic medium supplemented with 0.5% glucose; early exponential phase) the sake strains showed a surprisingly high mitochondrial metabolism compared to CEN.PK. Notably, sake yeast exhibited higher growth rates in glycerol-containing medium when compared to laboratory strains (data not shown). As a result the biomass yield of sake strains was very high for a *S. cerevisiae* strain, and the glycolytic flux, ethanol and glycerol production were significantly lower than in CEN.PK. Another striking metabolic feature of the sake strains was the high flux through the pentose phosphate pathway and the concomitantly lower glycolytic flux. The resulting increased NADPH production observed might have been acquired to cope with the harsh conditions observed during sake mash fermentation. Alternatively, the higher flux into PPP of sake yeast strain might reflect the long culture history of sake yeast strains in high glucose-containing media (sake brewing condition), which might have conferred sake yeast strain genetic characteristics to partly bypass the glycolytic flux. On the other hand it can be explained considering that more ATP is produced from mitochondria and hence glycolysis can be slowed down. Moreover the higher PPP flux with its increased NADPH regeneration of these strains correlates well with their higher NADPH demand for the increased biomass yields (Blank *et al.*, 2005).

Surprisingly also the effect of NaCl was very different between the sake strains and CEN.PK. For this laboratory strain NaCl led to important changes in the flux distribution, which are briefly summarized: decrease of the growth rate and of the glucose uptake rate, increase of the mitochondrial activity (oxidative TCA cycle), increase of the glycerol production as a result of higher osmolarity (Hohmann, 2002, Blank & Sauer, 2004). All of these effects are also present in

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the sake yeast strains but much more attenuated. This is probably related to the peculiar response to various stresses of modern sake yeast strains for which the dysfunction of key regulators of these pathways such as Msn2 and 4 and Rim15 (Watanabe et al., 2011, 2012) has been demonstrated. It is likely that a higher NaCl concentration is required to exert on sake yeast the same effects that 0.5 M NaCl has on CEN.PK 113-7D. Moreover the higher resistance of sake strains to NaCl is consistent with their higher mitochondrial metabolism, since it has been demonstrated that mitochondrial function is generally required for proper salt and osmotic stress adaptation (Pastor *et al.*, 2009).

It would be very interesting both for basic and applied research to find out which changes in the TCR7 genome determine the observed phenotype. TCR7 harbors 13 SNPs in the coding region of 13 different genes, as elucidated by next generation sequencing (Kitagaki et al. unpublished data). No differences were found in the *MPC1-3* coding regions. More mutations are found in the non coding regions. The metabolic change elucidated in this study is likely to be brought about by some of these mutations.

Consistent with the elevated mitochondrial activity and decreased pyruvate secretion of TCR7, inhibition of mitochondrial activity with the mitochondrial uncoupler FCCP increased pyruvate accumulation in the fermentation medium by sake yeast (Kitagaki *et al.*, 2008, Motomura *et al.*, 2012). In this study we have observed that in the shift from respiro-fermentative to fermentative conditions sake yeast retains a filamentous and tubular mitochondrial structure for a longer time than laboratory yeast (Figure 7). Mitochondria form a dynamic network whose overall shape is determined by the balance of fusion and fission events. Fission of the mitochondrial network is essential for normal oxidative phosphorylation function and perturbation of mitochondrial network dynamics is likely to induce the impairment of mitochondrial energy production (Benard *et al.*, 2007). Mitochondrial fragmentation has been observed in patients with alterations of mitochondrial energy production caused by genetic defects in respiratory-chain subunits (Koopman *et al.*, 2005), or in mitochondria treated with the uncoupler FCCP (Meeusen *et al.*, 2004). Furthermore in yeast various types of stresses (chemical, oxidative, etc.) generally trigger mitochondrial fragmentation (Meeusen *et al.*, 2004, Pozniakovsky *et al.*, 2005, Benard *et al.*, 2007, Kitagaki *et al.*, 2007). Fragmented mitochondria represent damaged and unfunctional mitochondria that can be eliminated by mitophagy (Youle & van der Bliek, 2012), whereas filamentous and branched structures reflect elevated activities of mitochondria (Skulachev, 2001). The results obtained in this study, together with the findings that mitochondria of sake yeast change from filamentous to fragmented structures during sake brewing (Kitagaki & Shimoi, 2007) and that during alcoholic fermentation mitochondrial morphology and activity determines the sake organic acid profile

(Kitagaki *et al.*, 2008, Motomura *et al.*, 2012), suggest a new role of mitochondria in brewer's yeast. Although mitochondrial activity decreases upon transfer to mostly anaerobic conditions such as those found in industrial fermentation, residual mitochondrial activity and its decrease over time can be a key factor in determining the organic acid profile during fermentation.

In conclusion these data provide evidence of a higher pyruvate oxidation in TCR7; although the molecular determinants of this phenotype are not known, we can hypothesize that a higher flux of carbon towards mitochondria determines a lower pyruvate secretion. Since PDH enzymatic activity is lower for TCR7 the increased pyruvate flux can be a result of a higher mitochondrial pyruvate uptake (pushing effect) or a higher respiratory dissimilation (pulling effect). The connection between mitochondrial activity and metabolic profile during fermentation of sake yeast suggested in this study, can provide a novel approach to manipulate fermentation profiles of other brewer's yeasts.

**ACKNOWLEDGEMENTS:** G.A. would like to thank the support of the DAAD and of the iAMB, RWTH Aachen University.

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## FIGURE LEGENDS

**Figure 1:** Extracellular pyruvate concentration during growth of TCR7 and its parent sake yeast, Kyokai no.7 (K7), in different growth conditions. A) Pyruvate concentration during anaerobic (static cultures) growth in YP supplemented with 10% glucose. B) Pyruvate concentration during aerobic growth (cultures shaken at 250 rpm) in YP supplemented with 2% lactate. Results indicate mean values  $\pm$  SEM of at least 3 independent experiments. The significance of the differences is indicated (\*P < 0.05, \*\*P < 0.01, Student's t-test).

**Figure 2:** Pyruvate plus malate oxidation, in the presence of 0.5 mM ADP by mitochondria isolated from the Kyokai no.7 and the TCR7 strains. Cells were grown in YP supplemented with 2% lactate YP. Values are mean  $\pm$  SEM of at least three independent experiments.

**Figure 3:** Pyruvate dehydrogenase activity of mitochondria isolated from the Kyokai no.7 and the TCR7 strains. Cells were grown in YP supplemented with 2% lactate. Values are mean  $\pm$  SEM of at least three independent experiments. The significance of the differences is indicated (\*\*P < 0.01, Student's t-test).

**Figure 4:** Respiratory TCA cycle activity (A) and relative flux of pentose phosphate pathway (PPP) vs. glycolysis (B) in the Kyokai no.7 (K7), TCR7, and CEN.PK 113-7D strains. Cells were cultivated in minimal medium supplemented with 5 g/l glucose. The addition of 0.5M NaCl is indicated. The respiratory TCA cycle activity was calculated as reported in (Kümmel *et al.*, 2010). The relative (PPP)/glycolysis flux ratio was calculated considering the fraction of PEP produced from the PPP using the software FIAT FLUX. The significance of the differences between K7 and the other two strains is indicated (\*P < 0.05, \*\*P < 0.01, one-way ANOVA followed by Bonferroni's t-test).

**Figure 5:** Relative distribution of absolute carbon fluxes in Kyokai no.7 (black), TCR7 (red) and CEN.PK113-7D (blue). Cells were cultivated in minimal medium supplemented with 5 g/l glucose. All fluxes are normalized to the specific glucose uptake rate, which is shown in the top inset, and are given in the same order in each box. The relative NADH dehydrogenase fluxes are the net reduction rates of cytosolic and mitochondrial NADH, assumed to be catalyzed by Nde1/2p and Ndi1p, respectively. The growth substrate and the secreted products are written in capital letters.

**Figure 6:** Relative distribution of absolute carbon fluxes in Kyokai no.7 (black), TCR7 (red) and CEN.PK113-7D (blue). Cells were cultivated in minimal medium supplemented with 5 g/l glucose in the presence of 0.5 M NaCl. All fluxes are normalized to the specific glucose uptake rate, which is shown in the top inset, and are given in the same order in each box. The relative NADH dehydrogenase fluxes are the net reduction rates of cytosolic and mitochondrial NADH, assumed to be catalyzed by Nde1/2p and Ndi1p, respectively. The growth substrate and the secreted products are written in capital letters.

**Figure 7:** Mitochondrial morphology of sake yeast and laboratory yeast during shift from aerobic to anaerobic culture. Yeast cells carrying mito-GFP, were transferred from shaking culture (aerobic condition) to static culture (anaerobic condition) and incubated for 12 h in synthetic complete medium containing 2% glucose, and mitochondrial morphologies were observed under a fluorescence microscope. (A,C) Sake yeast Kyokai no. 7 *his3/his3* + pRS413GPD-mitochondrial GFP. (B,D) Laboratory yeast BY4743 (MAT a/ $\alpha$  *his3 $\Delta$ 1/his3 $\Delta$ 1 leu2 $\Delta$ 0/leu2 $\Delta$ 0 lys2 $\Delta$ 0/LYS2 MET15/met15 $\Delta$ 0 ura3 $\Delta$ 0/ura3 $\Delta$ 0) + pRS413GPD-mitochondrial GFP (Motomura *et al.*, 2012). (A, B) 4 h (C, D) 12 h after the shift from aerobic to anaerobic culture.*

**Table 1:** Physiological parameters of the Kyokai no.7 (K7), TCR7, and CEN.PK 113-7D strains cultivated in minimal medium supplemented with 5 g/l glucose. The addition of 0.5M NaCl is indicated.

| Parameter                                                     | <i>Kyokai no.7</i> | <i>TCR7</i> | <i>CEN.PK 113-7D</i> |
|---------------------------------------------------------------|--------------------|-------------|----------------------|
| $r_{\text{glucose}}$ (mmol g <sup>-1</sup> h <sup>-1</sup> )  | 10.5 ± 1.4         | 10.4 ± 0.8  | 18.3 ± 1.5           |
| $r_{\text{glycerol}}$ (mmol g <sup>-1</sup> h <sup>-1</sup> ) | 0.9 ± 0.1          | 0.7 ± 0.1   | 1.7 ± 0.1            |
| $r_{\text{acetate}}$ (mmol g <sup>-1</sup> h <sup>-1</sup> )  | 0.4 ± 0.1          | 0.5 ± 0.1   | 0.8 ± 0.1            |
| $r_{\text{ethanol}}$ (mmol g <sup>-1</sup> h <sup>-1</sup> )  | 15.0 ± 1.4         | 14.6 ± 1.2  | 28.0 ± 1.8           |
| $\mu$ (h <sup>-1</sup> )                                      | 0.43 ± 0.05        | 0.42 ± 0.05 | 0.41 ± 0.02          |

| Parameter                                                     | <i>Kyokai no.7</i> + 0.5M NaCl | <i>TCR7</i> + 0.5M NaCl | <i>CEN.PK 113-7D</i> + 0.5M NaCl |
|---------------------------------------------------------------|--------------------------------|-------------------------|----------------------------------|
| $r_{\text{glucose}}$ (mmol g <sup>-1</sup> h <sup>-1</sup> )  | 10.0 ± 0.9                     | 9.5 ± 0.6               | 12.3 ± 1.3                       |
| $r_{\text{glycerol}}$ (mmol g <sup>-1</sup> h <sup>-1</sup> ) | 2.1 ± 0.2                      | 1.7 ± 0.1               | 3.2 ± 0.2                        |

|                                                              |                 |                 |                 |
|--------------------------------------------------------------|-----------------|-----------------|-----------------|
| $r_{\text{acetate}}$ (mmol g <sup>-1</sup> h <sup>-1</sup> ) | $0.1 \pm 0.05$  | $0.1 \pm 0.05$  | $1.0 \pm 0.1$   |
| $r_{\text{ethanol}}$ (mmol g <sup>-1</sup> h <sup>-1</sup> ) | $13.9 \pm 1.3$  | $13.3 \pm 1.1$  | $16.2 \pm 1.5$  |
| $\mu$ (h <sup>-1</sup> )                                     | $0.41 \pm 0.05$ | $0.38 \pm 0.04$ | $0.23 \pm 0.04$ |















