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Phenotypic analysis of mutant and overexpressing strains of lipid metabolism genes in *Saccharomyces cerevisiae*: Implication in growth at low temperatures

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ABSTRACT

The growing demand for wines with a more pronounced aromatic profile calls for low temperature alcoholic fermentations (10–15 °C). However, there are certain drawbacks to low temperature fermentations such as reduced growth rate, long lag phase and sluggish or stuck fermentations. The lipid metabolism of *Saccharomyces cerevisiae* plays a central role in low temperature adaptation. The aim of this study was to detect lipid metabolism genes involved in cold adaptation. To do so, we analyzed the growth of knockouts in phospholipids, sterols and sphingolipids, from the EUROSCARF collection *S. cerevisiae* BY4742 strain at low and optimal temperatures. Growth rate of these knockouts, compared with the control, enabled us to identify the genes involved, which were also deleted or overexpressed in a derivative haploid of a commercial wine strain. We identified genes involved in the phospholipid (*PSD1* and *OPI3*), sterol (*ERG3* and *IDI1*) and sphingolipid (*LCB3*) pathways, whose deletion strongly impaired growth at low temperature and whose overexpression reduced generation or division time by almost half. Our study also reveals many phenotypic differences between the laboratory strain and the commercial wine yeast strain, showing the importance of constructing mutant and overexpressing strains in both genetic backgrounds. The phenotypic differences in the mutant and overexpressing strains were correlated with changes in their lipid composition.

1. Introduction

Temperature fluctuations are an inevitable part of microbial life in exposed natural environments; however, sub-optimal temperatures are also common in industrial processes. Low temperatures (10–15 °C) are used in wine fermentations to enhance production and retain flavor volatiles. In this way, white and rosé wines of greater aromatic complexity can be achieved (Beltran et al., 2008; Torija et al., 2003). The optimum fermentation temperature for *Saccharomyces* is between 25 and 28 °C. Therefore, among the difficulties inherent to wine fermentation (high concentration of sugars, low pH, presence of ethanol, nutrient deficiency, etc.), we should add a sub-optimal temperature for the primary fermentation agent. Temperature affects both yeast growth and fermentation rate, with lower temperatures giving rise to a very long latency phase of up to one week or longer and sluggish fermentations (Bisson, 1999; Meurgues, 1996), dramatically lengthening alcoholic fermentation with the consequent energy expenditure.

Low temperature has several effects on biochemical and physiological properties in yeast cells: low efficiency of protein translation, low fluidity membrane, change in lipid composition, slow protein folding, stabilization of mRNA secondary structures and decrease in enzymatic activities (Aguilera et al., 2007; Hunter and Rose, 1972; Sahara et al., 2002; Schade et al., 2004). To date, most studies have mainly focused on the genome-wide transcriptional responses to cold-shock (Beltran et al., 2006; Homma et al., 2003; Murata et al., 2006; Sahara et al., 2002; Schade et al., 2004). In a decisive study, Tai et al. (2007) compared their transcriptomic results obtained in a steady-state chemostat culture with other previous genome-wide transcriptional studies of batch cultures at low temperature. Interestingly, lipid metabolism genes were the only ones whose activity was clearly regulated by low temperature. This is consistent with the notion that after a temperature downshift, homeoviscous adaptation of the membrane composition is essential for growth (Beltran et al., 2006, 2007; Hunter and Rose, 1972; Torija et al., 2003).

Biological membranes are the first barrier between the cell interior and its environment and a primary target for damage during cold stress. Major lipid components of eukaryotic membranes are phospholipids, sterols, sphingolipids and glycerolipids. The main species of fatty acids of *Saccharomyces cerevisiae* are C16 and C18, with or without a double bond. The composition of these lipid components is important for the

Abbreviations: FA, fatty acids; MCFA, medium chain fatty acids; UFA, unsaturated fatty acids; SFA, saturated fatty acids; ChL, chain lengths; TG, triacylglyceride; DG, diacylglyceride; PL, phospholipid; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; NL, neutral lipid; SE, sterol esters.

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physical properties of the membrane, such as fluidity. Incubation at low temperature increases the molecular order of membrane lipids by rigidification (Russell, 1990). Yeasts are known to have developed several strategies to maintain appropriate membrane fluidity. The most commonly studied involves the increase in unsaturation (mainly palmitoleic C16:1 and oleic C18:1 acids). Phospholipids with unsaturated fatty acids (UFA) have a lower melting point and greater flexibility than phospholipids with saturated acyl chains. Another way of increasing membrane lipid fluidity is to decrease the chain length (ChL) of these FA by increasing the synthesis of medium chain fatty acids (MCFA; C6 to C14) (Beltran et al., 2008; Torija et al., 2003). Recently, Redón et al. (2011) also reported new common changes in the lipid composition of different industrial species and strains of *Saccharomyces* after low temperature growth. Despite specific strains/species dependent responses, the results showed that at low temperatures the MCFA and triacylglyceride (TG) content increased, whereas the phosphatidic acid content (PA) and the phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratio decreased.

Reshaping the plasma membrane composition might be a good strategy for adapting yeast cells to low temperatures, reducing the lag-phase and speeding up fermentation onset. In this respect, knockout or overexpression of genes related with lipid metabolism can modify the architecture of this plasma membrane. In a preliminary study (Redón et al., 2012), we tested various phospholipid mutants from the EUROSCARF collection of *S. cerevisiae* BY4742 to ascertain whether the suppression of some genes could improve the fermentation vitality of the cells at low temperature. The aim of this study was to detect key genes in the lipid metabolism pathways which play an important role in the adaptation of *S. cerevisiae* to low temperature. To achieve this objective, we analyzed the growth of several knockouts of phospholipid, sterol and sphingolipid pathways at 12 °C and 28 °C and compared them to the wild type BY4742. This first screening of the laboratory strain enabled us to select genes for deletion and overexpression in the genetic background of a derivative haploid of the commercial wine strain, QA23. The phenotypic differences in the mutant and overexpressing strains were correlated with the changes in their lipid composition.

2. Material and methods

2.1. Strains and growth media

S. cerevisiae strains used in this study were: a total of 34 mutants of phospholipids, sterols and sphingolipids of the laboratory strain BY4742 (MATα, his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0), from the EUROSCARF collection (Frankfurt, Germany) and the derivative haploid of the commercial wine strain QA23 (Lallemand S.A., Canada), *ho*QA23 (Salvadó et al., 2012).

These strains were cultured on SC (6.7 g/L Difco Yeast Nitrogen Base (w/o amino acids), 20 g/L glucose, 0.83 g/L synthetic complete drop-out mix (2 g Histidine, 4 g Leucine, 2 g Lysine 1.2 g Uracil)). They were grown in Erlenmeyer flasks (250 mL) filled with 50 mL of medium, fitted with cotton and shaken at 200 rpm at 30 °C for 48 h. The population inoculated in every flask was 2×10^6 cells/mL from an overnight culture in YPD at 30 °C.

2.2. Construction of mutant and overexpressing strains

Mutated genes which showed growth insufficiency in the background of the laboratory strain BY4742 were deleted on the derivative haploid of a commercial wine strain, *ho*QA23. Genes were deleted using the short flanking homology (SFH) method based on the KanMX4 deletion cassette (Güldener et al., 1996). The primers used for amplification of the *loxP-KanMX4-loxP* cassette from the plasmid pUG6 have 50-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). The PCR fragments were used to transform the haploid *ho*QA23 strain using the lithium acetate procedure (Gietz and Woods, 2002). After transformation, strain selection was done using Geneticin (G418) added to YPD solid media at a concentration of 200 mg/L. Total DNA from transformants resistant to G418 Geneticin was analyzed by PCR using primers upstream and downstream of the deleted region combined with primers of the *KanMX* gene.

The genes, whose deletion significantly impaired growth in the *ho*QA23, were overexpressed by cloning into the centromeric plasmid pGREG505, as described in Jansen et al. (2005). All genes were amplified from approximately 600 nucleotides upstream of the start codon to 400 nucleotides downstream of the stop codon to ensure that the promoter and terminator regions were included. The PCR protocol involved an initial denaturation at 94 °C (2 min), followed by 30 cycles of 10 s at 94 °C, 30 s at 49–50 °C (depending on the different primers) and 3–4 min at 72 °C (depending on the different PCR product lengths). The last cycle was followed by a final extension step of 10 min at 72 °C. PCR fragments were generated with oligonucleotides that contained the short sequences rec5 (forward) and rec2 (reverse), which are homologous to the sequences in the plasmid (about 35 bp). The plasmid was linearized by *Sal*I digestion and digested with *Asl*I to avoid sticky ends and to make the recombination process easier (Jansen et al., 2005). The wine yeast *ho*QA23 was co-transformed with the digested pGREG505 plasmid together with the PCR amplified target gene, flanked by recombination sequence homologues to the plasmid ends. This co-transformation promotes an in vivo homologous recombination between both fragments. This recombination process also deleted the *GAL1* promoter of the plasmid (the genes were cloned with their own promoters). The transformants were selected by Geneticin resistance, which is encoded by the *KanMX* gene in the plasmid. Correct yeast transformations were verified by plasmid DNA isolation using a modification of the protocol described by Robzyk and Kassir (1992) and subsequently amplification with the Illustra TempliPhi Amplification Kit (GE Healthcare, UK). .Then, to verify the correct integration of the gene into the vector, plasmids were checked by PCR using primers specified for sequences rec5 and rec2. All the strains (mutants and overexpressing) constructed in this study are shown in Table 1.

2.3. Generation time (GT)

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 12 °C and 28 °C. Measurements were taken, after pre-shaking the microplate for 20 s, every half hour over 3 days. However at 12 °C the microplate had to be incubated outside the SPECTROstar spectrophotometer and then transferred inside to take measurements every 8 h during the lag phase and every 3 h during the exponential phase. The microplate wells were filled with 0.25 mL of SC medium, reaching an initial OD of approximately 0.2 (inoculum level of 2×10^6 CFU/mL). Uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal. All experiments were carried out in triplicate.

Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparameterized Gompertz equation proposed by Zwietering et al. (1990):

$$y = D * \exp\{-\exp[((\mu_{max} * e)/D) * (\lambda - t)) + 1]\}$$

where $y = \ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D = \ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h) (Salvadó et al., 2011). Generation time (GT) was calculated using the equation $GT = \ln 2/\mu_{max}$. To normalize, this value was divided by the GT of *S. cerevisiae* BY4742 and *ho*QA23 (control strains).