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Integration of transcriptomic and metabolic analyses for understanding the global responses of low-temperature winemaking fermentations

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wine fermentation; transcript profiling; metabolic regulation; flavours; yeast.

Abstract

Wine produced at low temperature is often considered to have improved sensory qualities. To investigate the effects of temperature on winemaking, the expression patterns during the industrial fermentation process carried out at 13 °C and 25 °C were compared, and correlated with physiological and biochemical data, including viability, fermentation byproducts and lipid content of the cells. From a total of 535 ORFs that were significantly differentially expressed between the 13 °C and 25 °C fermentations, two significant transcription programmes were identified. A cold-stress response was expressed at the initial stage of the fermentation, and this was followed by a transcription pattern of upregulated genes concerned with the cell cycle, growth control and maintenance in the middle and late stages of the process at 13 °C with respect to 25 °C. These expression patterns were correlated with higher cell viability at low temperature. The other relevant transcriptomic difference was that several genes implicated in cytosolic fatty acid synthesis were downregulated, while those involved in mitochondrial short-chain fatty acid synthesis were upregulated in the fermentation process conducted at 13 °C with respect to that at 25 °C. These transcriptional changes were qualitatively correlated with improved resistance to ethanol and increased production of short-chain (C₄–C₈) fatty acids and their corresponding esters at 13 °C as compared to 25 °C. While this increase of ethyl esters may account in part for the improved sensory quality of wine fermented at 13 °C, it is still unclear how the esterification of the short-chain fatty acids takes place. On the basis of its strong upregulation at 13 °C, we propose a possible role of *IAH1* encoding an esterase/ester synthase in this process.

Introduction

The wine yeast strains have been selected over hundred of years by winemakers and scientists to efficiently ferment grape-must sugars under rather stressful oenological conditions. These conditions are substantially different from laboratory conditions, especially with regard to the high sugar content (160–250 g L⁻¹), low pH (< 3.5), high alcohol production (> 10% v/v) and limited oxygen (hypoxia). Moreover, the grape musts are often unbalanced, with nitrogen sources, lipids and vitamins as limiting components (Bauer & Pretorius, 2000; Pretorius, 2000; Barre *et al.*, 2001). All these conditions lead to gene expression changes

and structural modifications of yeast cells as they adapt to these extreme conditions (Cavalieri *et al.*, 2000; Backhus *et al.*, 2001; Rossignol *et al.*, 2003).

Another important parameter that can drastically alter the winemaking process is the temperature. While the optimal rate of wine fermentation by *Saccharomyces cerevisiae* occurs close to 25 °C, there is increasing interest in conducting this process at lower temperatures. In fact, most white wine fermentations are carried out at 18 °C. Lowering the fermentation temperature to 13 °C or even below has some disadvantages, including an increase in the duration of the process and a greater risk of halted or sluggish fermentation (Bisson, 1999). Moreover, low temperature induces

cold-responsive genes, whose role in the winemaking process has not yet been addressed (Kondo & Inouye, 1991; Kondo *et al.*, 1992; Kowalski *et al.*, 1995; Rodriguez-Vargas *et al.*, 2002; Sahara *et al.*, 2002; Schade *et al.*, 2004). Conversely, fermentation at low temperature has advantages for taste, such as a restructuring of the flavour profiles with potential oenological applications (Feuillat *et al.*, 1997; CharoENCHAI *et al.*, 1998; Torija *et al.*, 2003). The aromatic complexity of a wine is dependent on primary flavours (those originating from the grapes), secondary or fermentative flavours (those that are produced by yeasts and bacteria during alcoholic and malolactic fermentation) and tertiary or postfermentative flavours (those appearing during the ageing process) (Schreier, 1979; Boulton *et al.*, 1996; Rapp, 1998). A simple interpretation could be that the retention of primary and secondary flavours is higher at low temperatures of 10–15 °C than at 25 °C. Thus, the improved 'bouquet' and taste of wines produced at low temperatures could be attributed to a greater retention of terpenoid compounds, an increase in the production of volatile esters and C₆–C₁₀ medium-chain fatty acid esters, and a reduction in higher alcohols and volatile acidity (Lambrecht & Pretorius, 2000; Torija *et al.*, 2003; Llaurodo *et al.*, 2004; Novo *et al.*, 2003a). However, our previous observations indicated that terpenoid compounds showed limited changes at low temperature, whereas fatty acid esters, which are produced by intracellular metabolic activity of the cells, increased considerably in amount at low temperature (Torija *et al.*, 2003; Llaurodo *et al.*, 2004).

To determine the molecular mechanisms behind the impact of low temperature on wine flavours, we compared the global gene response of yeast during the winemaking process at 13 °C with respect to 25 °C, using DNA microarray technology. The yeast strain used in this work was a wine strain that was originally isolated in a Portuguese vineyard. Technically, we used DNA microarrays bearing 6000 ORFs from the laboratory-sequenced strain S288c, since these are the only ones available and it was claimed that the sequence homology between wine and laboratory strains was over 98% (Hauser *et al.*, 2001). However, a recent transcriptomic study of a commercial wine yeast using serial analysis of gene expression (SAGE) revealed that 10% of transcripts at the end of the fermentation stage matched nonannotated ORF regions in the yeast genome (Varela *et al.*, 2005). Thus, our differential expression study was limited to well-annotated genes from the sequenced S288c yeast strain. In addition, to make any biological interpretation of transcriptomic data meaningful, we measured physiological and metabolic data such as cell viability, flavours, fermentation byproducts and cellular lipids during the winemaking process at both temperatures. The two datasets (transcriptomics and metabolic/physiological) helped us to identify relevant 'qualitative correlations' between expres-

sion changes and physiological modifications between 13 °C and 25 °C. Moreover, this functional analysis is the first study carried out under rigorous industrial fermentation conditions, as previous studies have been carried out under simulated oenological conditions (Backhus *et al.*, 2001; Marks *et al.*, 2003; Rossignol *et al.*, 2003).

Materials and methods

Strain and fermentation conditions

The *S. cerevisiae* wine strain QA23 (commercialized by Lallemant S.A., Canada) was cultured in a 100-L steel vat containing a muscat grape must (this medium was supplied by Mas dels Frares, Tarragona, Spain, and contained about 160–200 g L⁻¹ sugar) at a controlled temperature of 25 °C or at 13 °C. Before starting the fermentation, the must medium was clarified by natural settling to separate the clear juice from the sediments, and sulphur dioxide (50 mg L⁻¹) was added to the culture media. The fermentation was started with 2 × 10⁶ cells mL⁻¹ of dry yeast cells, which were rehydrated in water at 37 °C for 30 min prior to inoculation, according to the manufacturer's instructions. Yeast growth was monitored by counting the viable cells after plating on YEPD agar medium (yeast extract, 1% w/v; Bacto peptone 2% w/v; glucose 2% w/v; Difco agar 2% w/v) at an adequate dilution for 2 days at 28 °C. The values were expressed in CFU mL⁻¹. Consumption of sugars during fermentation was monitored by change in the medium density, which is correlated with levels of reducing sugar content as described in Ribéreau-Gayon *et al.* (2000). Fermentation was considered to be finished when the level of reducing sugars measured by enzymatic assay using an enzymatic kit from Roche Applied Science (Germany) was below 10 g L⁻¹. We used a restriction fragment length polymorphism method described elsewhere (Beltran *et al.*, 2002) to verify that the inoculated strain QA23 represented 80–100% of the yeasts isolated from day 2 to the end of the fermentation.

Preparation of mRNA and microarray analysis

Cell samples, corresponding to *c.* 2 × 10⁸ CFU (about 15 mg dry mass), from the fermentation tank were harvested in triplicate by centrifugation (5000 g for 5 min at 4 °C) at three points during the process, namely initial fermentation, mid-fermentation and final fermentation. The cell pellet was immediately frozen in liquid nitrogen, and stored at -80 °C until use. Total RNA was extracted using a commercial kit (RNAeasy mini kit, Qiagen). The quantity and the control quality of the extracted RNA were checked by microcapillary electrophoresis using a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE).

The DNA chips were manufactured at the Biochip platform of Toulouse-Genopole on dendrislides (LeBerre *et al.*,

2003) using 70-mer oligonucleotides representing *c.* 99% of the yeast genome purchased from Operon Inc. (list of corresponding genes accessible at <http://biopuce.insa-toulouse.fr/oligosets/>). Fluorescent-labelled cDNA was synthesized from 25 µg of total RNA using the CyScribe first-strand cDNA-labelling kit (Amersham Bioscience). Labelled cDNA was purified using the CyScribe GFX Purification kit (Amersham Pharmacia). Hybridization was carried out in an automatic hybridization chamber (Discovery, Ventana). Microarrays were prehybridized in a solution of 1% bovine serum albumin, 2 × SSC, 0.2% sodium dodecyl sulphate (SDS) for 30 min at 42 °C, and this was followed by addition of a mixture containing 200 µL of RiboHybe (Ventana), 10 µL of Cy3-labelled cDNA and 10 µL of Cy5-labelled cDNA. After 14 h of hybridization at 42 °C, the DNA chips were washed for 5 min in 2 × SSC, 0.1% SDS at room temperature, and four times in 0.1 × SSC buffer for 2 min at room temperature. The hybridization signal was detected by scanning using a GenePix 4000B laser Scanner (Axon Instruments), and the signal quantification was transformed to numerical values using the integrated GenePix software version 3.01. The analyses (from RNA extraction to image analysis) were performed twice by swapping the fluorescent dyes CY3 and CY5 to reduce the false-positive/negative values due to dye effects.

Data acquisition and data treatments

First, it should be acknowledged that statistical analysis was performed on the expression data from a single experiment (one fermentation performed at 13 °C and another at 25 °C), since it was technically impossible to repeat these fermentations, which were performed under strict industrial winemaking conditions. The raw data are presented at <http://biopuce.insa-toulouse.fr/jmflab/winegenomics/>, (Table S1), which provides full details of normalization and statistical regimes using our home-made Biplot software. This software is an online web service available to all users of Biochips platform. A complete user's guide is available at <http://biopuce.insa-toulouse.fr/ExperimentExplorer/doc/BioPLot/>. Raw intensities were corrected from the background, log transformed and normalized by the mean log-intensity of all spots. Log-ratios of normalized intensities from duplicate samples were tested for statistical significance using Student's paired bi-tailed *t*-test. To reduce the false discovery rate, we tested genes with at least a two-fold variation, and the *P*-value threshold in the Student's *t*-test was set at ≤ 0.05 . To determine the degree to which transcription of a particular gene was regulated under a given condition (mid-fermentation and final fermentation), the normalized value from that condition was divided by the corresponding value from the other condition (initial fermentation), and converted to \log_{10} . Positive and negative values defined,

respectively, upregulated and downregulated genes under the conditions studied. The differentially expressed genes were placed into the 16 functional classes defined by the Munich Information Center for Protein Sequences Yeast Genome database (<http://mips.gsf.de/proj/yeast/>). GeneSpring version 4.2 software (Silicon Genetics Inc.) was used for visualization and hierarchical clustering. The SGD (www.stanford.edu/Saccharomyces) and FunSpec (<http://funspec.med.utoronto.ca/>) databases were also consulted to detect nodes that were enriched in a particular cellular function (Genes Ontologies). Other details related to data processing as well as a query-based website for viewing specific gene fold change data are available online (<http://biopuce.insa-toulouse.fr/jmflab/winegenomics>).

Determination of fermentation byproducts, alcohols and esters

Wine samples (100 mL) were taken throughout the fermentation process for measurement of acetic acid and ethanol according to the method described (Garcia Barcelo, 1990). Other byproducts, including fusel alcohol, short-chain and medium-chain aliphatic esters, terpenes and free short-chain and medium-chain aliphatic fatty acids were extracted by liquid/liquid extraction with 200 µL of 1,1,2-trichlorotrifluoroethane (Fluka) and 0.5 g of NaCl using *n*-decanol (0.2 mg L⁻¹) as internal standard and according to the method of Ferreira *et al.* (Ferreira *et al.*, 1996). After 2 min of agitation and 2 min of centrifugation at 1500 g, the organic phase was extracted and 2 µL of this phase was injected into a TR-WAX column (60 m × 0.25 mm × 0.25 µm) with the automatic injector HP 7683 (Agilent Technologies, Wilmington, DE), and equipped with an FID detector. Aromatic volatile compounds were identified and quantified by comparison with standards.

Determination of cell fatty acid composition

Yeast cells (5–10 mg of dry mass or about 10⁸ CFU) were collected at the same stages of fermentation as for microarray analysis. Fatty acids were extracted from yeast cells and analysed according to the method of Rozes *et al.* (1992). Analytical gas chromatography was performed on a Hewlett-Packard 5890 connected to an HP Vectra computer with the ChemStation software (Agilent Technologies). A 2-µL cellular extract was injected (splitless, 1 min) into an FFAP-HP column (30 m × 0.25 mm × 0.25 µm from Agilent Technologies) with an HP 7673 automatic injector. The initial temperature was set at 140 and increased by 4 °C min⁻¹ up to 240 °C. Injector and detector temperatures were 250 °C and 280 °C, respectively. The carrier gas was helium at a flow rate of 1.2 mL min⁻¹. Heptanoic and heptadecanoic acids (1 and 4 mg mL⁻¹, respectively) were added as internal

standards. Relative amounts of fatty acids were calculated from their respective chromatographic peak areas. These values were related to the dry mass of cells and expressed as percentage of total fatty acids extracted. The mean fatty acid chain length (CL) was calculated as: $CL = \Sigma(PC)/100$, where P is the percentage of fatty acids and C is the number of carbon atoms.

Determination of sterol composition of the yeast cells

Yeast cells (5–10 mg of dry mass) collected as above were analysed for lipid and sterol content as follows. Cells were saponified by incubation at 90 °C for 30 min in a methanol solution containing 15% (w/v) KOH and 5- α -cholestane (1 mg mL⁻¹) was used as an external standard. Sterols were extracted with 30 μ L of hexane. Analytical gas chromatography was performed as described above, using a SAC-5 column (15 m \times 0.25 mm \times 0.25 μ m) (Supelco, Bellefonte, PA). The relative amount of a given sterol component was calculated from its respective chromatographic peak areas. From the same extracts, gas chromatography–mass spectrometry of sterols was conducted on an HP5890 series II coupled to an HP5972 mass selective detector. Electron impact GC/MS (70 eV, scanning from 42 to 600 atomic mass units at 1-s intervals) was performed under the following conditions: SAC-5 column (30 m \times 0.25 mm and 0.25- μ m film thickness), He as carrier gas (30 cm s⁻¹), detector temperature 250 °C, column temperature 125–250 °C (125 °C for 2 min, 20 °C min⁻¹ to 250 °C for 60 min), and injector temperature 250 °C. All injections were run in a splitless mode.

Lipid extraction and separation of yeast cells

Prior to lipid extraction, a solution of 100 μ L of cold methanol + 10 μ L of EDTA 0.1 mM was added to yeast cells (5–10 mg dry mass) with 1 g glass beads (0.5 mm, Biospec Products) in 2.0-mL conical screw-cap microtubes (Porex Bio Products), and then mixed for 5 min in a mini-beadbeater-8 (Biospec Products, Qiagen). Lipid extraction was performed with chloroform/methanol (2:1, v/v, two times and 1:1 v/v, once). The organic phase was transferred to a 15-mL glass screw-cap tube in the presence of KCl 0.88% (one-fourth of the total volume of the extract). After vortexing and cooling on ice for 15 min, the samples were centrifuged for 10 min at 1500 g. The organic phase was collected and dried by passing through a Na₂SO₄ column, and then concentrated to dryness under a nitrogen stream. The extract was dissolved in chloroform/methanol (2:1, v/v).

Individual lipid classes were separated by one-dimensional thin-layer chromatography (TLC) on silica gel 60F₂₅₄

plates (10 \times 20 cm, 250 μ m) (Merck, Germany) with solvent systems. For the analysis of neutral lipids (sterol, sterol ester, diacylglycerol, triacylglycerol, fatty acid ethyl ester (FAEE) and squalene), the solvents were hexane, *t*-butylmethyl ether (MTBE) and glacial acetic acid (70:30:2, v/v). For analysis of phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, cardiolipin and phosphatidic acid (PA)), the solvent system was chloroform/acetone/methanol/glacial acetic acid/water (50:15:10:10:5, v/v). The standard lipids lanosterol, ergosterol, squalene, cholesterol oleate, ethyl oleate, diolein, triolein, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, cardiolipin and PA were purchased from Sigma and used for calibration in standard solutions (0.5–4 μ g μ L⁻¹) that were applied as internal controls to each plate. Lipids on TLC plates were detected with 10% CuSO₄ in 8% H₃PO₄ and heated at 120 °C for 20 min. The TLC plate showing brown spots was photographed with a Kodak DC290 Zoom digital camera. For lipid quantification, each spot of the image on the TLC plate was processed with Quantity One software (Bio-Rad) and calculated from the calibration curves made with the lipid standards.

Results

Macrokinetic analyses of wine fermentation at 25 °C and 13 °C

Wine fermentations were carried out at 25 °C and 13 °C in 100-L tanks with fresh rehydrated industrial yeast cells inoculated at 2×10^6 CFU mL⁻¹ in muscat grape must containing approximately 200 g L⁻¹ sugars (*c.* 50% glucose and *c.* 50% fructose) (Fig. 1). At the two fermentation temperatures, the exponential phase was relatively short in duration, and was followed by a lengthy nonproliferating phase that lasted for 6 days at 25 °C and for 14 days at 13 °C, during which > 70% of the sugars initially present in the must were consumed. The fermentation was considered to be finished when the grape-must density dropped below 1000 units, *i.e.* when the residual reducing sugar in the medium was below 10 g L⁻¹. At this point, the final ethanol titre reached about 11% (v/v) for the two fermentations (Table 1). It is interesting to note that the fermentation profiles were roughly similar at both temperatures, although, as expected, the rate of the process was about two-fold lower at 13 °C than at 25 °C. In addition, the CFU mL⁻¹ of medium reached maximum values after 3 days and 6 days at 13 °C and 25 °C, and subsequently decreased gradually at 25 °C, while it continued to increase very weakly during the remaining 12 days of fermentation at 13 °C.

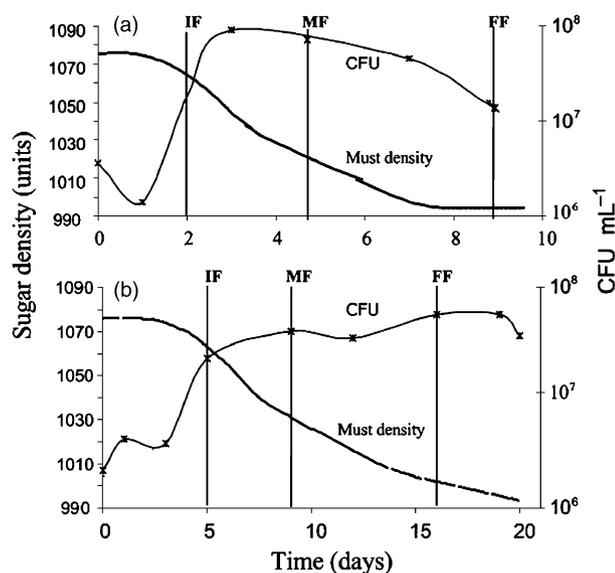


Fig. 1. Fermentation kinetics of *Saccharomyces cerevisiae* strain QA23 on grape must at 13 °C (a) and 25 °C (b). The samples for RNA extraction, analysis of cell lipid and sterol composition and measurement of fermentation byproducts are represented by dotted lines and termed IF (initial fermentation), MF (middle fermentation) and FF (final fermentation).

Table 1. Effects of temperature of fermentation on ethanol content, volatile acidity and aromatic volatile compounds of wines

| Flavour compounds (mg L ⁻¹) | 13 °C | 25 °C |
|---|-------|-------|
| Fusel alcohols | 161.6 | 217.6 |
| Fusel alcohol acetate | 1.8 | 1.5 |
| Terpenes | 0.63 | 0.60 |
| Fatty acid ethyl esters | 3.4 | 2.3 |
| Fatty acids | 9.4 | 5.1 |
| Ethanol (% v/v) | 10.9 | 10.7 |
| Volatile acidity (g acetic acid L ⁻¹) | 0.36 | 0.45 |

Values are given for this typical wine fermentation carried out at 13 °C and 25 °C. They are the average of two determinations. The coefficient of variation (CV in %) was close to 10%, except for decanoic acid (18%), dodecanoic acid (38%), ethyl octanoate (16%) and ethyl decanoate (29%). Note that very comparable values have been obtained in other wine fermentation over the years (Novo *et al.*, 2003a; Torija *et al.*, 2003; Llauro *et al.*, 2004).

Transcription profiles at 13 °C and 25 °C and comparison of expression profiles between the two temperatures

Changes in the global expression of genes during wine fermentation at 13 °C and 25 °C were analysed with microarrays containing 70-mer oligonucleotides corresponding to 5915 ORFs from the best annotated genome of the *S. cerevisiae* S288c strain (Talla *et al.*, 2003). In order to compare gene expression changes during the wine fermentation at two different temperatures, we arbitrarily defined

three stages based on similar macrokinetic data (Fig. 1), assuming that cells experienced comparable glucose consumption and ethanol production. The 'growing phase' (initial fermentation) corresponded to a period during which yeast cells were growing exponentially, when the production of ethanol was barely detectable. Two other stages were chosen, the mid-fermentation and final fermentation. The mid-fermentation corresponded to a period in which yeast cultures were at the start of the nonproliferating phase, with about 50% of the sugar consumed and ethanol production at approximately 5–6% (v/v). The final fermentation corresponded to the arrest of wine fermentation with the medium containing residual sugars (< 10 g L⁻¹) and high ethanol levels (> 10% v/v).

The kinetics of transcription profiles during fermentation at 25 °C and 13 °C were determined by hybridizing labelled cDNA from the mid-fermentation and final fermentation relative to the initial fermentation, used as the reference. The analyses were performed twice by swapping the fluorescent CY3 and CY5 dyes using RNAs extracted from two different samples taken from the same fermentation tank. Although it was not possible for technical reasons to perform two independent wine fermentations under these specific industrial conditions, this mode of analysis had the merit of reducing most of the systematic errors associated with DNA microarray technology (Yang *et al.*, 2002). Overall, c. 5200 ORFs (88% of the whole genome) were qualitatively detected after filtering procedures (see Materials and methods), from which 1561 genes at 25 °C and 1226 at 13 °C showed a two-fold difference for at least one stage of the fermentation. On the basis of our data quality settings (i.e. ≥ 2 -fold variation and statistical significance with *P*-value set at < 0.05 from Student's *t*-test), 519 and 379 genes were retained for the fermentations at 25 °C and 13 °C, respectively (see supplementary online Tables S2 and S3).

In a second analysis, we compared gene expression at each stage of the fermentation process at 13 °C with gene expression at the corresponding phases of fermentation at 25 °C. We decided to perform this direct stage-to-stage comparison of yeast cultures on the assumption that they exhibited comparable macrokinetic parameters, i.e. same amount of sugars consumed and ethanol produced. Therefore, labelled transcripts from the initial fermentation at 13 °C were hybridized against labelled transcripts from the initial fermentation at 25 °C, and so on for the mid-fermentation and final fermentation. From a total of 2275 genes that exhibited a greater than two-fold change in signal intensity between 13 °C and 25 °C, only 535 genes (20%) were retained on the basis of a second filtering test (Student's *t*-test with *P*-value < 0.05) (see supplementary online Table S4). The differentially expressed genes from these filtered datasets were organized by hierarchical clustering (Eisen & Brown, 1999) to search for correlations between expression profiles of the

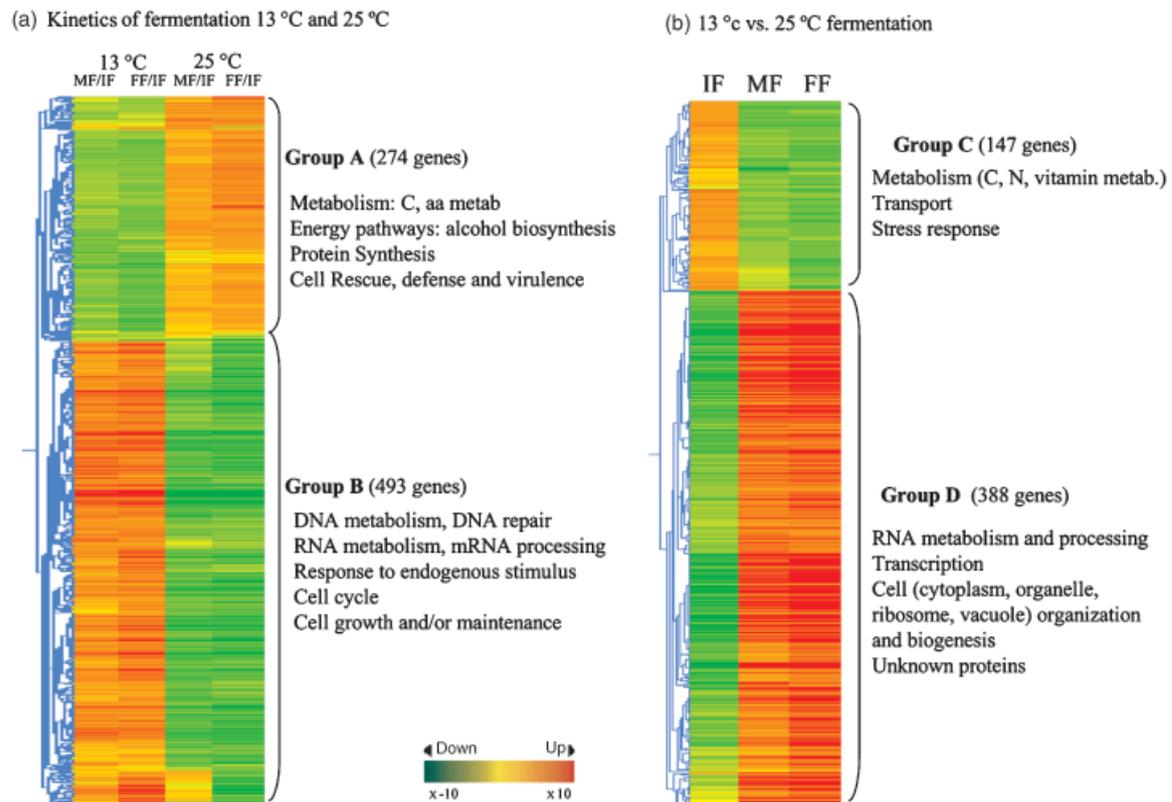


Fig. 2. Hierarchical clustering analysis of differentially expressed genes during fermentation at 13 °C and 25 °C (four datasets) and 13 °C vs. 25 °C, at different points of the fermentation (13 °C vs. 25 °C, three datasets). Upregulated and downregulated genes (at least two-fold variation and a *P*-value threshold in Student' *t*-test < 0.05) were analysed by a clustering method as described in Materials and methods. This analysis identified the main clusters enriched in genes in specific MIPS; GO functional categories listed on the right (obtained from Funspec analysis, see Materials and methods). The colour scale at the bottom represents the expression ratio: x-fold repressed in green, and x-fold activated in red, with a maximum level of 10-fold.

seven datasets (Fig. 2a and b). Genes were clustered in two main groups. Interestingly, genes that were downregulated during fermentation at 25 °C were those that were upregulated at 13 °C (group B), and they were the same genes (cluster of 388 genes) that were more expressed in the middle to late phases of fermentation at 13 °C than at 25 °C (13 °C vs. 25 °C; see group D in Fig. 2b). The main categories of this cluster (groups B and D) were genes that encode proteins implicated in DNA and RNA metabolism, in transcription and in growth-associated functions (cell growth and maintenance, cell cycle, nucleic acid metabolism; see also supplementary online Tables S2–S4). Conversely, genes that showed coordinated upregulation during fermentation at 25 °C were those that were downregulated during the same fermentation stages at 13 °C (groups A and C in Fig. 2), and hence these were the same genes that were more expressed in the middle to late stationary phase of growth at 25 °C relative to 13 °C (13 °C vs. 25 °C, mid-fermentation and final fermentation). The main functional categories of this cluster (groups A and C) were the biological processes of carbon, energy reserve and vitamin

metabolism, energy pathways, glucose and amino acid transport, and general stress response. Taken together, these expression data were consistent with higher metabolic activity of cells during the early growth phase at 25 °C than at 13 °C, and with the observation that cell viability was higher in the nonproliferating phase at 13 °C than at 25 °C (Fig. 1).

Upregulation of genes implicated in cold-stress response, cell viability and cell defence characterized the fermentation at 13 °C relative to 25 °C

About 10% of genes in the category of 'cell rescue, defence and virulence' were differentially expressed at one of the stages during fermentation at 13 °C relative to 25 °C. Of these, a group of genes including *TIR1*, *PAU1*, *ZEO1* and *SRL1* showed higher transcript levels at the initial stage of the fermentation at 13 °C than at 25 °C, and then showed lower transcript levels at the middle and late stages of fermentation at 13 °C than at 25 °C (Fig. 3a). Since the

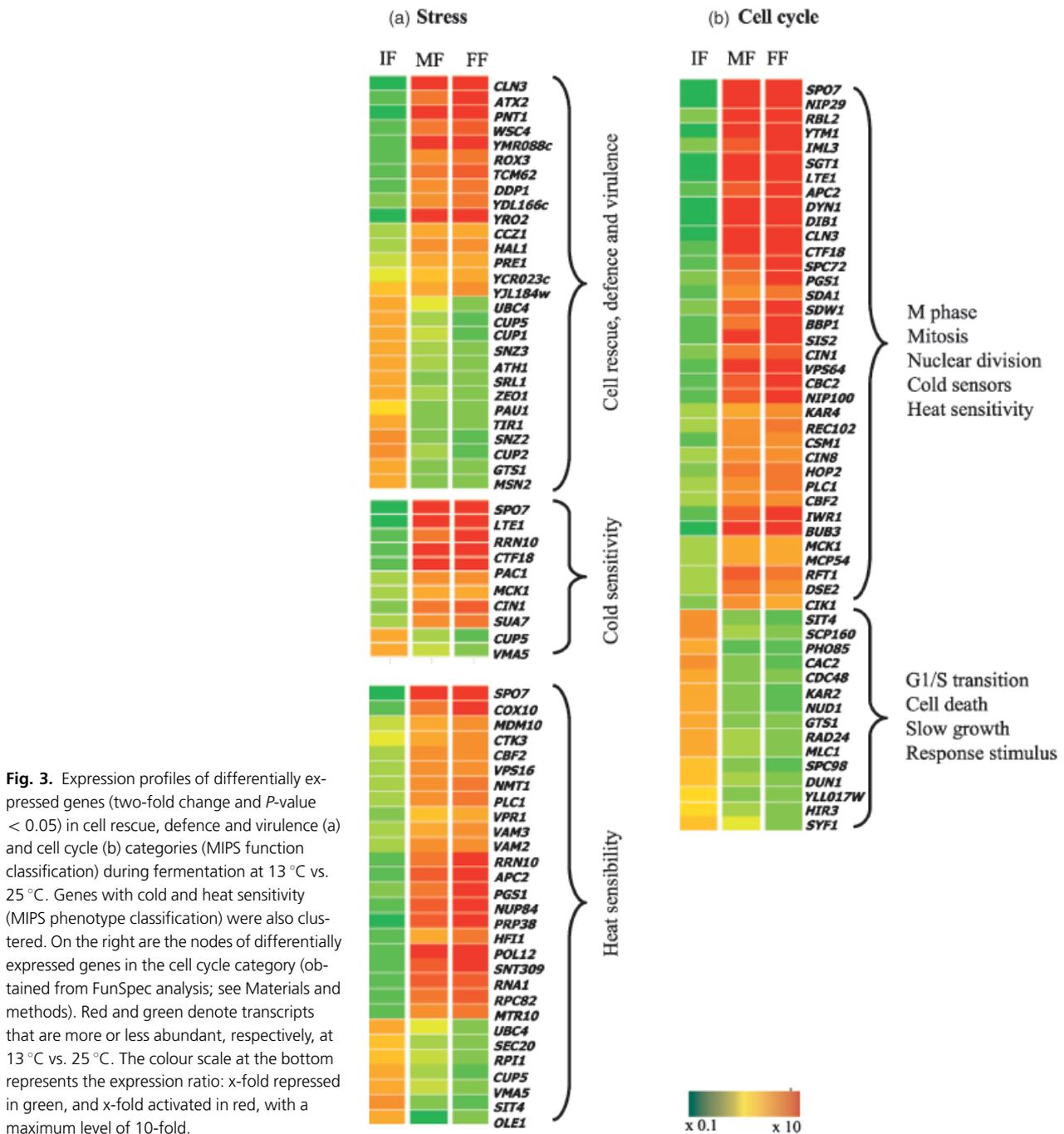


Fig. 3. Expression profiles of differentially expressed genes (two-fold change and P -value < 0.05) in cell rescue, defence and virulence (a) and cell cycle (b) categories (MIPS function classification) during fermentation at 13 °C vs. 25 °C. Genes with cold and heat sensitivity (MIPS phenotype classification) were also clustered. On the right are the nodes of differentially expressed genes in the cell cycle category (obtained from FunSpec analysis; see Materials and methods). Red and green denote transcripts that are more or less abundant, respectively, at 13 °C vs. 25 °C. The colour scale at the bottom represents the expression ratio: x-fold repressed in green, and x-fold activated in red, with a maximum level of 10-fold.

expression of these genes is also known to be induced during hypoxia and cold shock (Kowalski *et al.*, 1995; Kwast *et al.*, 2002), it is expected that their expression changes may influence cell wall porosity and may be required for the change in membrane fluidity (see below). We also found that transcript levels of *MSN2*, which encodes the master transcription factor of the general stress response (Martinez-Pastor *et al.*, 1996; Estruch, 2000), were higher in the initial stage of fermentation at 13 °C than at 25 °C. This higher

expression was accompanied by activation of a set of stress response element-responsive genes in this stage of fermentation at 13 °C (see supplementary online Table S1). These results were consistent with the recent work of Schade *et al.* (2004), who found that some of the late cold-responsive genes are those involved in the *MSN2/4*-dependent stress response. Conversely, the transcript levels of a series of genes encoding proteins implicated in drug resistance, metal detoxification (*PNT1*, *ATX2*, *YMR088c*, *DDP1*, *CCZ1*,

YCR023c and *HAL1*), and cell wall stress sensor (*WSC4*), and two genes encoding proteins with chaperone properties (*YRO2* and *TCM62*), reported to be induced very late in response to a cold stress (Schade *et al.*, 2004), were more abundant in the middle and final stages of the fermentation at 13 °C relative to 25 °C. A small 'cold-sensitive cluster' that comprised 10 genes was also identified (Fig. 3a). However, apart from *LTE1*, which encodes a GDP/GTP exchange factor required for growth at low temperatures (Shirayama *et al.*, 1994), and *MCK1*, encoding a Ser/Thr kinase that is involved in cold-stress tolerance (Jesus Ferreira *et al.*, 2001), the other genes were classified as 'cold-sensitive genes' because their loss of function leads to either very slow growth or no growth at 4 °C (see SGD at <http://www.yeast-genome.org/>). Some of these genes, namely *SPO7*, *RRN10*, *PAC1* and *SUA7*, are implicated in DNA synthesis and chromosome segregation, suggesting that this process is temperature sensitive. The third subgroup of the cell rescue and defence category included a set of genes reported to be 'heat sensitive'. A quick overview of this subgroup indicates that most of those whose expression was increased during late fermentation at 13 °C rather than at 25 °C encode products that are associated with cell growth and whose deficiency causes growth defects at temperatures above 37 °C. Note that the MIPS classification placed *OLE1* in the heat-sensitive class, although it is also known to be induced immediately in response to hypoxia and low temperature (Nakagawa *et al.*, 2002; Sahara *et al.*, 2002; Schade *et al.*, 2004), as can be seen in the initial growth phase at 13 °C (Fig. 3).

An interesting cluster of 51 genes implicated in the cell cycle was extracted (Fig. 3b). This cluster of genes, the transcript of which became more abundant in the middle and late phases of the fermentation at 13 °C than at 25 °C, encompassed functional categories related to chromosome segregation (*APC2*, *CTF18*, *CIN1* and *CSM1*) and the connection of cellular polarity/spindle position with cell cycle progression (*LTE1*, *DYN1*, *DIB1*, *IML3*, *CTF18*, *CIN1*, *CIN8* and *BUB3*). It is worth noting that the coregulation of several of these genes was consistent with their genetic interaction as identified by synthetic genetic array (SGA) analysis (Tong *et al.*, 2001). Another set of genes that showed a similar trend at 13 °C vs. 25 °C were those that encoded products involved in mating and sporulation (*SPO7*, *FUS1*, *KAR4*, *OPY2*, *SST2* and *SPS4*) as well as in pseudohyphae formation and invasive growth (*DFG16*, *DIG1* and *DFG10*) (see supplementary online Tables S2 and S3). However, the upregulation of these genes at the lower temperature was not accompanied by visible differences in the morphology of yeast cells between the two temperatures under a regular optical microscope (data not shown). Conversely, down-regulated genes of 'the cell cycle cluster' were those implicated in G1 to S transition, among which the most affected

were *PHO85*, *SIT4* and *GTS1*, which encode, respectively, a cyclin-dependent protein kinase (Measday *et al.*, 1997), a catalytic subunit of protein phosphatase type 2A (Sutton *et al.*, 1991) and a putative transcription factor implicated in the timing of bud emergence and regulation of lifespan and ageing (Mitsui *et al.*, 1994). Taken together, these transcriptional changes suggested a better capacity of yeast cells at 13 °C than at 25 °C to continue to grow and to survive, while they were apparently not able to divide. Consistent with this idea, one should bear in mind that the viability of the yeast cells during prolonged cultivation was higher at 13 °C than at 25 °C (Fig. 1).

Enhanced expression of genes implicated in aroma production at low temperature correlated with increased production of volatile and fatty acid ethyl esters

Since fermentation temperature affects wine sensory qualities (Lambrecht & Pretorius, 2000; Pretorius & Bauer, 2002), we measured several fermentation byproducts that may contribute to the sensory aspect of wines at the end of the process. Fusel alcohols, volatile fatty acids and ethyl esters were measured at the final stage of the process (i.e. day 9 for the fermentation at 25 °C and day 20 for that at 13 °C; see Table 1). It can be seen that levels of acetate esters (sum of isoamyl acetate+hexyl acetate) and fatty acid ethyl esters were 20–50% higher at 13 °C than at 25 °C, whereas levels of volatile acidity and fusel alcohols were lower by about 25%. Also, lowering the fermentation temperature resulted in an 84% increase in the production of short-chain (C₄–C₈) fatty acid ethyl esters. It should be stressed that these flavour profiles were similar to those reported in other independent industrial fermentations over the years (Novo *et al.*, 2003a; Torija *et al.*, 2003; Llauro *et al.*, 2004). At low temperature the levels of varietal aromas (terpenes, not synthesized by yeast) were also maintained, which permitted us to more easily discriminate the taste of the wine fermented at 13 °C from those fermented at 25 °C. Taken together, these results illustrated the importance of the fermentation temperature in the final bouquet composition of the wine, which was linked not only to temperature-dependent aroma retention but also to significant metabolic intervention by the yeast cells.

The enhanced production of flavours at 13 °C may be due in part to differential expression of genes implicated in their synthesis. As shown in Fig. 4, genes involved in the catabolism of amino acids by the Ehrlich pathway, such as *BAT2* (which encodes the cytosolic transaminase isoform, Kispal *et al.*, 1996), *PDC6* (which encodes a minor pyruvate decarboxylase isoform, Dickinson *et al.*, 2000) and *ADH6* (which encodes an NADP-dependent alcohol dehydrogenase, Larroy *et al.*, 2002), as well as those implicated in the

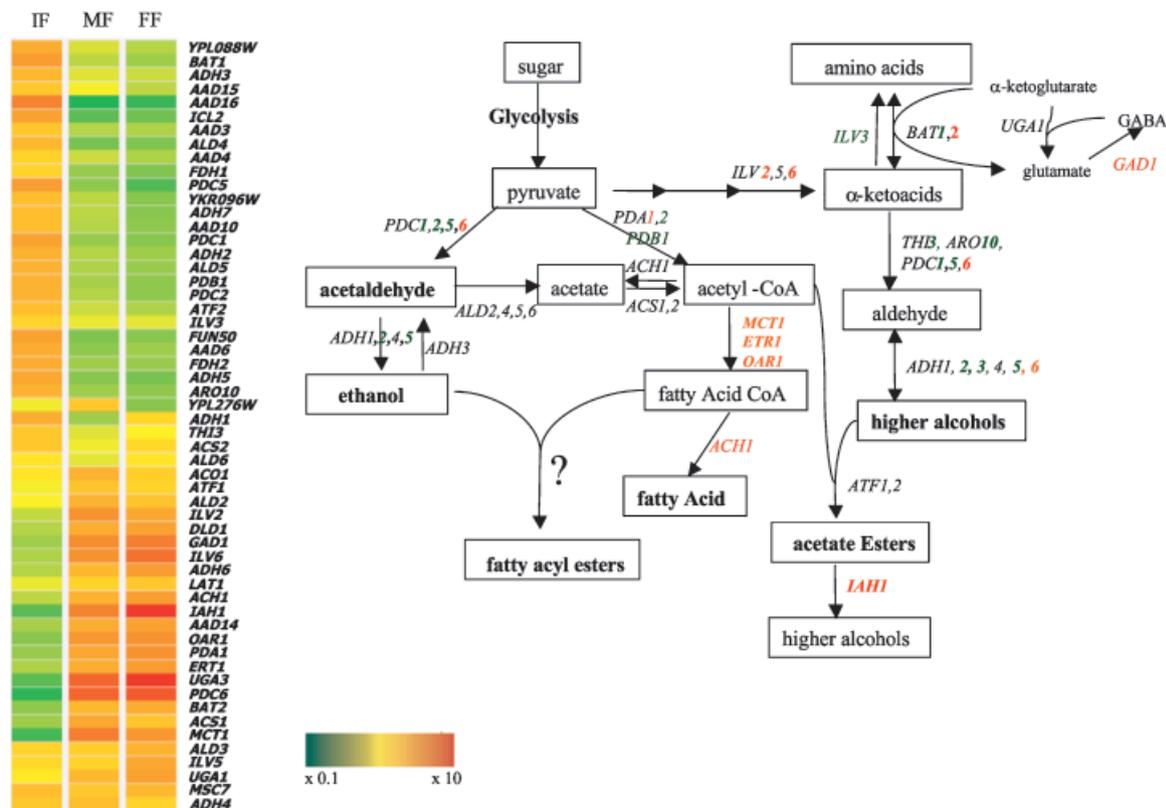


Fig. 4. Expression profiles of differentially expressed genes whose products are implicated in volatiles and fatty acid ester pathways during fermentation at 13 °C vs. 25 °C. The genes used to make this cluster were from the expression profile shown in Fig. 2. On the right is a simplified metabolic map of aroma formation from glucose. Genes upregulated at 13 °C vs. 25 °C are in red; genes downregulated at 13 °C vs. 25 °C are in green. The question mark indicates uncertainty about the enzyme that catalyses this reaction.

degradation of the branched-chain amino acids (*ILV2*, *ILV5* and *ILV6*), were expressed a higher level in the middle and late stages of the fermentation at 13 °C than at 25 °C. Also, it is possible that glutamate, 2-ketoglutarate and 4-aminobutyrate (GABA) supplied the Ehrlich pathway, as indicated by enhanced expression of *UGA1*, *UGA3* and *GAD1*, which encode, respectively, GABA transaminase, a transcription factor that regulates the GABA degradation system, and glutamate decarboxylase (Coleman *et al.*, 2001). In spite of this possible activation of the Ehrlich pathway at 13 °C, there was a lower production of fusel alcohols at this temperature relative to 25 °C. In addition, a slight but reproducible increase in the levels of two volatile esters (isoamyl acetate and hexyl acetate), which are made by the condensation of alcohols with acetyl-CoA, was not accompanied by increased expression of *ATF1* and *ATF2*, which encode the redundant alcohol acetyltransferase (Malcorps & Dufour, 1992; Fujii *et al.*, 1994). On the contrary, we found a 5- to 10-fold higher expression of *IAH1*, which encodes an esterase that is supposed to act antagonistically to the alcohol acetyltransferases (Mason & Dufour, 2000), at the end of fermentation at 13 °C relative to 25 °C.

With respect to higher levels of short-chain fatty acids and their corresponding ethyl esters at 13 °C, it is interesting to correlate these data with the fact that genes involved in mitochondrial short-chain fatty acid synthesis were significantly upregulated at 13 °C (see below). Moreover, the increased expression of *ACH1* at 13 °C relative to 25 °C could also account for greater production of free fatty acids, since the product of this gene, which is an acetyl-CoA hydrolase, is also active on fatty acyl-CoA (Mason & Dufour, 2000). However, it is unclear how fatty acyl esters are produced, since the enzyme responsible for the esterification reaction is still poorly characterized (Malcorps & Dufour, 1992; Mason & Dufour, 2000; Fig. 4). Finally, our comparative transcriptomic analysis also showed that the expression of most of the genes that belong to the aryl alcohol dehydrogenase family (*AAD3*, *AAD4*, *AAD6*, *AAD10*, *AAD15*, *YFL057c* and *YPL088w*) was lower at the end of fermentation at 13 °C than at 25 °C. Assuming that a putative function of the products of these genes in yeast is to degrade the complex aromatic compounds from grapes into their corresponding unpleasant alcohols (Delneri *et al.*, 1999), lower expression of these genes at 13 °C as compared

to 25 °C may also favour an increased aromatic quality of wine produced at 13 °C.

Differential cellular fatty acid and phospholipid composition partially correlated with differential expression of their corresponding genes between fermentation at 13 °C and 25 °C

Fermentation temperature has been reported to affect lipid composition and the membrane fluidity of cells (Alexandre *et al.*, 1994, 1996; Torija *et al.*, 2003), but data have been contradictory. Thus, we reconsidered this analysis and found that the major changes observed at low temperature were an increase in the degree of unsaturation at the beginning of fermentation and a decrease in the chain length as fermentation progressed. As also shown in Table 2, cells fermenting at 25 °C had higher levels of saturated fatty acids than cells fermenting at 13 °C. On the other hand, the proportion of medium-chain fatty acids remained at approximately 27% of total fatty acid content throughout the fermentation at 13 °C, but dropped from 35% at the initial stage to 11% at the final stage of the process at 25 °C. These results are in agreement with previous studies carried out by our research group (Torija *et al.*, 2003), where the fatty acid composition was analysed in several yeast strains fermenting at 13 °C and 25 °C. All yeast strains analysed showed a higher percentage of unsaturated fatty acids and a lower percentage of saturated fatty acids at low temperature.

The expression of lipid-related genes was different in the two fermentation processes. Since it is likely that oxygen availability was similar in the two fermentations, this differential gene expression may be due to the effect of temperature. As shown in Table 3, the expression profiles of key genes required for the cytosolic synthesis of long-chain fatty acids (*FAA1*, *FAA2*, *FAA3* and *SUR4*) were higher in the early phase and lower in the middle and late phases of the fermentation at 13 °C than in the corresponding phases at

25 °C. On the other hand, the expression profile of the genes required for type II mitochondrial (short-chain) fatty acid synthesis (*OARI*, *ETRI* and *MCT1*) was opposite to that of genes involved in long-chain fatty acid synthesis. It is therefore tempting to associate this relative difference in medium-chain fatty acid content between the fermentations at 13 °C and 25 °C with the differential expression of genes involved in mitochondrial fatty acid synthesis (Table 2), even though the role of this pathway in the biosynthesis of short-chain and medium-chain fatty acids is still a matter of discussion (Daum *et al.*, 1998).

Table 2 also shows that the proportion of unsaturated fatty acids at the initial stage of the fermentation at 13 °C was higher than at the same stage at 25 °C. This may be consistent with higher expression levels of *OLE1* at this stage of fermentation (Table 3). *OLE1* was one of the genes found in the principle component analysis (PCA) whose expression is significantly modified by temperature (see also Appendix S1, Table S5 and Fig. S1). We also measured a comparable amount of C18:2 in the lipid fraction of the yeast during the fermentations at 13 °C and 25 °C. Since yeast is unable to synthesize this polyunsaturated fatty acid (Taylor *et al.*, 1979), this indicates that there was no effect of temperature on the uptake and incorporation of this compound in yeast cells.

We also analysed the squalene and sterol composition of yeast cells fermenting at 13 °C and 25 °C (Table 2). The levels of squalene were higher at the initial fermentation stage at 13 °C than at 25 °C, but, as fermentation progressed, the proportion of squalene increased more clearly at 25 °C and was higher at this temperature in the final stages of fermentation. The sterol content decreased during the fermentation at both temperatures in a similar way. This could be in contradiction with the transcriptomic data, because *IDII*, which encodes the isopentenyl-diphosphate isomerase and which is implicated in sterol biosynthesis, was strongly upregulated at the middle to late phases of the

Table 2. Fatty acid, squalene and sterol composition (%) measured by gas chromatography in yeast cells at three stages of the fermentation at 13 °C and 25 °C

| | 13 °C | | | 25 °C | | |
|--------------------------|-------|-------|-------|-------|-------|-------|
| | IF | MF | FF | IF | MF | FF |
| Medium-chain fatty acids | 25.3 | 26.8 | 27.1 | 35.3 | 26.0 | 10.7 |
| Saturated fatty acids | 31.8 | 38.5 | 43.6 | 33.3 | 40.8 | 58.8 |
| Unsaturated fatty acids | 37.9 | 30.7 | 26.3 | 26.7 | 30.0 | 24.9 |
| Linoleic acid (C18:2) | 5.0 | 4.0 | 3.0 | 4.7 | 3.2 | 5.6 |
| Average chain length* | 14.95 | 14.96 | 14.98 | 14.39 | 15.07 | 16.19 |
| Squalene | 48.9 | 54.2 | 58.9 | 38.9 | 59.3 | 62.6 |
| Sterols | 38.7 | 34.2 | 28.9 | 47.6 | 27.7 | 23.6 |
| Phytosterols | 12.4 | 11.6 | 12.2 | 13.5 | 13.0 | 13.8 |

Values are from two measurements from the 13 °C and 25 °C fermentation. The coefficient of variation (CV in %) varied from 2.4% to 5.6% for fatty acids, except for octanoic acid (11%) and squalene and sterols (7–12.5%).

*Average chain length (CL) = $\Sigma(PC)/100$, as described in Materials and methods.

Table 3. List of differentially expressed genes at 13 °C and 25 °C, the products of which are implicated in fatty acid, phospholipid and sterol metabolism

| ORF | Gene name | Fold change of gene expression between 13 °C and 25 °C | | | Gene description |
|-------------------------|--------------|--|--------|--------|--|
| | | IF | MF | FF | |
| Fatty acid metabolism | | | | | |
| <i>YGL055w</i> | <i>OLE1</i> | 2.08 | − 5.88 | − 2.17 | Stearoyl-CoA desaturase |
| <i>YOR317w</i> | <i>FAA1</i> | 1.76 | − 1.69 | − 2.38 | Long-chain fatty acid-CoA ligase |
| <i>YER015w</i> | <i>FAA2</i> | 2.07 | 1.18 | − 1.60 | Long chain fatty acid-CoA ligase |
| <i>YIL009w</i> | <i>FAA3</i> | 2.44 | − 2.50 | − 3.57 | Acyl-CoA synthase |
| <i>YLR372w</i> | <i>SUR4</i> | 1.80 | − 1.72 | − 2.13 | Sterol isomerase, fatty acid elongase |
| <i>YCL026c-a</i> | <i>FMR2</i> | − 4.76 | 5.70 | 5.87 | Involved in fatty acid regulation |
| <i>YBR026c</i> | <i>ETR1</i> | − 1.67 | 1.83 | 2.19 | Mitochondrial respiratory function protein |
| <i>YER061c</i> | <i>CEM1</i> | 1.49 | − 1.41 | − 1.56 | β-Keto-acyl-ACP synthase, mitochondrial |
| <i>YKL055c</i> | <i>OAR1</i> | − 2.17 | 2.30 | 2.52 | Putative 3-oxoacyl-(acyl carrier protein) reductase |
| <i>YKL192c</i> | <i>ACP1</i> | − 1.05 | 1.02 | 1.12 | Mitochondrial acyl carrier protein |
| <i>YOR221c</i> | <i>MCT1</i> | − 4.76 | 3.38 | 2.43 | Malonyl-CoA:ACP transferase |
| Phospholipid metabolism | | | | | |
| <i>YHL020c</i> | <i>OPI1</i> | 1.82 | − 1.75 | − 2.78 | Negative regulator of phospholipid biosynthesis |
| <i>YAL013w</i> | <i>DEP1</i> | − 2.78 | 2.83 | 5.93 | Regulator of phospholipid metabolism |
| <i>YCL004w</i> | <i>PGS1</i> | − 2.63 | 3.52 | 6.00 | Phosphatidylglycerophosphate synthase |
| <i>YDL142c</i> | <i>CRD1</i> | − 2.38 | 2.23 | 4.63 | Cardiolipin synthase |
| <i>YPL268w</i> | <i>PLC1</i> | − 2.00 | 2.67 | 3.19 | 1-Phosphatidylinositol-4,5-bisphosphatase |
| <i>YDL052c</i> | <i>SLC1</i> | − 2.50 | 2.46 | 3.41 | Fatty acyltransferase |
| <i>YBR029c</i> | <i>CDS1</i> | − 1.13 | 1.35 | 1.73 | CDP-diacylglycerol synthase |
| <i>YER026c</i> | <i>CHO1</i> | 1.60 | − 1.56 | − 1.85 | CDP-diacylglycerol serine O-phosphatidyltransferase |
| Sterol metabolism | | | | | |
| <i>YPL172c</i> | <i>COX10</i> | − 4.55 | 3.64 | 9.14 | Farnesyl transferase |
| <i>YMR202w</i> | <i>ERG2</i> | − 2 | 2.66 | 3.45 | Sterol C-8 isomerase |
| <i>YLR056c</i> | <i>ERG3</i> | − 2.62 | − 2.56 | − 1.51 | Sterol C-5 desaturase |
| <i>YMR208w</i> | <i>ERG12</i> | − 3.22 | 3.88 | 5.81 | Mevalonate kinase |
| <i>YGR060w</i> | <i>ERG25</i> | 2.47 | − 2.57 | − 1.25 | Sterol C-4 methyloxidase |
| <i>YGL001c</i> | <i>ERG26</i> | 2.84 | − 2.12 | − 2 | Sterol C-3 dehydrogenase |
| <i>YGL162w</i> | <i>SUT1</i> | − 3.03 | 2.51 | 2.7 | Hypoxic protein involved in sterol metabolism |
| <i>YPL117c</i> | <i>ID11</i> | − 9.09 | 11.81 | 24.18 | Isopentenil-diphosphate isomerase |
| <i>YJL167w</i> | <i>ERG20</i> | 1.9 | 2.5 | 1.65 | Farnesyl-diphosphate synthetase |
| <i>YOR274w</i> | <i>MOD5</i> | − 4.5 | 6.12 | 11.56 | δ-2-Isopentenyl pyrophosphate:tRNA isopentenyl transferase |
| Sterol uptake | | | | | |
| <i>YIL013c</i> | <i>PDR11</i> | 2.15 | − 1.82 | − 2.04 | ABC transporter involved in sterol uptake |
| <i>YOR011w</i> | <i>AUS1</i> | 1.47 | − 1.42 | − 1.20 | Transporter of the ATP-binding cassette family, involved in uptake of sterols and anaerobic growth |

IF, initial stage of fermentation; MF, middle stage of fermentation; FF, final stage of fermentation.

fermentation at 13 °C (Table 3). However, the product of *ID11*, dimethylallylpyrophosphate (DMPP), is also implicated in tRNA biosynthesis. In fact, the strong upregulation of *MOD5*, which encodes a tRNA isopentenyltransferase that catalyses the isopentenylation of adenosine on tRNAs using DMPP (Benko *et al.*, 2000), together with the downregulation of *ERG20*, which encodes the farnesyl pyrophosphate synthetase that converts DMPP into farnesyl-pyrophosphate (FPP), may suggest that the upregulation of *ID11* is probably not related to sterol biosynthesis, but rather to supplying more substrate to Mod5p. The meaning of this upregulation of *MOD5* is still unclear, but it may be linked with the cell's metabolic activity, since this gene is implicated in the protein synthesis machinery through its efficiency in suppressing nonsense mutations by tRNA modification

(Benko *et al.*, 2000). Although it has not been demonstrated that the viability of a *mod5* mutant is affected by temperature, another report stated that loss of function of this gene leads to severe growth defects on YED after several generations (Giaever *et al.*, 2002). Finally, the incorporation of phytosterols that occurs during wine fermentation (Luparia *et al.*, 2004) was not altered by temperature, although we observed a higher expression of *PDR11* and *AUS1*, two genes suspected to be involved in sterol uptake (Wilcox *et al.*, 2002; Rossignol *et al.*, 2003), at the beginning of fermentation at 13 °C than at 25 °C (Table 3).

In contrast to genes involved in long-chain fatty acid metabolism, the transcripts of genes of phospholipid metabolism were relatively more abundant in the middle and late stages of fermentation at 13 °C than at 25 °C. This was

Table 4. Phospholipid composition measured by thin-layer chromatography in yeast cells at three phases of the wine fermentation carried out at 13 °C and 25 °C

| Composition of phospholipid in the cell (% total lipid) | 13 °C | | | 25 °C | | |
|---|-------|------|------|-------|------|------|
| | IF | MF | FF | IF | MF | FF |
| Phosphatidylserine | 2.1 | 10.1 | 9.6 | 7.2 | 2.7 | 2.2 |
| Phosphatidylethanolamine | 12.8 | 13.0 | 8.2 | 13.3 | 16.8 | 17.8 |
| Phosphatidylcholine | 20.2 | 13.0 | 13.7 | 21.1 | 15.4 | 14.6 |
| Phosphatidylinositol | 11.7 | 10.1 | 8.2 | 10.6 | 14.1 | 11.9 |
| Phosphatidic acid | 33.0 | 39.1 | 41.1 | 24.9 | 23.5 | 29.2 |
| Phosphatidylglycerol | 9.6 | 11.6 | 15.1 | 10.0 | 15.4 | 13.0 |
| Cardiolipin | 10.6 | 2.9 | 4.1 | 13.9 | 12.1 | 11.4 |
| Total phospholipids (% of cellular lipid content) | 9.4 | 6.9 | 7.3 | 18 | 15 | 18.5 |

Values are from two measurements obtained from the same fermentation at 25 °C and 13 °C. The coefficient of variation (CV in %) calculated for phospholipids were: 10.2 for phosphatidylserine, 9.6 for phosphatidylethanolamine, 13.3 for phosphatidylcholine, 10.2 for phosphatidylinositol, 6.3 for phosphatidic acid, 7.5 for phosphatidylglycerol and 6.1 for cardiolipin. IF, initial stage of fermentation; MF, middle stage of fermentation; FF, final stage of fermentation.

particularly the case for mRNA levels of *SLC1*, *CDS1*, *PGS1* and *CRD1* (Table 3). Increased phospholipid metabolism in the middle and late phases of fermentation at 13 °C could also be inferred from the upregulation of *DEP1*, which encodes the positive regulator of the phospholipid synthesis pathway, and downregulation of *OPI1*, which encodes a negative regulator of phospholipid biosynthesis. The repression of *OPI1* was further consistent with increased levels of medium-chain fatty acids (Table 2), and agreed with a previous report that indicated a correlation between *OPI1* repression and increased content of medium-chain fatty acids (Furukawa *et al.*, 2003). However, the higher expression of this class of genes at 13 °C compared to 25 °C was not accompanied by an increase in cardiolipin, which is an endproduct of this metabolic pathway (Table 4), but by an increase in phosphatidic acid, the obligate intermediate in the synthesis of several phospholipids, including sphingolipid (Daum *et al.*, 1998). Another inconsistency was that the expression of *CHO1*, which encodes phosphatidylserine synthase, was lower at 13 °C than at 25 °C, while the proportion of phosphatidylserine in the middle to late phases of fermentation at 13 °C was four times higher than at 25 °C. Despite this important differential gene expression between the two temperatures, which could suggest a reorientation of the metabolic flux in the phospholipid synthesis branch, the total content of phospholipids in yeast cells cultivated at 13 °C was actually half that in yeast cultivated at 25 °C (Table 3). As a consequence, the significant reduction of total phospholipid in yeast cells cultivated at 13 °C accompanied by minor changes in sterol content led to a sterol/phospholipid ratio that changed from *c.* 1.3 in yeast cells cultivated at 25 °C to *c.* 3.0 in those cultivated at 13 °C. This difference is consistent with reduced membrane fluidity and with a higher resistance to ethanol of yeast cultured at low temperature (Alexandre *et al.*, 1994).

Effect of low temperature on energy production and carbohydrate reserve metabolism

Our analysis also highlighted that the expression of genes involved in glycogen and trehalose metabolism in the fermentation at 13 °C was different from that in the fermentation at 25 °C (Table 5). Breakdown of trehalose and glycogen was much less efficient at the beginning of the fermentation at 13 °C than at the beginning of fermentation at 25 °C (Novo *et al.*, 2003b). However, our transcriptomic data showed that genes implicated in the catabolism (*NTH1*, *NTH2* and *GPH1*) and the biosynthesis (*TPS1*, *TPS2*, *GLG1*, *GLG2* and *GSY2*) of these two glucose stores were respectively upregulated and downregulated at the beginning of fermentation at 13 °C relative to 25 °C (Table 5). Therefore, the higher rate of degradation at 25 °C than at 13 °C could be mainly due to a direct effect of temperature on the catalytic activity of the enzymes. As fermentation progressed, this expression profile was reversed, with genes of the catabolic pathways being less expressed and those in the biosynthesis pathways more expressed at 13 °C. Moreover, the remodelling of expression level of this subset of genes correlated with levels of trehalose and glycogen, which were 15–20% higher in the middle to late phases of the fermentation at 13 °C than at 25 °C (data not shown).

Discussion

Previous studies from our research group (Torija *et al.*, 2003; Llauro *et al.*, 2004) showed that fermentations carried out at lower temperatures (13 °C) improved certain sensory attributes of the wines that are related not only to primary aroma retention, but also to an increase in the biosynthesis of flavour-active compounds, such as acetates and ethyl esters, and to a decrease in unpleasant ones, such as acetic acid and fusel alcohols. It is obvious that a direct effect of lowering temperature is to slow down the metabolic activity

Table 5. Changes in expression levels of genes involved in carbohydrate reserve metabolism during the fermentations at 13 °C and 25 °C

| ORF | Gene | (13 vs. 25 °C) fold changes | | |
|----------------------|-------------|-----------------------------|-------|-------|
| | | IF | MF | FF |
| Trehalose metabolism | | | | |
| <i>YBR126c</i> | <i>TPS1</i> | -1.96 | 2.15 | 2.89 |
| <i>YDR074</i> | <i>TPS2</i> | -2.22 | 2.57 | 2.75 |
| <i>YMR261c</i> | <i>TPS3</i> | -1.53 | 1.73 | 1.74 |
| <i>YDR001c</i> | <i>NTH1</i> | 1.14 | 1.05 | -1.40 |
| <i>YBR001c</i> | <i>NTH2</i> | 1.52 | -1.61 | -1.75 |
| <i>YPRO26w</i> | <i>ATH1</i> | 1.67 | -1.92 | -2.13 |
| Glycogen metabolism | | | | |
| <i>YKR058w</i> | <i>GLG1</i> | -2.00 | 2.63 | 2.61 |
| <i>YJL137c</i> | <i>GLG2</i> | -1.29 | 1.66 | 1.69 |
| <i>YFR015c</i> | <i>GSY1</i> | 1.08 | 1.07 | -1.67 |
| <i>YLR258w</i> | <i>GSY2</i> | -8.33 | 6.27 | 11.86 |
| <i>YEL011w</i> | <i>GLC3</i> | 1.57 | -2.50 | -1.79 |
| <i>YPR160w</i> | <i>GPH1</i> | 1.31 | -1.50 | -1.67 |
| <i>YPR184w</i> | <i>GDB1</i> | -2.56 | 2.56 | 3.45 |
| <i>YILO99w</i> | <i>SGA1</i> | -1.09 | -1.69 | -2.27 |
| Others | | | | |
| <i>YKL127w</i> | <i>PGM1</i> | 1.19 | -1.25 | -1.05 |
| <i>YMR105c</i> | <i>PGM2</i> | -1.92 | 1.93 | 2.36 |
| <i>YFR053c</i> | <i>HXK1</i> | 1.17 | -1.14 | -1.45 |
| <i>YGL253w</i> | <i>HXK2</i> | 1.62 | -1.69 | -2.22 |
| <i>YKL035w</i> | <i>UGP1</i> | 1.53 | -1.41 | -1.72 |

A metabolic map for glycogen and trehalose metabolism is shown. Only genes implicated in their biosynthesis and biodegradation whose expression level was increased at 13 °C relative to 25 °C are shown.

of yeast cells, which explains the lower growth rate and the longer fermentation process at 13 °C. However, this explanation cannot be invoked to account for changes in metabolite production. These metabolic changes could be due to temperature-mediated transcriptional and/or post-transcriptional effects on yeast. Thus, we used the DNA microarray technology to examine on a genomic scale whether these metabolic changes caused by lowering temperature from 25 °C to 13 °C could be in part explained by changes in gene expression.

From a methodological viewpoint, our study was carried out under strict industrial winemaking conditions. Thus, these conditions were notably different from those used in previous genome-wide studies, which investigated expression profiles by mimicking oenological processes (Backhus *et al.*, 2001; Marks *et al.*, 2003; Rossignol *et al.*, 2003). Also, our genome-wide analysis differed from two previous reports that studied the effects of an abrupt drop of temperature from 30 °C to 10 °C on gene expression (Sahara *et al.*, 2002; Schade *et al.*, 2004). As a caveat, the transcriptomic analysis of wine fermentation at 13 °C and 25 °C was done in a single vintage season, and hence the expression data and biochemical analyses obtained must be taken with

some caution. Nevertheless, an indication that this work provided reliable results was that our transcriptional data obtained during wine fermentation at 25 °C overlapped with some of the data reported by Rossignol *et al.* (2003), who carried out transcriptome analyses of yeast cultivated under conditions that mimicked wine fermentation.

However, the expression profiles during wine fermentation at 13 °C contrasted significantly with those at 25 °C. In particular, genes of the cell cycle, cell growth, cell fate and maintenance categories were less expressed in the exponential growth phase than during the nonproliferating phase of the fermentation at 13 °C, whereas genes whose expression was activated during the exponential phase of growth at 13 °C were essentially those involved in the environmental stress response (Gasch *et al.*, 2000). This indicates, in agreement with previous reports (Sahara *et al.*, 2002; Schade *et al.*, 2004), that a stress response was induced early during fermentation at 13 °C, while it was activated much later at 25 °C, as cells entered the nonproliferating growth phase. During fermentation at 25 °C, this general stress response is primarily associated with the stationary phase (Gasch *et al.*, 2000; Rossignol *et al.*, 2003). Still, this response was detected much earlier at 13 °C than at 25 °C. According to the list of genes that were found to be activated at the initial stage of fermentation at 13 °C, this stress is comparable to the LCR (late cold response) identified by Schade *et al.* (2004). It could be argued that the induction of this 'LCR' is not a direct effect of low temperature but rather a consequence of the slow growth induced by low temperature. This is clearly an important question that deserves further investigation. Nonetheless, this cold-stress response probably prepared the cells for better resistance to subsequent unfavourable situations that may occur later during the growth and fermentation, such as ethanol toxicity. In addition, genes that are upregulated in the stationary phase of the 13 °C fermentation are involved in cell fate, growth control, and maintenance. As a consequence of this cross-protection, the cell viability is expected to be increased. This is actually what was concluded from the finding that the CFU value remained high during the long nonproliferating phase of fermentation at 13 °C, whereas it gradually decreased during this period at 25 °C (Fig. 1).

As reported in a previous study (Torija *et al.*, 2003), low temperature modified the lipid composition of yeast cells, increasing the degree of unsaturation at the beginning of fermentation and decreasing the chain length as fermentation progressed, with a decrease of long-chain saturated fatty acids. This modification could be related in part to a lower expression of *FAA3*, *FAA1* and *SUR4* at the 13 °C fermentation. On the other hand, the expression of genes that encode enzymes required for mitochondrial short-chain fatty acid synthesis was upregulated, together with higher production of short- and medium-chain fatty acids

(carbon chain < 12). These short- and medium-chain fatty acids could then be exported into the medium directly or converted into ethyl esters to improve the aromatic quality of the wine at low temperature. Another significant difference was that total cellular phospholipid at 13 °C was two-fold lower than at 25 °C. However, the expression of genes involved in phospholipid synthesis was relatively higher at 13 °C than at 25 °C. This change could be a compensatory response resulting from a decrease of the endproducts of this pathway, as it occurs in response to depletion of an amino acid (ter Schure *et al.*, 2000; Beltran *et al.*, 2004), or it could be due to some post-transcriptional regulation of the corresponding enzymes. Whatever the exact mechanism, the lower content of phospholipid in yeast cells at 13 °C together with no change in the levels of sterols between the two fermentation temperatures may explain the decrease in the membrane fluidity and the higher resistance to ethanol of yeast cultured at low temperature (Watson, 1987; Alexandre *et al.*, 1996; Chi & Arneborg, 1999; Torija *et al.*, 2003).

Although upregulation of the two genes, *ATF1* and *ATF2*, that encode alcohol acetyltransferases (Mason & Dufour, 2000) was shown to correlate with increased formation of volatile esters (Fukuda *et al.*, 1998; Lilly *et al.*, 2000), this was probably not the explanation for the higher production of acetate esters in wines fermented at 13 °C than in those at 25 °C, since the expression of these two genes was not altered by temperature. The upregulation of *IAH1*, which encodes an esterase reported to act antagonistically to alcohol acetyltransferase (Fukuda *et al.*, 1998; Mason & Dufour, 2000), at 13 °C may be an alternative explanation for this increased acetate ester production at low temperature, since this enzyme may function in the ester synthase direction, as previously suggested by Dufour and coworkers (Malcorps & Dufour, 1992; Verstrepen *et al.*, 2003). Moreover, the higher production of aromatic compounds at low temperature may also be a consequence of higher viability of the cells, and therefore of higher metabolic activity of yeast cells in the later stage of fermentation at 13 °C.

To conclude, this genome-wide analysis, which was carried out for the first time under strict industrial conditions and with a commercial yeast strain, revealed major differential gene expression both during the course of the wine fermentation and between two fermentation temperatures. With regard to industrial output, one advantage of wine fermentation at 13 °C is the induction of an early cold-stress response that apparently does not adversely affect the whole process, but rather boosts the viability of the cells and keeps the fermentation process very effective for a longer period of time.

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Supplementary material

The following material is available for this article online:

Appendix S1. Multivariate analysis data.

Fig. S1. Plot values of the first and third principal component analysis (76.3% of the dispersion) according to the growth conditions of the 1.8-fold significant change in signal intensity of gene expression (1111 genes). Distribution of the up-regulated genes on the plot.

Table S1. All data. List of all 5915 genes, showing the Ratio and *P*-value obtained from the comparison between

fermentation phases at 13 and 25 °C (three datasets) and from the kinetics at 13 and 25 °C (four datasets).

Table S2. Kinetic 25 °C. List of differentially expressed genes (two-fold variation and statistical significance by the Student's *t*-test with a *P* value < 0.05, for at least one stage of the fermentation) during fermentation at 25 °C (MF vs IF and FF vs. IF), classified as functional genomic (MIPS classification).

Table S3. Kinetic 13 °C. List of differentially expressed genes (two-fold variation and statistical significance by the Student's *t*-test with a *P* value < 0.05, for at least one stage of

the fermentation) during fermentation at 13 °C (MF vs. IF and FF vs IF), classified as functional genomic (MIPS classification).

Table S4. Comparison 13 vs. 25 °C. List of differentially expressed genes (two-fold variation and statistical significance by the Student's *t*-test with a *P* value < 0.05, for at least one stage of the fermentation) at 13 °C vs. 25 °C fermentation (IF, MF and FF), classified as functional genomic (MIPS classification).

Table S5. Global results of multivariable data analysis (1111 genes statistically significant with *p* value set at < 0.05 from the Student's *t*-test).