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# Global gene expression in recombinant and non-recombinant yeast *Saccharomyces cerevisiae* in three different metabolic states

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ABSTRACT

Global gene expression of two strains of *Saccharomyces cerevisiae*, one recombinant (P+), accumulating large amounts of an intracellular protein Superoxide Dismutase (SOD) and one non-recombinant (P−) which does not contain the recombinant plasmid, were compared in batch culture during diauxic growth when cells were growing exponentially on glucose, when they were growing exponentially on ethanol, and in the early stationary phase when glycerol was being utilized.

When comparing the gene expression for P− (and P+) during growth on ethanol to that on glucose (Eth/Gluc), overexpression is related to an increase in consumption of glycerol, activation of the TCA cycle, degradation of glycogen and metabolism of ethanol. Furthermore, 97.6% of genes (80 genes) involved in the central metabolic pathway are overexpressed. This is similar to that observed by DeRisi et al. [DeRisi, J.L., Iyer, V.R. & Brown, P.O. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278:680–686.] but very different from was observed for Metabolic Flux Analysis (MFA), where the specific growth rate is lowered to ca. 40%, the fluxes in the TCA cycle are reduced to ca. 40% (to 30% in P+), glycolysis is reduced to virtually 0 and protein synthesis to ca. 50% (to 40% in P+). Clearly it is not possible to correlate in a simple or direct way, quantitative mRNA expression levels with cell function which is shown by the Metabolic Flux Analysis (MFA).

When comparing the two strains in the 3 growth stages, 4 genes were found to be under or overexpressed in all cases. The products of all of these genes are expressed at the plasma membrane or cell wall of the yeast. While comparing the strains (P+/P−) when growing on glucose, ethanol and in the early stationary phase, many of the genes of the central metabolic pathways are underexpressed in P+, which is similar to the behaviour of the metabolic fluxes of both strains (MFA). Comparing the gene expression for P− (and to some extent P+) during the early stationary phase to growth on ethanol (Stat/Eth), underexpression is generalized. This shows that the switch in metabolism between ethanol and early stationary phases has an almost instantaneous effect on gene expression but a much more retarded effect on metabolic fluxes and that the "early stationary" phase represents a "late ethanol" phase from the metabolic analysis point of view since ethanol is still present and being consumed although at a much slower rate.



## 1. Introduction

When and where a gene is expressed gives a good indication of its biological role. Similarly, the pattern of genes expressed in a cell can give useful information about its state. Although there are several other factors, in addition to mRNA regulation that affect the abundance and function of proteins in a cell (Hatzimanikatis and Lee, 1999), many of the differences in the type or state of a cell are correlated with the changes in the levels of mRNA of many genes. DNA microarrays, consisting of thousands of individual sequences of genes printed over an array of high density on a glass slide are an extremely practical, valuable and affordable tool to study gene expression on a very large scale (Lockhart et al., 1996; DeRisi et al., 1997).

*Saccharomyces cerevisiae* is a particularly interesting organism to carry out a systematic investigation of gene expression. Amongst other characteristics, the genes of *S. cerevisiae* are easy to recognize in the genome sequence since this has been totally sequenced for a few years. Extensive studies have been carried out in *S. cerevisiae* using the technique of DNA microarrays (cDNA and/or biochips), which range

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from wide studies of gene expression in native strains and/or mutants, exploration of metabolic control and gene regulation of gene expression, determination of gene expression under different types of stress, resistance to drugs and to agents that damage DNA in mutant and native strains; to the determination of the specific function of certain genes or groups of genes in metabolic routes of interest.

Notwithstanding the vast range of studies that have used cDNA microarrays of *S. cerevisiae*, no studies have been reported so far that investigate the differential expression of genes in strains synthesizing large amounts of a recombinant protein. At the moment, only two studies have analyzed, using cDNA microarrays, the differential expression of genes in a mutant strain of *S. cerevisiae* (TMB 3400) with increased ability to use xylose (Wahlbom et al., 2003), whereas Bro et al. (2004) studied the transcriptional response of a strain of *S. cerevisiae* (CEN.PK448-3B–Δ*gdh1*), which had an altered oxide-reduction metabolism.

Such studies could be an important element in the optimization of the production of heterologous proteins in *S. cerevisiae*, as this species is an important host for the industrial production of important recombinant proteins. Yeast are even able to carry out some post-transductional modifications required in eucaryotic proteins. Therefore, the determination of genes important for the production of a recombinant protein in *S. cerevisiae* is important in several aspects regarding production of these proteins.

Previously we studied the synthesis of human Superoxide Dismutase (SOD) in a recombinant strain using Metabolic Flux Analysis (MFA) and also compared the behaviour of this yeast strain to the non-recombinant (or wild type) strain (Gonzalez et al., 2003). A simplified stoichiometric model was used that included 78 reactions following the metabolic pathways operative in these strains during respirofermentative and oxidative metabolism. In this paper we describe the use of the high density oligonucleotide arrays (HDOA) GeneChip® Yeast Genome S98 Array (Affymetrix Inc., Santa Clara, CA) to characterize the changes in the expression of the total genome genes that take place during respirofermentative growth (growth on glucose/ethanol/glycerol and initial stationary phase) of native and recombinant *S. cerevisiae* strains, that synthesize large amounts of human Superoxide Dismutase (SOD), to study the metabolic changes and the gene network that regulates the synthesis of this recombinant protein.

## 2. Materials and methods

### 2.1. Strains, culture media and fermentations

The recombinant strain *Saccharomyces cerevisiae* rhSOD 2060 411 SGA122 (MATα, *leu2*), a gift from Chiron Corporation (Emeryville, California, USA), was utilized. This strain contains the plasmid pC1/1PGAPSOD (with promotor and terminator of the enzyme glyceraldehyde phosphate dehydrogenase (GAP) and a marker for leucine), which allows the production of high levels of the human protein Superoxide Dismutase (SOD); and a second strain derived from the *S. cerevisiae* rhSOD 2060 411 SGA122, which does not contain the expression vector (and does not produce SOD) and requires leucine in the growth medium. Following the denomination previously used (Gonzalez et al., 2003), the strain that contains the plasmid is called P+, and the strain without plasmid, P−. Both strains were stored at −80 °C in YEP medium (Rose et al., 1990) with 20% v/v glycerol. The growth media and fermentation conditions have been described in detail by Gonzalez et al. (2003).

Fermentations were carried out in a 1 L Applikon fermenter at 30 °C and pH 5, fully controlled for pH, temperature, dissolved oxygen (D.O.) and foam formation. Aeration was 2 vvm and agitation 650 rpm, hence D.O. was ca. 100% throughout. A water condenser was used to avoid ethanol loss by evaporation.

### 2.2. Analytical procedures

Samples were taken from the fermentor for the measurement of cell mass, total RNA, total protein, total carbohydrates, glucose, ethanol, and SOD. Total RNA was extracted with perchloric acid and quantified using orcinol as suggested by Herbert et al. (1971). Total protein was extracted with hot NaOH and quantified with Folin–Ciocalteu reagent (Lowry's method, Peterson, 1977). Total carbohydrates were measured using the anthrone method (Herbert et al., 1971). SOD measurement involved cell disruption (1 OD (600 nm) pellet resuspended in load buffer) by 3 cycles of freezing (−80 °C) and thawing in a boiling water bath (Sambrook et al., 1989). The cell extract was subjected to polyacrylamide gel electrophoresis (SDS-PAGE, 12%), where molecular weight markers and SOD standards were included. SOD spots were quantified using SigmaGel (Jandel Scientific Co.) software. Cell concentration was measured as dry weight. Total lipids were calculated using total RNA, total protein, total carbohydrates, SOD, and cell concentration measurements, and considering a 3% ash content (Nielsen and Villadsen, 1994). Glucose and ethanol concentration were measured enzimatically using Sigma kits (procedure number 510 and 332-UV respectively, Sigma Diagnostics, USA). Glycerol concentration was measured enzymatically using a kit from Boehringer Mannheim/ R-Biopharm (10148270035).

### 2.3. Sampling and total RNA extraction

Quadruplicate samples of approximately 20 ml were taken from the fermentor during each growth phase and used to measure biomass, glucose and ethanol concentrations and for RNA extraction as described by Hayes et al. (2002) and Hauser et al. (1998).

### 2.4. Microarray GeneChip®

For the analysis of genome expression the GeneChip® Yeast Genome S98 Array was used (Affymetrix Inc., Santa Clara, CA). A description of these high density oligonucleotide arrays is found in Wodicka et al. (1997).

The synthesis of cDNA from total RNA was done using the Superscript RNA Amplification System (Invitrogen Inc., UK), and the cDNA was washed using the GeneChip® reagent (Affymetrix Inc., Santa Clara, CA).

### 2.5. Washing of cRNA, fragmentation, washing and drying

The detail of these methodologies is found in http://www.cogeme.man.ac.uk/tsf-training%20course.pdf and in Díaz (2006).

### 2.6. Analysis and quantification

The GeneChips were scanned using a GeneChip® Scanner 3000 (Affymetrix Inc., Santa Clara, CA). The intensity data obtained were normalized using the RMA Express programme v. 0.3 alpha 3, which works with the data of the GeneChip® of Affymetrix (Bolstad et al., 2003). A first approach to the classification of the expression data was carried out using the software maxdView v. 1.2 (http://bioinf.man.ac.uk/microarray/maxd/download.html). For the determination of statistically significative differences in the gene expression levels a Variance Analysis model, ANOVA (Baldi and Long, 2001; Churchill, 2004) was used with a Bonnferroni correction (Dudoit et al., 2000). For detailed analysis of the data the software GeneSpring v 7.1 (Silicon Genetics, USA) was used as well as Statistica v.6.0 (Statsoft Inc., USA). For gene ontology the data bases of Affymetrix were used (http://www.affymetrix.com/support/technical/byproduct.affx?product=yeast) as well as Saccharomyces Genome Database (http://www.yeastgenome.org/), the Comprehensive Yeast Genome Database (http://mips.gsf.de/

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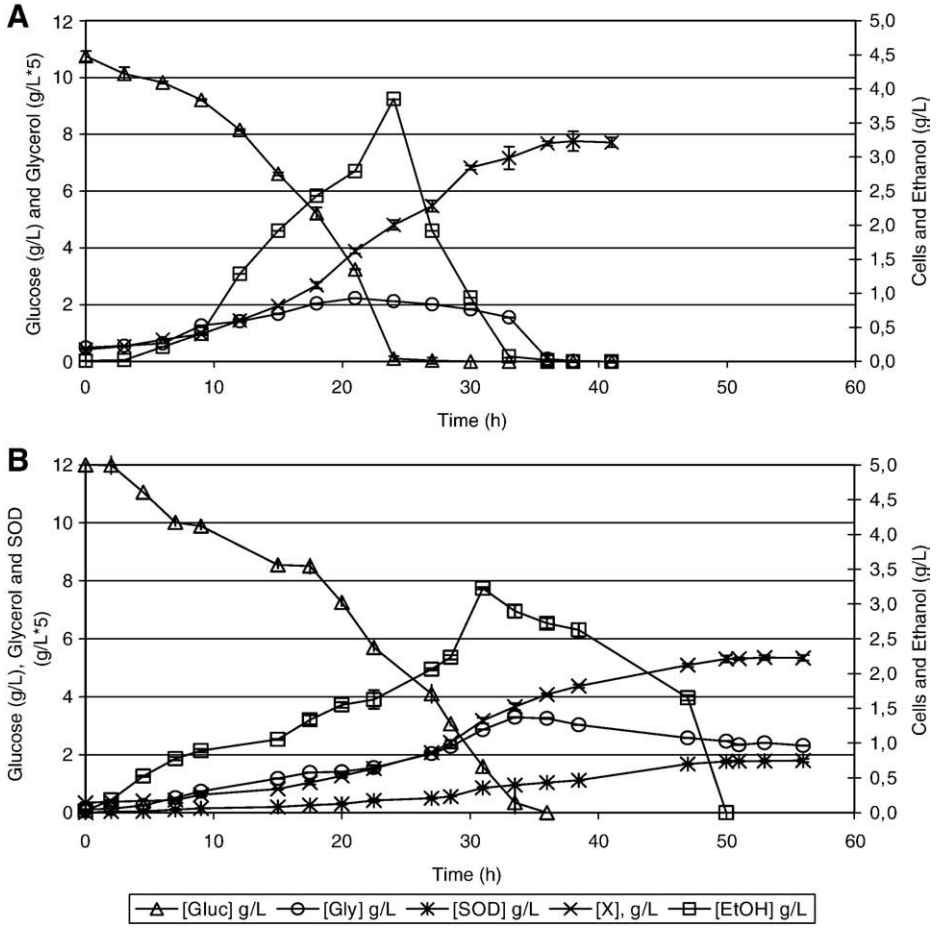


**Fig. 1.** Growth of *S. cerevisiae*, wild type strain (A) and recombinant strain (B). x = cells.

genre/proj/yeast/) and the Stanford Microarray Database (http://genome-www5.stanford.edu/).

## 3. Results and discussion

### 3.1. Fermentation profiles

Fermentations were carried out to measure cell growth and SOD production and to enable direct comparison with the previous results of Gonzalez et al. (2003). They were sampled in each of the three growth phases, as described in Materials and methods for total RNA extraction in quadruplicate samples. Fig. 1 shows time profiles of cell growth, glucose consumption, ethanol, glycerol and SOD production for the P+ and P− strains. Both strains show diauxic growth, a first phase on glucose followed by a second growth phase. During growth on glucose ethanol is synthesized along with other compounds of which glycerol is quantitatively the most important. Glycerol is mainly involved in osmotic regulation of the cell (Blomberg and Adler, 1992), specifically in the intracellular redox balance (Gancedo and Serrano, 1989), transforming the excess NADH generated during the formation of biomass to NAD+. In the case of the strain P−, the glycerol is produced during the consumption of glucose along with the formation of alcohol, and is consumed in the late exponential/early stationary phase. In the strain P+, although the formation of glycerol coincides with that of ethanol, the consumption dynamics are different, the alcohol is totally consumed and the glycerol is only partially consumed. Also, the maximum concentration of glycerol in the recombinant strain (0.658 g/L) is almost 68% greater than the maximum produced by the native strain (0.445 g/L) which is an indication of the redox stress in the recombinant strain.

The growth rate on glucose and ethanol was different in the P+ and P− strains, being evidently much greater in both cases for the P− strain. When all the available glucose has been consumed, the cells enter the second growth phase consuming ethanol and glycerol produced during growth on glucose. The concentrations of ethanol produced similar for both strains, (3.85 g/L in P− and 3.23 g/L for P+). At the beginning of the stationary phase, the most notable result is cell concentration, which is around 69% higher in P− with respect to P+. The larger cell concentrations reached for the P− strain is related with the larger biomass/substrate yield and the absence of oxidative stress

**Table 1**
Comparison of macromolecular composition (%) of wild-type (P−) and recombinant strain (P+).

| Component | This study | | | Gonzalez et al. (2003) | | Ostergaard et al. (2001) |
|---|---|---|---|---|---|---|
| | P+ strain | P− strain | *p*-value | P+ strain | P− strain | |
| Protein | 49.00 ± 0.93 | 45.61 ± 4.02 | 0.004 | 49.23 ± 0.68 | 45.69 ± 2.30 | 41.0 |
| Carbohydrates | 32.74 ± 0.85 | 35.41 ± 2.40 | 0.022 | 32.37 ± 0.69 | 34.28 ± 1.51 | 41.3 |
| RNA | 11.52 ± 1.04 | 6.78 ± 0.88 | 0.000 | 11.96 ± 1.00 | 8.70 ± 1.12 | 6.3 |
| Lipids | 3.74 ± 1.13 | 10.62 ± 1.73 | 0.000 | 3.44 ± 1.40 | 8.34 ± 1.70 | 8.0 |

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for copper. The glucose to ethanol yields are similar in both strains as seen previously by Gonzalez et al. (2003).

The results of Fig. 1 show that SOD is produced constitutively during all of the phases of growth of *S. cerevisiae* and reached 30% of total protein as reported by Gonzalez et al. (2003). These profiles are similar to those found for synthesis of recombinant proteins in *S. cerevisiae* using the promotors PGK1 y ADH2 (Dickson and Brown, 1998; Georgens et al., 2001), but are somewhat different from those shown by Gonzalez et al. (2003), where SOD levels remain almost constant in the exponential growth phase in ethanol.

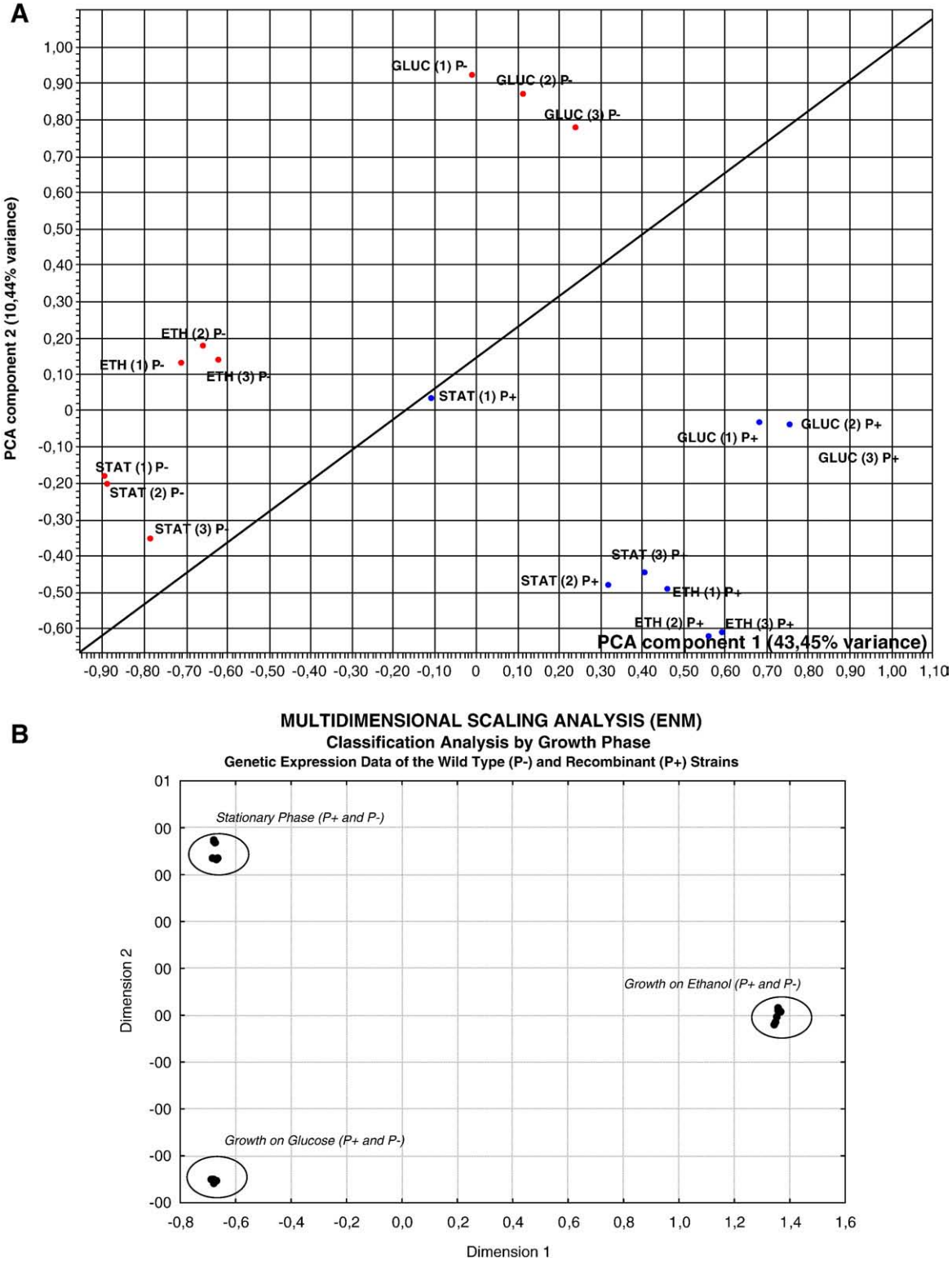


**Fig. 2.** A. Principal Component Analysis (PCA) of the microarray essay results. Gluc: Growth on glucose, EtOH: Growth on ethanol, Stat: Growth in stationary phase, P+: Recombinant strain (in yellow), P−: Wild type strain (in red). Three replicas for each strain at each stage of growth are included. B. Multidimensional scaling for each of the microarrays essay results. P+: Recombinant strain, P−: Wild type strain. Three replicas for each strain at each stage of growth were included. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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The distribution of the different cell fractions in the total dry cell weight is shown in Table 1. These were calculated using data on cell concentration, total protein, total carbohydrates and total RNA (data not shown), and a 3% ash content was assumed for the calculation of the lipid fraction (Ostergaard et al., 2001; Gonzalez et al., 2003). The levels of total protein and RNA are much greater in P+, while the lipid and carbohydrate fractions are greater in P−. These results are similar to those obtained by Gonzalez et al. (2003). A statistical analysis indicates that the differences observed in the cell fractions between the strains P+ y P− are, in all cases, statistically significant. The cellular composition is very similar to that obtained by Gonzalez et al. (2003) but has differences when compared to the data of Ostergaard et al. (2001), especially with respect to the carbohydrate fraction for P − and all fractions for the recombinant strain.

### 3.2. Specific growth rates

The specific growth rates were different during the different growth phases for both strains. The P− strain showed a specific growth rate on glucose (0.13 $h^{-1}$) 23.3% higher than the strain P+ (0.10 $h^{-1}$). The specific growth rate on glucose is similar to that obtained by Gonzalez et al. (2003), for P+ (0.1049 $h^{-1}$), for P− the value reported by Gonzalez is larger (0.1586 $h^{-1}$). During growth on ethanol, the specific growth rate of both strains was smaller than that on glucose (0.04 $h^{-1}$ for P+ and 0.08 $h^{-1}$ for P−).

Although other studies have found important decreases in specific growth rates in *S. cerevisiae* during the expression of recombinant proteins (Da Silva and Bailey, 1991; Dequin and Barre, 1994; Georgens et al., 2001), the variations observed here are much larger. In the present study, the largest differences observed between the two strains and the different growth phases, could be related to the production of large amounts of SOD. However, one of the most important finding of studies of gene expression, is the strong repression of gene *ctr3* and the strong induction of the genes *cup1–2* in the recombinant strain, which indicates stress due to the presence of copper in the medium. The influence of specific growth rate on the transcriptome of yeast has been studied previously (Chu et al., 1998, Wodicka et al., 1997).

### 3.3. Analysis of transcription profiles

#### 3.3.1. Comparison of global expression levels: different growth phases in native and recombinant strains of S. cerevisiae

The gene expression data were analyzed using a principal component analysis, PCA (Wall et al., 2003; Alter et al., 2000; Holter et al., 2000). Only with the expression data obtained with both strains it was possible to distinguish the strains and the different growth phases clearly, as shown in Fig. 2A.

The two axes that separate the different samples of the PCA, together explain 62.9% of the variance (43.5% of component 1 and 19.4% of component 2). The PCA allows "intuitively" to identify the strain P+ (recombinant) from P− (native) as the data of P− are all above the diagonal line drawn and those of P+ below. This is an unsupervised analysis.

Fig. 2A also shows that the replicas of each growth phase (glucose, ethanol and stationary) are clearly grouped together and only 1 point (stat (1) P+) "falls" out of line. It thus appears that with the methodology used, 3 replicas was an adequate choice to obtain reliable and reproducible results.

Fig. 2B shows a Multidimensional Scaling Analysis (MSA) of the gene expression data which corresponds to an unsupervised order analysis. It can be seen that, independently of the strain used, the 3 phases of the respirofermentative growth are clearly separated. The data used correspond to those of over or underexpression of the total number of genes. The capacity of MSA to separate and group genetic expression data has been discussed by Tzeng et al. (2008).

A global comparison of the gene expression data of both strains P+ and P− in the different stages of respirofermentaitve growth show that of the total number of genes analyzed (6871 genes) only 344 (5% of the total) were under or overexpressed in a statistically significant manner (according to the statistical model utilized, see Materials and methods) when growing on glucose. Similarly only 55 genes (0.8%) were differentially expressed in both strains in the ethanol phase and 480 genes (6.9%) are differentially expressed in the early stationary phase. It is worth noting that only 7 genes (0.1%) show differential expression in both the glucose and ethanol phase and 18 genes (0.3%) show differential expression in the ethanol and early stationary phases and 39 genes (0.6%) are shared between growth on glucose and the early stationary phases. There are only 4 genes (0.06%) that show statistically significant differences in expression in all the phases of growth studied. The ontology of these genes (genes *ctr3, muc1, pdr15* and *pst1*) is shown in Table 2.

In all these genes the product is expressed at the plasma membrane of the cell wall of yeast (Ontology Data Mining of FatiGo (http://www.fatigo.org/) and Saccharomyces Genome Database http://www.yeastgenome.org/). It is known that many environmental changes result in changes in membrane permeability in yeast cells inducing or repressing genes involved in the ion transport mechanisms (Causton et al., 2001).

Two genes directly related with transport through the plasma membrane are *pdr15* and *ctr3*. *pdr15* is a transporter with ATPase activity and is actively coupled to the movement of transmembrane substances. The protein expressed by this gene has been related to the yeast response to stress particularly involved in cell detoxification

**Table 2**
Ontology of the 4 genes that, after comparison of strains P+ and P−, show statistically different expression levels (up or down regulated), in the three stages of growth of *S. cerevisiae*.

| Gene | |
|---|---|
| | *CTR3* |
| Gene product | Copper transporter. |
| Description | High-affinity copper transporter of the plasma membrane acts as a trimer; gene is disrupted by a Ty2 transposon insertion in many laboratory strains of *S. cerevisiae*. |
| Biological process | Copper ion import. |
| Molecular function | Copper uptake transporter activity. |
| Cellular component | Integral to plasma membrane. |
| | *PDR15* |
| Gene product | Multidrug resistance transporter (putative) |
| Description | ATP binding cassette (ABC) transporter of the plasma membrane; general stress response factor implicated in cellular detoxification; target of Pdr1p, Pdr3p and Pdr8p transcription regulators; promoter contains a PDR responsive element. |
| Biological process | Transport. |
| Molecular function | ATPase activity, coupled to transmembrane movement of substances. |
| Cellular component | Integral to membrane. |
| | *MUC1* |
| Gene product | Cell surface flocculin with structure similar to serine/threonine-rich GPI-anchored cell wall proteins |
| Description | GPI-anchored cell surface glycoprotein required for diploid pseudohyphal formation and haploid invasive growth, transcriptionally regulated by the MAPK pathway (via Ste12p and Tec1p) and the cAMP pathway (via Flo8p). |
| Biological process | Cell–cell adhesion/filamentous growth /invasive growth (sensu *Saccharomyces*)/pseudohyphal growth. |
| Molecular function | Molecular function unknown. |
| Cellular component | Plasma membrane. |
| | *PST1* |
| Gene product | The gene product has been detected among the proteins secreted by regenerating protoplasts. |
| Description | Cell wall protein that contains a putative GPI-attachment site; secreted by regenerating protoplasts; up-regulated by activation of the cell integrity pathway, as mediated by Rlm1p; upregulated by cell wall damage via disruption of FKS1. |
| Biological process | Cell wall organization and biogenesis. |
| Molecular function | Molecular function unknown. |
| Cellular component | Cell wall (sensu Fungi). |

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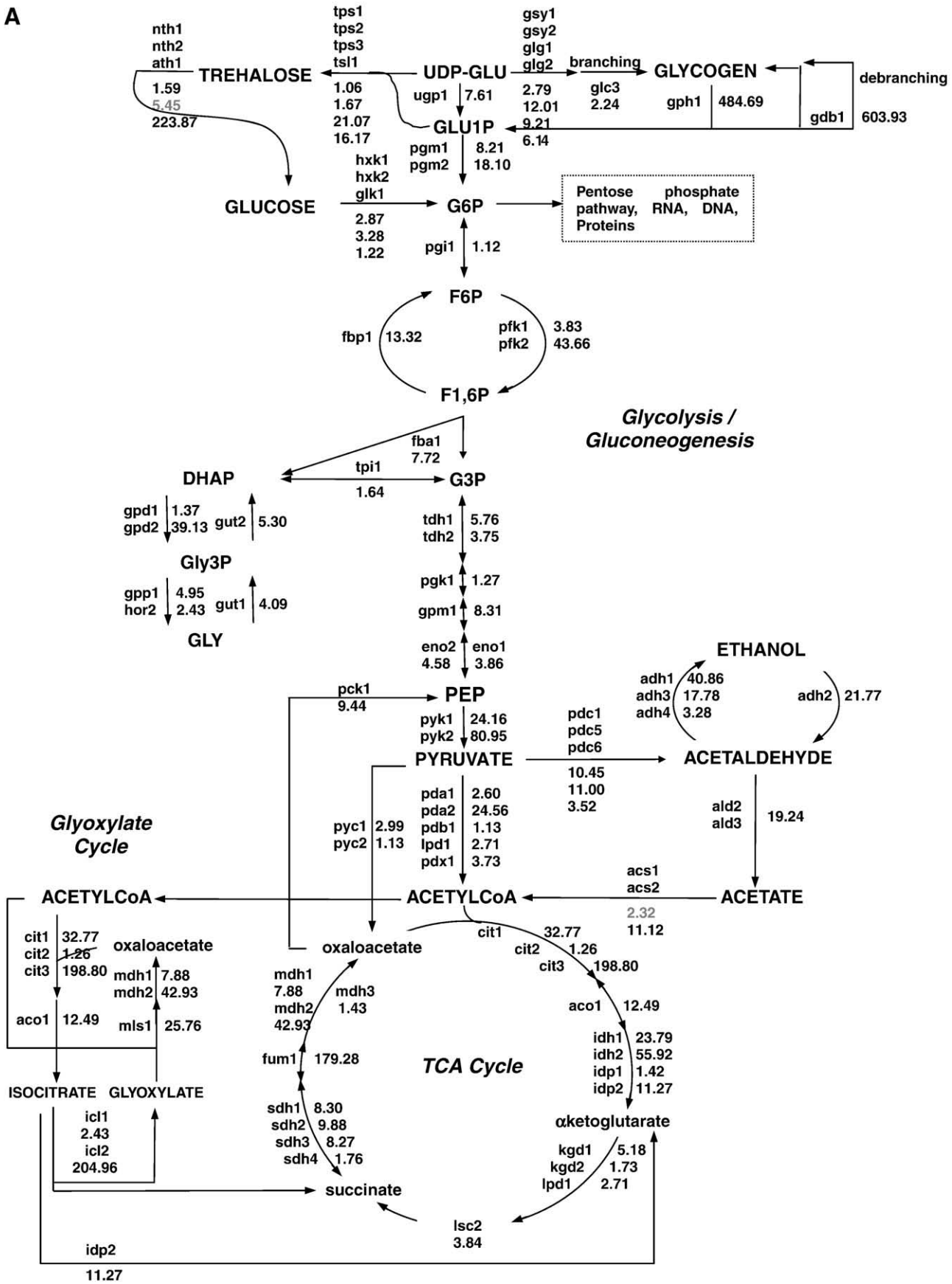


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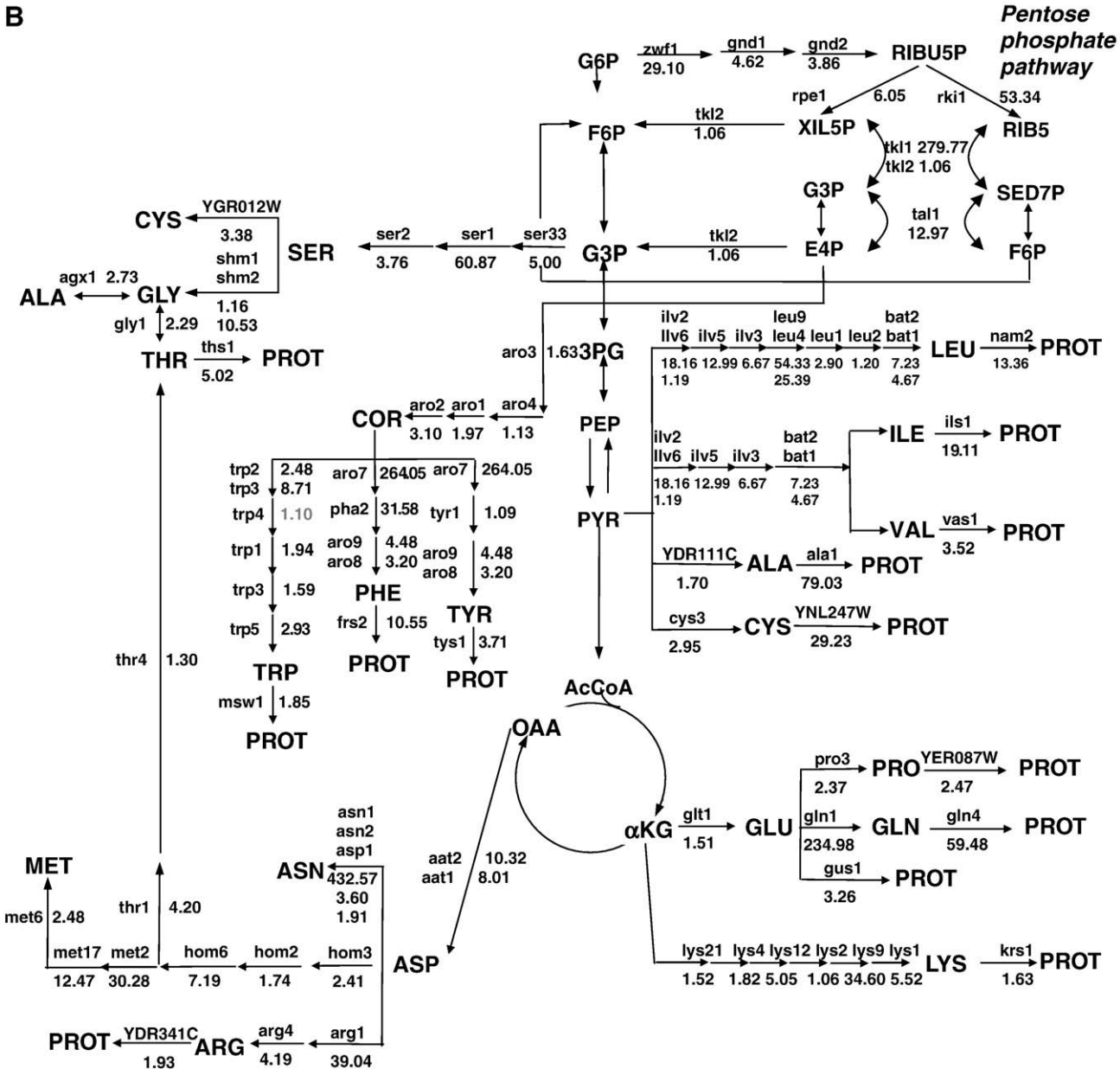


**Fig. 3**. A. Ratios of the levels of transcribed (fold change) of the genes of the wild type strain (P−) growth from glucose to ethanol (Eth/Gluc), of the central metabolic pathways. Names of the genes are shown on the arrows. The abbreviations correspond to: uridine biphosphoglucose (UDP-GLU), glucose-1-phosphate (GLU1P), glucose-1-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-phosphate (F1,6P), glyceraldehyde-3-phosphate (G3P) and phosphoenolpiruvate (PEP). Bold represents genes whose expression increases in the recombinant yeast when grown on ethanol and grey, genes whose expression is decreased in this phase. B. Ratios of the levels of transcribed (fold change) of the genes of the wild type strain (P−) growth from glucose to ethanol (Eth/Gluc), of the pentose phosphate pathway and the most important biosynthetic pathways. The abbreviations correspond to: 3-phosphoglycerate (3PG), phosphoenolpyruvate (PEP), pyruvate (PYR), acetyl-CoA (AcCoA), corismate (COR), ribose-5-phosphate (RIB5P), ribulose-5-phosphate (RIBU5P), xilulose-5-phosphate (XIL5P), pseudoheptulose-7-phosphate (SED7P), eritrose-5-phosphate (E4P), oxaloacetate (OAA), α-ketoglutarate (αKG) and proteins (PROT). Symbols of three letters have been used for the amino acids (blue). Other abbreviations as in Fig. 3A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Wolfger et al., 2004). The targets of this protein are the transcriptional regulators Pdr1p, Pdr2p and Pdr3p.

The other gene directly related to ion transport is *ctr3*. The protein expressed is integrated into the plasma membrane and is related to copper ion incorporation. As will be discussed later this gene is strongly repressed in strain P+ (as compared with P−). Only the recombinant strain P+ has copper incorporated in the medium necessary for SOD synthesis. Thus, although copper is necessary for producing SOD, its concentration in the medium should be regulated as it causes stress effects in the recombinant strain which is expressed at the gene level with the repression of *ctr3* to avoid copper incorporation into the cell.

A crucial element in the use of Gene Microarrays is to evaluate the quality and integrity of the samples of RNA isolated from the cells (see Materials and methods). To do this, the electrospherograms of the corresponding electrophoresis for each of the samples obtained in the different growth phases was carried out for both strains (Díaz, 2006). In all cases all the peaks that correspond to the rRNA 18s and 28s respectively showed that the RNA extracted was of excellent quality (Díaz, 2006).

### *3.3.2. Comparison of the transcription profiles in the different respirofermentative growth phases of non recombinant yeast S. cerevisiae (strain P−)*

*3.3.2.1. Change in expression profiles in the shift from exponential growth on glucose to growth on ethanol (diauxic "shift").* Of the total number of genes studied (6871), 44% (3041 genes) were overexpressed. If we

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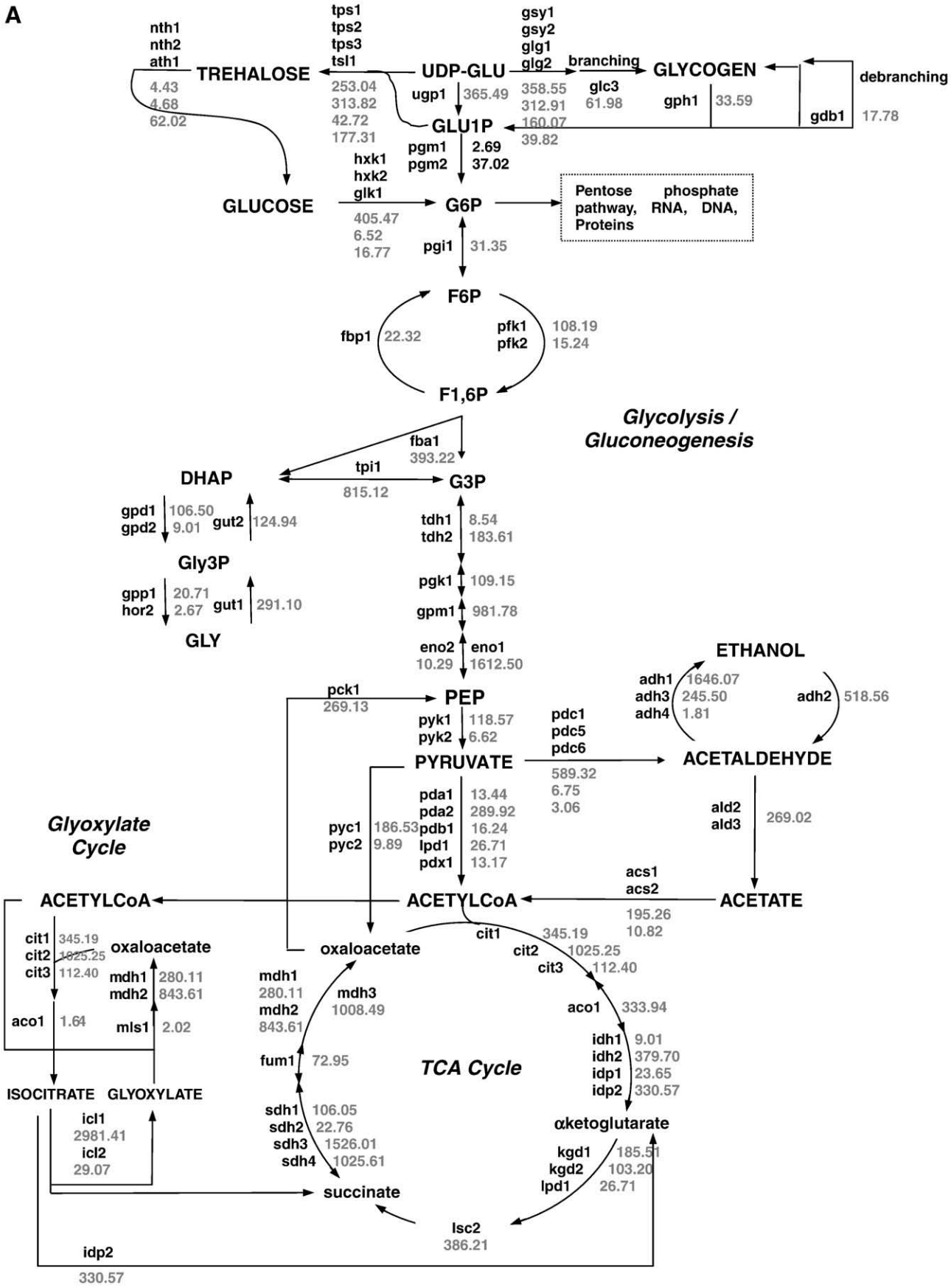


**Fig. 4.** A. Ratios of the levels of transcribed (fold change) of the genes of the wild type strain (P−) growth from ethanol to stationary phase ethanol (Sta/Eth), of the central metabolic pathways. Abbreviations as in Fig. 3A. B. Ratios of the levels of transcribed (fold change) of the genes of the wild type strain (P−) growth from ethanol to stationary phase ethanol (Sta/Eth), of the pentose phosphate pathway and the most important biosynthetic pathways. Abbreviations as in Fig. 3A and B.

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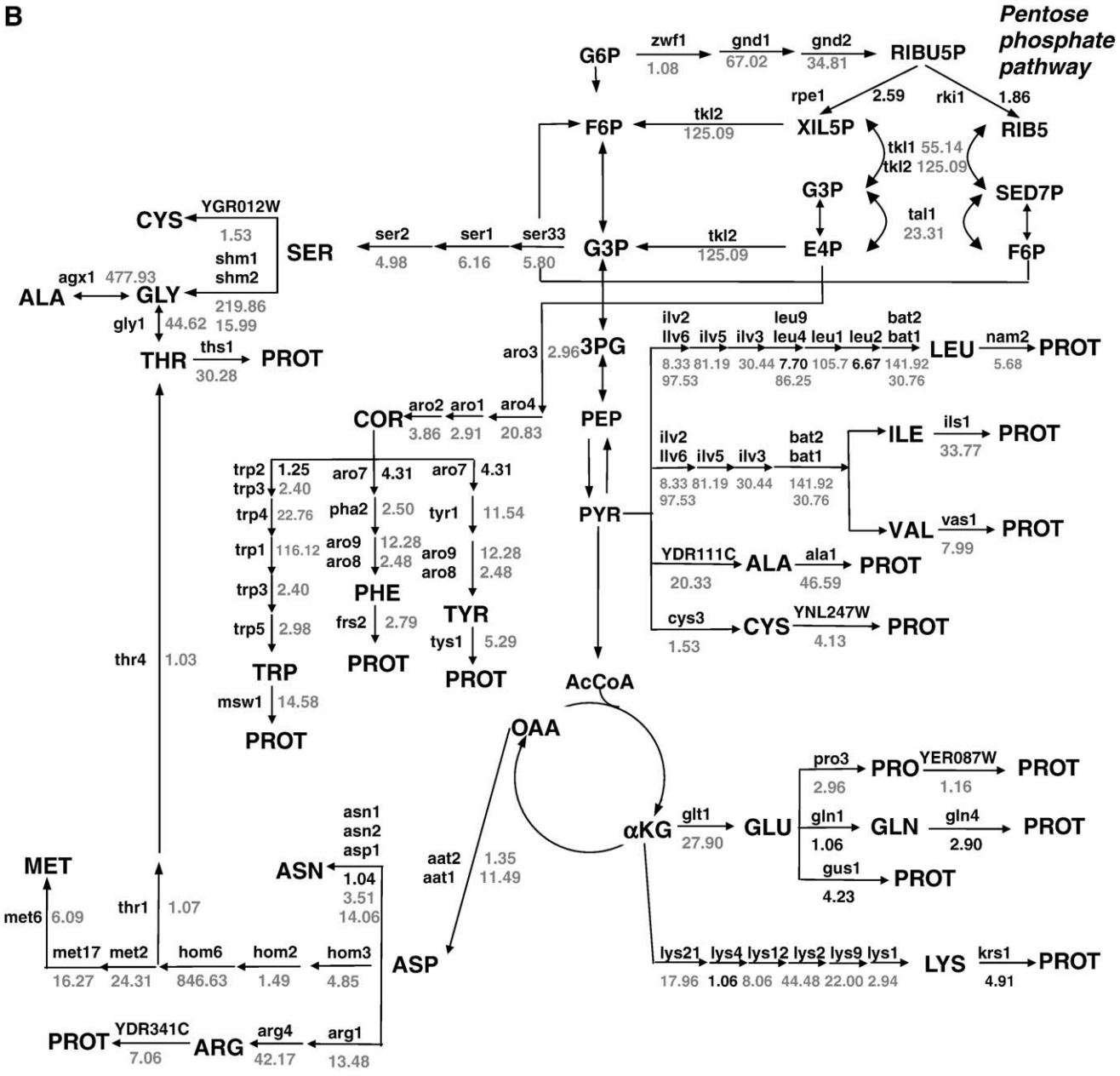


**Fig. 4** (*continued*).

use the criteria of DeRisi et al. (1997) and Causton et al. (2001) where differential expression of genes was considered using a factor of 2 fold, in strain P−, 2982 genes were overexpressed. This result contrasts with work of DeRisi et al. (1997) who observed 710 overexpressed genes and 1030 underexpressed genes (out of a total of 6400 analyzed). Part of the difference can be explained by the fact that they used microarrays based on glass slides ("spotted arrays") where we used Affymetrix gene biochips based on photolithography which have a higher degree of accuracy.

Fig. 3A and B show the central metabolic pathways and the pentose phosphate pathway (PPP) as well as the most important biosynthetic pathways (aminoacid synthesis) of *S. cerevisiae*. The numbers shown for each gene next to the pathways correspond to the ratio of gene expression in the ethanol phase over the glucose phase. Numbers in bold show overexpression and numbers in grey show underexpression. 82 genes have been considered in the central pathways (Fig. 3A) and 80 genes in the PPP and biosynthetic pathways (Fig. 3B).

Fig. 3A shows that in the P− strain in the diauxic shift 97.6% (80 genes) of genes involved in the central metabolic pathways are overexpressed. This is consistent with what was observed by DeRisi et al. (1997) who found that virtually all enzymes in the central metabolic pathway of yeast were overexpressed in the shift from glucose to ethanol. His values were somewhat smaller than those reported here but that could be due to the methodology used to measure gene expression.

A detailed analysis of the genes that show a high degree of overexpression in the central metabolism show that in all pathways involved some genes are highly overexpressed. For instance, in glycolysis, genes *pyk2* (of pyruvate kinase) and *pfk2* (of 6-phosphofructo kinase) are 81 and 44 times overexpressed in the shift from glucose to ethanol. These results appear related with the increase in glycerol consumption and the activation of the TCA cycle. In this cycle genes *cit3* (citrate synthase), *fum1* (fumarate hydrase), *idh2* (isocitrate dehydrogenase), *mdh2* (L-malate dehydrogenase) and *cit1* (citrate synthase), amongst others, show overexpression levels of 199, 179, 56, 43 and 33 fold in the shift from glucose to ethanol respectively. Other genes that show a high overexpression are genes *gdb1* (604 fold) involved in glycogen degradation (Teste et al., 2000), *gph1* (485 fold) whose gene product

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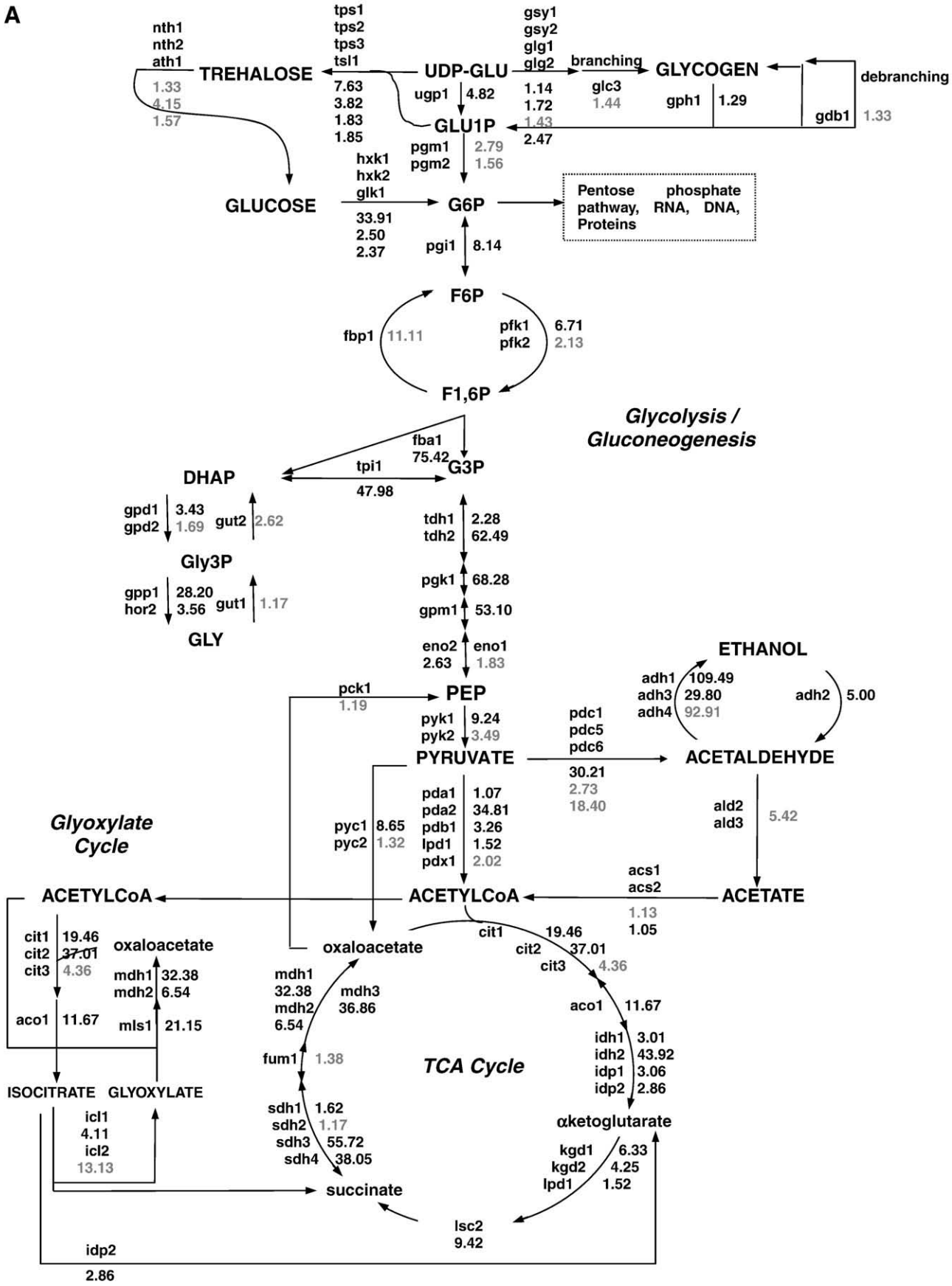


**Fig. 5.** A. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) growth from glucose to ethanol (Eth/Gluc), of the central metabolic pathways. Abbreviations as in Fig. 3A. B. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) growth from glucose to ethanol (Eth/Gluc), of the pentose phosphate pathway and the most important biosynthetic pathways. Abbreviations as in Fig. 3A and B.

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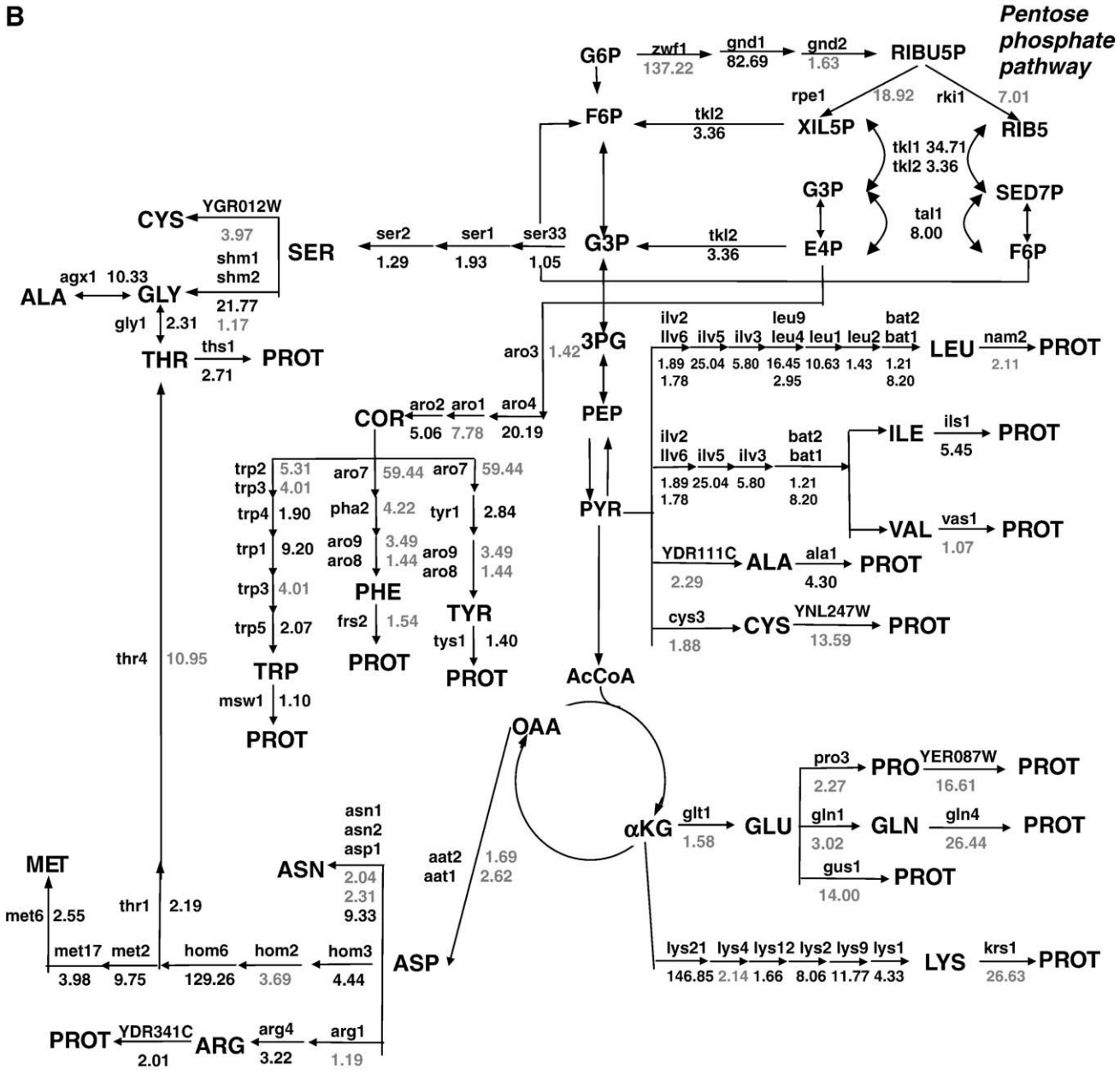


**Fig. 5** (*continued*).

is required for the transport of glycogen (its activity is regulated by the phosphorylation which is mediated by cyclic AMP and its expression is regulated by elements of response to stress and the path of the HOG MAP kinase (Hwang et al., 1989; Sunnarborg et al., 2001)) and gene *ath1* (224 fold) implied in trehalose catabolism, and gene *adh1* (41 fold) that takes part in ethanol metabolism. The activation of these genes and others shown in Fig. 3A imply the concerted action of the enzymes that allow the conversion of ethanol to Acetyl CoA, which is utilized to "fuel" the TCA and glyoxylate cycle. The induction of important genes such as *pck1*, responsible for phosphoenolpyruvate carbokinase, and *fbp1* that codes for fructose-1, 6-biphosphate, directs the flux to two irreversible pathways, which are key for glycolysis, reverting the flow of metabolites towards essential biosynthetic precursors such as glucose -6-phosphate. The induction of genes responsible for the synthesis of trehaloses and the synthesis of glycogen redirect the flux of glucose-6-phosphate towards the storage of carbohydrates. Finally, a group of genes related to protein synthesis, inducing ribosomal proteins, tRNA synthases and translation, elongation and initiation factors, showed a "coordinated" underexpression (Díaz, 2006). 97% of the genes related to ribosomal protein showed underexpression during the diauxic shift.

A similar behaviour to that observed for the central metabolic pathways can be observed in Fig. 3B for the pentose phosphate pathway (PPP) and the most important biosynthetic pathways (aminoacid synthesis). 98.8% (79 genes) involved are overexpressed and only 1 gene is underexpressed in the diauxic shift. For instance, gene *asn1* (asparginine synthase) involved in the metabolism of asparginine (433 fold), gene *aro7* involved in the synthesis of phenylalanine, trypsin and tryptophan (264 fold) and genes *gln1* and *gln4* involved in the synthesis of glutamine amongst others (235 and 59 fold, respectively). Genes of the pentose phosphate pathway such as *tkl1* (transketolase) necessary for aromatic aminoacid synthesis and *rki1* (ribose-5-phosphate isomerase) are overexpressed 280 and 63 fold when growing on ethanol, respectively.

*3.3.2.2. Change in expression profile in the shift from exponential growth on ethanol to early stationary phase.* Of the total number of genes studied (6871), 56% (3869 genes) were overexpressed in the early

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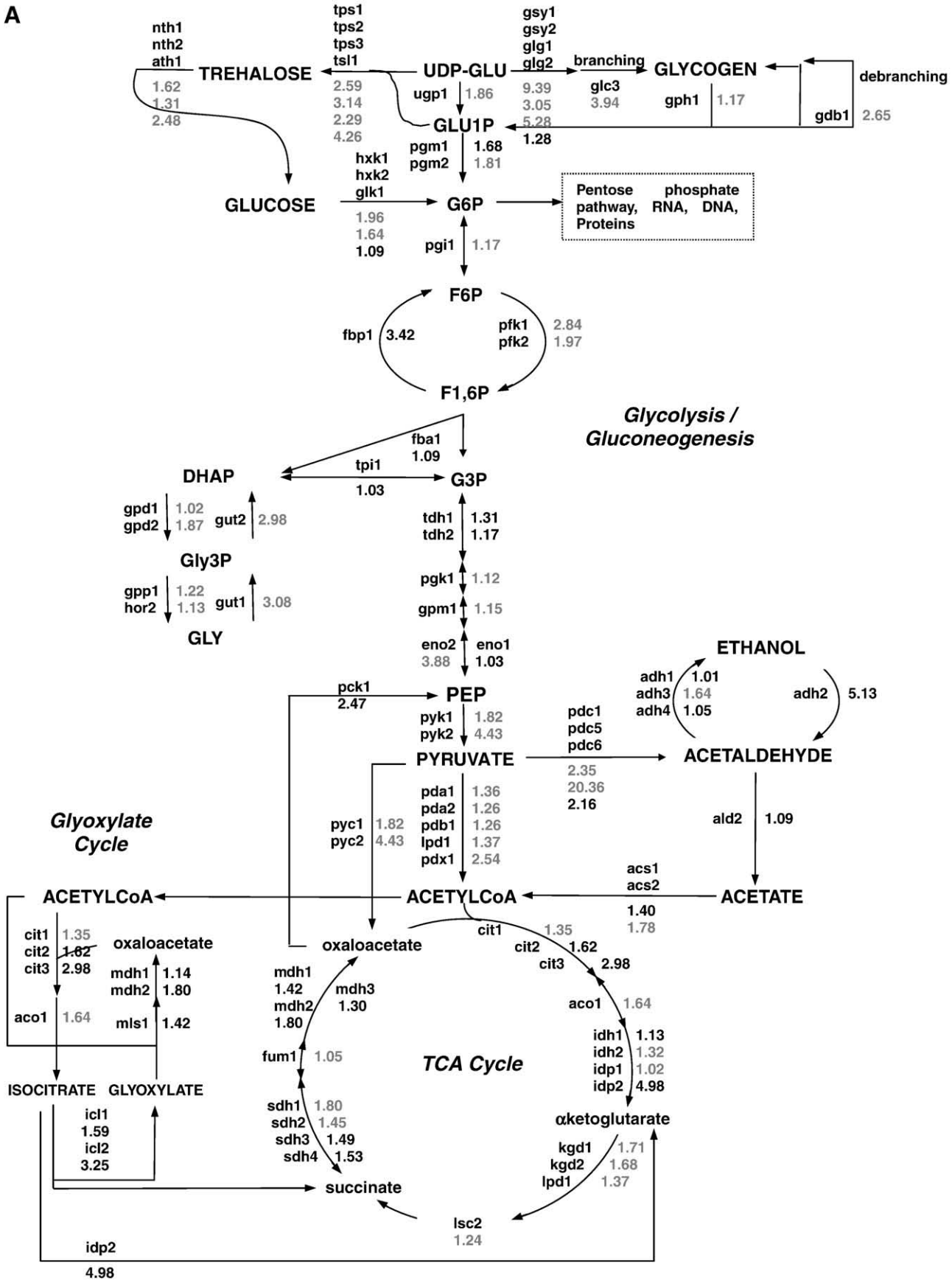


**Fig. 6.** A. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) compared to the wild type strain (P−) growth on glucose (P+/P−), of the central metabolic pathways. Abbreviations as in Fig. 3A. B. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) compared to the wild type strain (P−) growth on ethanol (P+/P−), of the central metabolic pathways. Abbreviations as in Fig. 3A.

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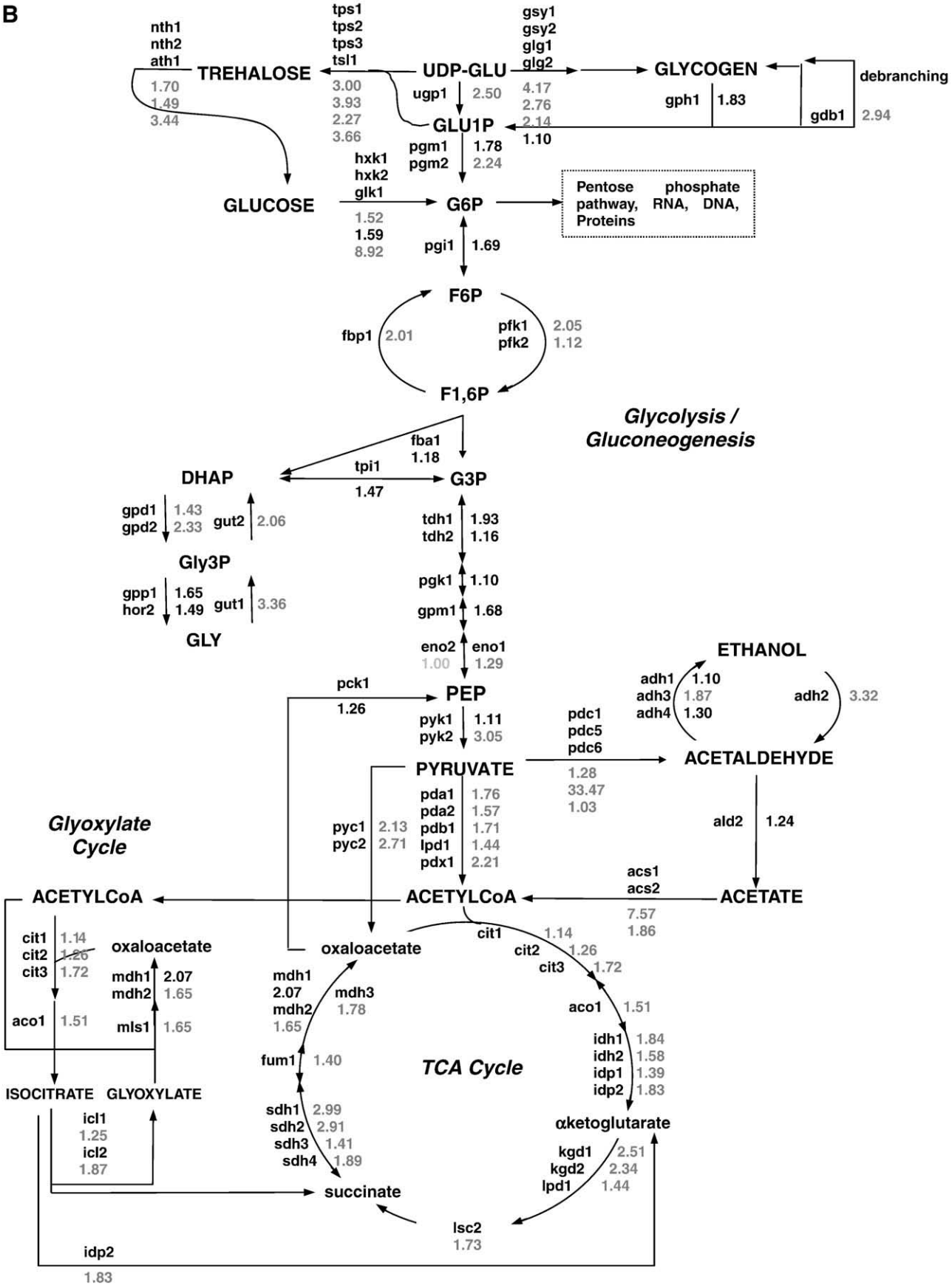


**Fig. 6** (*continued*).

**Table 3**
Genes induced by SOD expression on glucose.

| ID | Gene | Description | Fold-increase |
|---|---|---|---|
| RDN37-1 | – | Structural constituent of ribosome: 25S ribosomal RNA, component of the large (60S) ribosomal subunit; encoded in the rDNA repeat (RDN1) as part of the 35S primary transcript. | 28.56 |
| SNR67 | – | Nucleic acid binding: guides 2′-O-methylation on large subunit rRNA at G2616 and U2721; small nucleolar RNA snR67 (Activity that provides specificity to a methylase by using base complementarity to guide site-specific 2′-O-ribose methylations to a small nuclear RNA molecule). Suppressor of Glycerol Defect. | 16.60 |
| YHR053C | *CUP1-1* | Ion binding: metallothionein, binds copper and mediates resistance to high concentrations of copper and cadmium; locus is variably amplified in different strains, with two copies, CUP1-1 and CUP1-2, in the genomic sequence reference strain S288C. | 10.40 |
| YNL043C | – | Molecular function unknown (ATP synthase subunit h) | 10.05 |
| YPL223C | *GRE1* | Hydrophilin of unknown function; stress induced (osmotic, ionic, oxidative, heat shock and heavy metals); regulated by the HOG pathway. Gamma-glutamyltransferase homolog | 8.96 |
| YILCDELTA2 | – | Hypothetical ORF (Ty2 LTR) | 8.74 |
| YFRWDELTA10 | – | Hypothetical ORF (Ty1 LTR) | 8.51 |
| RDN25-1 | – | Structural constituent of ribosome: 25S ribosomal RNA, component of the large (60S) ribosomal subunit; encoded in the rDNA repeat (RDN1) as part of the 35S primary transcript. Phosphatidylinositol-4-kinase\|similar to VPC34 | 7.65 |
| YFL020C | *PAU5* | Enzyme that mediates the conjugation of Rub1p, a ubiquitin-like protein, to other proteins; related to E2 ubiquitin-conjugating enzymes. Part of 23-member seripauperin multigene family encoded mainly in subtelomeric regions, active during alcoholic fermentation, regulated by anaerobiosis, negatively regulated by oxygen, repressed by heme. | 7.54 |
| YMR118C | – | Hypothetical ORF (chitin deacetylase) | 7.53 |
| YMR175W | *SIP18* | Protein involved in vesicular transport, mediates transport between an endosomal compartment and the Golgi, contains a Golgi-localization (GRIP) domain that interacts with activated Arl1p-GTP to localize Imh1p to the Golgi. Protein whose expression is induced by salt, response to desiccation and to osmotic stress. Interacting selectively with phospholipids. | 7.38 |
| YOR031W | *CRS5* | Ion binding: copper-binding metallothionein, required for wild-type copper resistance. | 7.29 |
| RDN25-1 | – | Spa2p homolog. Protein involved in shmoo formation and required for bipolar bud site selection, homologous to Spa2p, localizes to sites of polarized growth. | 7.22 |
| YDR536W | *STL1* | Septin: sugar transporter-like protein | 7.14 |
| SNR51 | – | Nucleic acid binding: guides 2′-O-methylation on small subunit rRNA at A100 and on large subunit rRNA at U2726; small nucleolar RNA snR51 (Activity that provides specificity to a methylase by using base complementarity to guide site-specific 2′-O-ribose methylations to a small nuclear RNA molecule). | 7.05 |
| YKL161C | *MLP1* | Mpk1-like protein kinase; associates with Rlm1p | 7.03 |
| YGLCDELTA5 | – | Hypothetical ORF (Ty1 LTR). Component of the hexameric MCM complex, which is important for priming origins of DNA replication in G1 and becomes an active ATP-dependent helicase that promotes DNA melting and elongation when activated by Cdc7p-Dbf4p in S-phase. | 6.80 |
| YOR010C | *TIR2* | Putative cell wall mannoprotein of the Srp1p/Tip1p family of serine–alanine-rich proteins; transcription is induced by cold shock and anaerobiosis. U1 snRNP protein of the Sm class. | 6.80 |
| YMR040W | *YET2* | Protein of unknown function that may interact with ribosomes, based on co-purification experiments; homolog of human BAP31 protein. | 6.68 |

This list includes the 20 most over expressed genes in the recombinant yeast compared to the wild type strain. The average fold-increase in mRNA levels measured in the three experiments is indicated.

stationary phase as compared to the ethanol phase and 44% (2988 genes) were underexpressed.

Fig. 4A and B show the central metabolic pathway and the pentose phosphate pathway (PPP) as well as the most important biosynthetic pathways (aminoacid synthesis). 82 genes have been considered in the central pathway and 80 genes in the PPP and biosynthetic pathways.

Fig. 4A shows that in the P− strain in the shift from the ethanol phase to the early stationary 100% of the genes involved in the central metabolic pathways are underexpressed.

A detailed analysis of the degree of underexpression in the central metabolic pathways shows that some genes involved in all pathways are highly underexpressed and that the level of change is much larger that the previous case shown in Fig. 3. For instance gene *icl1* shows an underexpression of 2981 fold in the shift form the ethanol to the early stationary phase. This gene is responsible for the translation of isocitate lyase, catalyzes the synthesis of succinate and glyoxylate from isocitrate, a key reaction in the glyoxylate cycle and thus in providing succinate for the TCA cycle. Gene *icl1* is overexpressed when *S. cerevisiae* grows on ethanol and underexpressed when growing on glucose (Fernandez et al., 1992; Luttik et al., 2000). Also genes *eno1* (phosphopyruvate hydratase) and *gpm1* (phosphoglycerate mutase) that take part both in glycolysis and in gluconeogenesis show a very high level of underexpression (1613 and 982 fold, respectively). Other genes highly underexpressed in the shift from ethanol to early stationary phase are those responsible for the TCA cycle such as *cit2* (1026 fold), *sdh3* and *sdh4* (succinate dehydrogenases) (1526 and 1026 fold respectively) and *mdh3* (malate dehydrogenase also involved in the glyoxylate cycle, 1009 fold). Also strongly underexpressed are the genes *tpi1* (815 fold) that participates in glycolysis and glycerol catabolism and *fba1* (fructose-1,6-biphosphate aldolase, 393 fold) that takes part in glycolysis and gluconeogenesis. All this reflects the environmental conditions that the yeast is subjected to in this phase, namely a lack of carbon source: glucose, ethanol and glycerol.

A similar situation to that observed in Fig. 4A is shown in Fig. 4B for the PPP and the biosynthetic pathways (aminoacid synthesis). Here (Fig. 4B) 85% (68 genes) of the genes involved are underexpressed. However, the few that are overexpressed (15%) show a very low level, most values being very near 1.0.

A detailed analysis of Fig. 4B shows that in the synthesis pathways of all aminoacids (except for glutamine) the genes are highly underexpressed in the shift from ethanol to early stationary phase. For instance the genes *hom6* (homoserine dehydrogenase) involved in the metabolism of homoserine, methionine and threonine (847 fold), *shm1* involved in the synthesis of glycine from serine (220 fold) and genes *bat2, ilv6* and *ilv5* involved in the synthesis of leucine, isoleucine and valine (142, 98 and 81 fold respectively). Another gene is *glt1* (glutamate synthetase) involved in the synthesis of glutamate, and subsequently of proline and glutamine (28 fold). Other genes highly underexpressed are those of the PPP such as *tkl2*, which is responsible for transketolase that catalyzes the conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and gluteraldyhde-3-phosphate (125 fold). This enzyme is also necessary for aromatic aminoacid

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**Table 4**
Genes repressed by SOD expression on glucose.

| ID | Gene | Description | Fold-decrease |
|---|---|---|---|
| YLR411W | *CTR3* | Copper uptake transporter activity. High-affinity copper transporter of the plasma membrane, acts as a trimer; gene is disrupted by a Ty2 transposon insertion in many laboratory strains of *S. cerevisiae*. Actin. | 34.54 |
| YIR019C | *MUC1* | GPI-anchored cell surface glycoprotein required for diploid pseudohyphal formation and haploid invasive growth, transcriptionally regulated by the MAPK pathway (via Ste12p and Tec1p) and the cAMP pathway (via Flo8p). Actin. | 25.17 |
| YIL080W | – | Protein binding. TyB Gag-Pol protein; proteolytically processed to make the Gag, RT, PR, and IN proteins that are required for retrotransposition. Actin. | 22.56 |
| YLR134W | *PDC5* | Minor isoform of pyruvate decarboxylase, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde, regulation is glucose- and ethanol-dependent, repressed by thiamine, involved in amino acid catabolism TFIID subunit. | 20.36 |
| YILWTY3-1 | – | Retrotransposon. Full length Ty3. TFIID subunit | 16.10 |
| YNLCTY1-1 | – | Retrotransposon. Full length Ty1. TFIID subunit | 14.35 |
| YMRCTY1-3 | – | Retrotransposon. Full length Ty1. RNA polymerase II holoenzyme/mediator subunit | 12.48 |
| YGRCTY1-3 | – | Retrotransposon. Full length Ty1. RNA polymerase II holoenzyme/mediator subunit | 12.33 |
| YBLWTY1-1 | – | Retrotransposon. Full length Ty1. Orotidine-5′-phosphate decarboxylase | 11.73 |
| YORWTY1-2 | – | Retrotransposon. Full length Ty1. Esa1p-associated factor, subunit of the NuA4 acetyltransferase complex | 10.96 |
| YGRWTY1-1 | – | Retrotransposon. Full length Ty1. Oligosaccharyl transferase glycoprotein complex, beta subunit | 10.95 |
| YBRWTY1-2 | – | Retrotransposon. Full length Ty1. | 10.70 |
| YLL067C | *YRF1-7* | Hypothetical ORF. Helicase activity (catalysis of the unwinding of a DNA or RNA duplex) | 10.07 |
| YFR015C | *GSY1* | Glycogen synthase with similarity to Gsy2p, the more highly expressed yeast homolog; expression induced by glucose limitation, nitrogen starvation, environmental stress, and entry into stationary phase. | 9.39 |
| YARCTY1-1 | – | Retrotransposon. Full length Ty1. | 9.38 |
| YJRWTY1-2 | – | Retrotransposon. Full length Ty1. | 9.25 |
| YDRCTY1-1 | – | Retrotransposon. Full length Ty1. | 9.19 |
| YDRWTY1-5 | – | Retrotransposon. Full length Ty1. Interleukin 4 | 9.04 |
| YMLWTY1-1 | – | Retrotransposon. Full length Ty1. Interleukin 10 | 8.61 |

This list includes the 20 most under-expressed genes in the recombinant yeast compared to the wild type strain. The average fold-decrease in mRNA levels measured in the three experiments is indicated.

synthesis. The changes shown in Fig. 4 reflect the fall in the biosynthetic machinery for proteins in the cell during early stationary phase.

### *3.3.3. Comparison of the transcription profiles in the different respirofermentative growth phases of recombinant yeast S. cerevisiae (strain P+)*

*3.3.3.1. Change in expression profiles in the shift from exponential growth on glucose to growth on ethanol (diauxic "shift").* Of the total number of genes studied (6871), 56% (3882 genes) were overexpressed, 43% (2988) were underexpressed and only 2 of them did not show a difference in expression. The discussion of these results with those obtained by other authors has already been done for the non-recombinant strain (P−).

Fig. 5A and B show the central metabolic pathways and the pentose phosphate pathway (PPP) as well as the most important biosynthetic pathways (aminoacid synthesis) of *S. cerevisiae*. 82 genes have been considered in the central pathway (Fig. 5A) and 80 genes in the PPP and biosynthetic pathways (Fig. 5B).

The global situation for the recombinant strain (P+) in the expression levels in the diauxic shift (from glucose to ethanol) is similar to that observed in the P− strain, however in this case (P+) the number of genes overexpressed is somewhat smaller and the fold-change of expression of the genes is lower than is the P− strain. Fig. 5A shows that in the P+ strain in the diauxic shift 68% (56 genes) of the genes involved in the central metabolic pathways are over-expressed compared to 98% in the P− strain. 32% (26 genes) are underexpressed, but the larger numbers for "fold-expression changes" were observed with overexpressed genes. As for the P− strain, this behaviour is similar to that observed by DeRisi et al. (1997) but different from that observed in MFA (Gonzalez et al., 2003). Although the MFA studies correspond to calculated fluxes, they show that there is an important synthesis of glycerol during growth on ethanol particularly in the P+ strain. In the present study glycerol was measured and the accumulation of glycerol was confirmed experimentally as shown in Fig. 1. This clearly shows the value of MFA and that the fluxes predicted are indicative of real behaviour.

A detailed analysis of the genes that take part in the central metabolic pathway shows that in all of them these are genes highly overexpressed. For instance in the metabolism of ethanol, the gene *adh1* (alcohol dehydrogenase) is 110 times overexpressed and genes fba1 (fructose 1,6-diphosphate aldolase), *tdh2* (glyceraldehyde-3-phosphate dehydrogenase), *pgk1* (3 phosphoglycerate kinase) and *gpm1* (phosphoglycerate mutase), all taking part in glycolysis and gluconeogenesis (and in the catabolism of glycerol in the case of *tdh2*) show overexpressions of 75, 62, 68 an 53 fold respectively; the enzyme 3-phosphoglycerate kinase is considered to be a key enzyme in glycolysis and gluconeogenesis (Blake and Rice, 1981; Hitzeman et al., 1980). In the TCA cycle genes *sdh3* and *idh2* show an overexpression of 56 and 44 fold in the glucose to ethanol shift. The only gene that shows an important underexpression in the diauxic shift in P+ is *adh4* (93 fold) for alcohol dehydrogenase whose transcription is regulated by zinc (Yuan, 2000). As will be discussed later, in contrast to what was observed with strain P−, where all genes of the TCA cycle were overexpressed in the glucose to ethanol shift, here 2 genes (*cit3* and *fum1*) were underexpressed in the shift.

Fig. 5B shows a similar behaviour for the pentose phosphate cycle and the most important biosynthetic routes. Here 55% (44 genes) were overexpressed and 45% (36 genes) underexpressed. However in contrast with Fig. 5A here there are both high level of overexpression and high levels of underexpression. A detailed analysis of Fig. 5B shows the highest level of overexpression for gene *lys21* (147 fold), gene of the isozyme hemocitrate synthase, which catalyzes the condensation of acetyl CoA and α-ketoglutarate to the homocitrate "form" which constitutes the first step to lysine biosynthesis. The highest underexpression was obtained in gene *zwf1* (137 fold) that transcribes for glucose-6-phosphate dehydrogenase involved in the pentose phosphate pathway. Other genes with high overexpression were *gnd1* (83 fold) and *tkl1* (35 fold) both involved in the pentose phosphate pathway. High underexpression was observed in genes *aro7* (59 fold) involved in the synthesis of phenylalanine, tyrosine and tryptophan an in *g1n4* (26 fold) involved in the synthesis of glutamine.

*3.3.3.2. Change in expression profile in the shift from exponential growth on ethanol to early stationary phase.* Of the total number of genes studied (6871), 45% (3091 genes) were overexpressed in the early stationary phase as compared to the ethanol phase and 55% (9778 genes) were underexpressed.

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**Table 5**
Genes induced by SOD expression on ethanol.

| ID | Gene | Description | Fold-increase |
|---|---|---|---|
| SNR66 | – | Nucleic acid binding: guides 2′-O-methylation on large subunit rRNA at U2415; small nucleolar RNA snR66 (Activity that provides specificity to a methylase by using base complementarity to guide site-specific 2′-O-ribose methylations to a small nuclear RNA molecule). | 62.87 |
| YLR367W | *RPS22B* | Structural constituent of ribosome: protein component of the small (40S) ribosomal subunit; nearly identical to Rps22Ap and has similarity to *E. coli* S8 and rat S15a ribosomal proteins. | 41.67 |
| SNR67 | – | Nucleic acid binding: guides 2′-O-methylation on large subunit rRNA at G2616 and U2721; small nucleolar RNA snR67 (Activity that provides specificity to a methylase by using base complementarity to guide site-specific 2′-O-ribose methylations to a small nuclear RNA molecule). | 30.40 |
| YLR264c-a | – | Molecular function unknown | 18.63 |
| YOR277C | – | Hypothetical ORF. | 14.54 |
| YDL055C | *PSA1* | GDP-mannose pyrophosphorylase (mannose-1-phosphate guanyltransferase), synthesizes GDP-mannose from GTP and mannose-1-phosphate in cell wall biosynthesis; required for normal cell wall structure. | 12.74 |
| YLR136C | *TIS11* | Possible transcription factor, contains two zinc fingers; homolog of mammalian TIS11; transcription is glucose repressed. | 12.20 |
| YHR053C | *CUP1-1* | Ion binding: metallothionein binds copper and mediates resistance to high concentrations of copper and cadmium; locus is variably amplified in different strains, with two copies, CUP1-1 and CUP1-2, in the genomic sequence reference strain S288C. | 11.65 |
| YMR294W-A | – | Hypothetical ORF. | 11.26 |
| YPL142C | – | Protein required for cell viability. | 10.85 |
| YLR367W | *RPS22B* | Structural constituent of ribosome: Protein component of the small (40S) ribosomal subunit; nearly identical to Rps22Ap and has similarity to *E. coli* S8 and rat S15a ribosomal proteins. | 10.59 |
| YDR524w-a | – | ORF, uncharacterized. | 10.13 |
| YCR013C | – | Protein required for cell viability. | 9.39 |
| YLR372W | *SUR4* | Fatty acid elongase activity: elongase III synthesizes 20–26-carbon fatty acids from C18-CoA primers; involved in fatty acid biosynthesis. | 8.72 |
| YNL289W | *PCL1* | Pho85 cyclin of the Pcl1,2-like subfamily, involved in entry into the mitotic cell cycle and regulation of morphogenesis, localizes to sites of polarized cell growth. G1 cyclin associates with PHO85. Modulates the activity of a cyclin-dependent protein kinase, enzymes of the protein kinase family that are regulated through association with cyclins and other proteins. | 8.54 |
| YLR076C | – | Protein required for cell viability. | 8.24 |
| YJL188C | *BUD19* | Dubious open reading frame, unlikely to encode a protein; not conserved in closely related Saccharomyces species; 88% of ORF overlaps the verified gene RPL39; diploid mutant displays a weak budding pattern phenotype in a systematic assay. | 8.19 |
| YGL102C | – | Protein required for cell viability. | 8.08 |
| YBL002W | *HTB2* | DNA binding: one of two nearly identical (see HTB2) histone H2B subtypes required for chromatin assembly and chromosome function; Rad6p-Bre1p-Lge1p mediated ubiquitination regulates transcriptional activation, meiotic DSB formation and H3 methylation. | 8.01 |

This list includes the 20 most over expressed genes in the recombinant yeast compared to the wild type strain. The average fold-increase in mRNA levels measured in the three experiments is indicated.

Fig. 7A and B (Appendix) show the central metabolic pathway and the pentose phosphate pathway (PPP) as well as the most important biosynthetic pathways (aminoacid synthesis). The numbers shown for each gene next to the pathways correspond to the ratio of gene expression in the early stationary phase over the ethanol consumption phase.

The recombinant strain shows expression levels very similar to those of the P− strain. The levels of underexpression are generally higher in the P+ strain compared to P− in this growth phase. Fig. 7A (Appendix) shows that in the recombinant (P+) strain in the shift from the ethanol phase to the early stationary 99% of the genes (81 genes) involved in the central metabolic pathways are underexpressed.

The detailed analysis of these genes shows that some of them are highly underexpressed. The highest underexpression is again observed for gene *icl1* (isocitrate lyase that catalyzes a key reaction in the glyoxylate cycle) which is 1489 fold underexpressed in the ethanol to stationary shift (compared to 2981 fold in the P− strain). A high underexpression is also shown for genes *adh1* and *adh3* (910 and 208 fold), both, alcohol dehydrogenases involved in the ethanol metabolism (the first in the reduction of acetaldehyde to ethanol, the last step in the glycolytic pathway at the cytosol level; and the second one involved in the exchange of mitochondrial NADH to the cytosol under anaerobic conditions and ethanol synthesis). Other genes that also show high underexpression are *fba1* (610 fold) and *tpi1* (369 fold) which take part in glycolysis, gluconeogenesis and in the catabolism of glycerol, as well as genes *pgk1* and *gpm1* (432 and 423 fold) all related to these pathways. Other genes strongly underexpressed in the shift to stationary phase are those involved in the TCA cycle such as *sdh3, cit2, mdh2, idh2, sdh4, mdh3, mdh1* and *cyt1* (606, 591, 556, 556, 439, 339, 317 and 315 fold, respectively).

A similar situation as that described for the P− strain can be observed in Fig. 7B (Appendix) for the pentose phosphate cycle and for the biosynthetic routes for aminoacids. Here 85% (68 genes) of the genes involved are underexpressed while only 15% (12 genes) are overexpressed. Nevertheless the level of overexpression of these last genes is extremely low.

The detailed analysis of Fig. 7B (Appendix) shows that genes responsible for the synthesis of all aminoacids and the pentose phosphate pathway genes are highly underexpressed in the shift to the stationary phase. Genes *hom6* (847 fold) and *shm1* (151 fold) as well as gene *agx1* (149 fold) which corresponds to glyoxylate aminotranferase that catalyzes the synthesis of glycine from glyoxylate one of the 3 paths for the synthesis of alanine. Other genes of importance in aminoacid synthesis highly underexpressed are *lys21, lys2* and *lys9* (79, 81 and 49 fold, respectively) all involved in lysine synthesis, as well as genes in the pentose phosphate pathway such as *gnd1* (278 fold) and *tkl1* (286 fold).

### *3.3.4. Comparison of the transcription profiles of the recombinant (P+) and non recombinant (or wild type, P−) strains of the yeast S. cerevisiae in the different respirofermentative phases*

*3.3.4.1. Genes under- and over-expressed during the exponential growth phase on glucose.* Figs. 6A and 8A (Appendix) show the ratios of the gene expression levels (fold change) in the recombinant strain in relation to the non-recombinant or native strain (P+/P−). Numbers in bold correspond to overexpression and numbers in grey to under-expression. Fig. 6A corresponds to the central metabolic pathways and Fig. 8A (Appendix) the PPP and biosynthetic pathways.

Fig. 6A shows that when both strains are compared most of the genes of the central metabolic pathways (53 genes, 65%) are underexpressed in P+. However, the values of underexpression are very much smaller than those observed in Figs. 3A–5B, and 7A and B (Appendix) when different growth phases are compared for each of the two strains. Hence, very low levels of underexpression are observed.

A detailed analysis of Fig. 6A shows that the gene with the highest underexpression in P+ is *pdc5* (206 fold). This gene transcribes

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**Table 6**
Genes repressed by SOD expression on ethanol.

| ID | Gene | Description | Fold-decrease |
|---|---|---|---|
| YIR019C | *MUC1* | Cell surface flocculin with structure similar to serine/threonine-rich GPI-anchored cell wall proteins. GPI-anchored cell surface glycoprotein required for diploid pseudohyphal formation and haploid invasive growth, transcriptionally regulated by the MAPK pathway (via Ste12p and Tec1p) and the cAMP pathway (via Flo8p). | 53.85 |
| YLR411W | *CTR3* | Copper transporter. High-affinity copper transporter of the plasma membrane, acts as a trimer; gene is disrupted by a Ty2 transposon insertion in many laboratory strains of *S. cerevisiae*. | 46.00 |
| YOR348C | *PUT4* | Proline specific permease (also capable of transporting alanine and glycine); putative proline-specific permease. | 38.53 |
| YLR134W | *PDC5* | Pyruvate decarboxylase. Minor isoform of pyruvate decarboxylase, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde, regulation is glucose- and ethanol-dependent, repressed by thiamine, involved in amino acid catabolism. | 33.47 |
| YIL080W | – | TyB Gag-Pol protein; proteolytically processed to make the Gag, RT, PR, and IN proteins that are required for retrotransposition. | 19.64 |
| YILWTY3-1 | – | Retrotransposon, full length Ty3.TyB Gag-Pol protein; proteolytically processed to make the Gag, RT, PR, and IN proteins that are required for retrotransposition. | 18.04 |
| RDN37-1 | – | 35S ribosomal RNA transcript, encoded by the RDN1 locus, that is processed into the 25S, 18S and 5.8S rRNAs (represented by the RDN25, RDN18, and RDN58 loci). | 15.33 |
| YLL067C | – | Putative protein of unknown function with similarity to helicases. | 13.99 |
| YNL194C | – | Hypothetical ORF | 13.85 |
| YPR124W | *CTR1* | Copper transport protein. High-affinity copper transporter of the plasma membrane, mediates nearly all copper uptake under low copper conditions; transcriptionally induced at low copper levels and degraded at high copper levels. | 12.56 |
| YCR010C | *ADY2* | Transmembrane protein. Accumulation of DYads; member of the TC 9.B.33 YaaH family of putative transporters; Protein involved in Accumulation of DYads. Enables the directed movement of acetate into, out of, within or between cells. | 11.05 |
| YHL050C | *YRF1-7* | Protein of unknown function, potential Cdc28p substrate.Y′-helicase protein 1. | 10.40 |
| YLR214W | *FRE1* | Cupric reductase\|ferric reductase. Ferric reductase and cupric reductase, reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low copper and iron levels. | 9.04 |
| YCL040W | *GLK1* | Glucokinase, catalyzes the phosphorylation of glucose at C6 in the first irreversible step of glucose metabolism; expression regulated by non-fermentable carbon sources | 8.92 |
| YARCTY1-1 | – | Retrotransposon, full length Ty3.TyB Gag-Pol protein; proteolytically processed to make the Gag, RT, PR, and IN proteins that are required for retrotransposition. | 8.76 |
| YAL040C | *CLN3* | G1 cyclin. Role in cell cycle START; involved in G(sub)1 size control; G(sub)1 cyclin. | 8.02 |
| YDL185W | *TFP1* | Vacuolar ATPase V1 domain subunit A containing the catalytic nucleotide binding sites. | 7.94 |
| YOL152W | *FRE7* | Putative ferric reductase with similarity to Fre2p; expression induced by low copper levels but not by low iron levels. | 7.93 |
| YNLCTY1-1 | – | Retrotransposon, full length Ty3. TyB Gag-Pol protein; proteolytically processed to make the Gag, RT, PR, and IN proteins that are required for retrotransposition. | 7.85 |
| YNL142W | *MEP2* | Ammonia transport protein. Ammonia permease; belongs to a ubiquitous family of cytoplasmic membrane proteins that transport only ammonium (NH4+). Expression is under the nitrogen catabolite repression regulation. | 7.80 |

This list includes the 20 most under-expressed genes in the recombinant yeast compared to the wild type strain. The average fold-decrease in mRNA levels measured in the three experiments is indicated.

the minor isoform of pyruvate decarboxylase, a key enzyme in alcoholic fermentation, it decarboxilates pyruvate to acetaldehyde (Hohmann and Cederberg, 1990; Muller et al., 1999). Other genes that show underexpression in the P+ strain are those involved in the reaction of uridine diphosphoglucose (UDP-GLU) to glycogen: *gsy1* (9 fold), *glg1* (5 fold) and *gsy2* (3 fold). *gsy1* codes for a glucose synthetase.

Tables 3 and 4 show the 20 genes most overexpressed (Table 3) and underexpressed (Table 4) in P+ when growing on glucose. Of the 20 genes most overexpressed 5 are hypothetical ORFs and their molecular function of one of them is unknown (YNLO43C). The genes with highest overexpression in P+ are *rdn37-1* (29 fold) and *snr67* (17 fold) which are "related" to ribosomes and to the "union" to nucleic acids, respectively, and that would be involved in protein synthesis, including the recombinant one. Three of the genes are very important in this study, *cup1-1, gre1* and *crs5*. *cup1-1* codes for proteins that bind copper and participate in the resistance to high concentrations of copper and cadmium. Gene *gre1* transcribes for proteins induced under osmotic, ionic, oxidative and thermal stress and in this case due to the presence of (heavy) metals such as copper. Finally *crs5* codes for a metalothionine that binds copper and is needed in wild type strains for resistance to this metal. Hence, these three genes are related to the resistance and response to a high level of copper. Only the medium used for the P+ strain contained copper necessary for SOD synthesis.

Table 4 shows that of the 20 most underexpressed genes in P+, 65% correspond to retrotranposons (13 genes) and one of them is a hypothetical ORF (YLLO67C). Gene *ctr3* (35 fold) codes a protein related to copper transport, hence its underexpression would mean that it is necessary to control copper transport into the cell.

Clearly it can be seen that when comparing the values of under-expression of (P+/P−) with those that have been observed for MFA in the same pathways, the values obtained for gene expression are qualitatively similar, but give several fold higher values than those observed for metabolic fluxes (Gonzalez et al., 2003).

*3.3.4.2. Genes under and overexpressed during exponential growth on ethanol.* Figs. 6B and 8B (Appendix) show the ratios of the gene expression levels (fold change) in the recombinant strain in relation to the non-recombinant or native strain (P+/P−). Fig. 6B corresponds to the central metabolic pathways and Fig. 8B (Appendix) the PPP and biosynthetic pathways.

Fig. 6B shows a similar behaviour to that during growth on glucose as most genes are underexpressed in P+ (63 genes, 77% in the central pathways and 64% in the pentose phosphate and biosynthetic pathways). It can be seen that the levels of underexpression are relatively low compared to the differences observed when the carbon source is changed. For the genes that show overexpression these values tend to be even smaller and very close to 1.0. A similar behaviour has been observed in the analysis of the metabolic fluxes of P+/P− (Gonzalez et al., 2003). Again, fluxes in the TCA cycle were 60% lower and protein synthesis was ca. 50% in the P+ and no SOD was synthesised in this phase. Ethanol consumption was ca. 80%.

Similarly to the behaviour when growing on glucose the gene that showed the largest underexpression in P+ was *pdc5* (35 fold). Other genes that were underexpressed are *gsy1* (4 fold) involved in the reaction of UDP-GLU to glycogen and a group of genes related to the conversion of UDP-GLU to trehaloses: genes *tps2, tsl1* and *tps1* (3.9, 3.7 and 3.0 fold respectively). The genes *acs1* and *glk1* are expressed during growth on a non fermentable carbon source such as ethanol (Herrero et al., 1995; Kratzer and Schuller, 1997). However in P+ these genes are underexpressed when compared to P− which shows a slowing down of the respiratory metabolism which translates in an underexpression of virtually all the enzymes in the TCA cycle. This is

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**Table 7**
Genes induced by SOD expression in stationary phase.

| ID | Gene | Description | Fold-increase |
|---|---|---|---|
| YLR264c-a | – | ORF, uncharacterized. Identified by SAGE. | 21.69 |
| SNR67 | – | Nucleic acid binding: guides 2′-O-methylation on large subunit rRNA at G2616 and U2721; small nucleolar RNA snR67 (Activity that provides specificity to a methylase by using base complementarity to guide site-specific 2′-O-ribose methylations to a small nuclear RNA molecule). Suppressor of glycerol defect. | 21.32 |
| YLR367W | *RPS22B* | Structural constituent of ribosome: protein component of the small (40S) ribosomal subunit; nearly identical to Rps22Ap and has similarity to *E. coli* S8 and rat S15a ribosomal proteins. Ribosomal protein S22B (S24B) (rp50) (YS22). | 19.73 |
| YNR065C | *YSN1* | ORF, uncharacterized. Sortilin homolog, interacts with proteins of the endocytic machinery. | 17.57 |
| YOR310C | *NOP58* | Protein involved in pre-rRNA processing, 18S rRNA synthesis, and snoRNA synthesis; component of the small subunit processosome complex, which is required for processing of pre-18S Rrna. | 15.88 |
| YLR175W | *CBF5* | Pseudouridylate synthase activity. Major low affinity 55 kDa centromere/microtubule binding protein. | 12.20 |
| YDR055W | *PST1* | Cell wall protein that contains a putative GPI-attachment site; secreted by regenerating protoplasts; up-regulated by activation of the cell integrity pathway, as mediated by Rlm1p; upregulated by cell wall damage via disruption of FKS1. | 11.34 |
| YER070W | *RNR1* | Ribonucleotide-diphosphate reductase (RNR), large subunit; the RNR complex catalyzes the rate-limiting step in dNTP synthesis and is regulated by DNA replication and DNA damage checkpoint pathways via localization of the small subunits. | 11.18 |
| YHR053C | *CUP1-1* | Ion binding: metallothionein, binds copper and mediates resistance to high concentrations of copper and cadmium; locus is variably amplified in different strains, with two copies, CUP1-1 and CUP1-2, in the genomic sequence reference strain S288C. | 11.10 |
| YEL026W | *SNU13* | Pre-mRNA splicing factor activity. Part of small (ribosomal) subunit (SSU) processosome (contains U3 snoRNA); RNA binding protein (putative), similar to Nhp2p; U4/U6.U5 snRNP component. | 9.91 |
| YER131W | *RPS26B* | Structural constituent of ribosome: protein component of the small (40S) ribosomal subunit; nearly identical to Rps26Ap and has similarity to rat S26 ribosomal protein. | 9.35 |
| YDL083C | *RPS16B* | Structural constituent of ribosome: protein component of the small (40S) ribosomal subunit; identical to Rps16Ap and has similarity to *E. coli* S9 and rat S16 ribosomal proteins. | 9.33 |
| YDL208W | *NHP2* | RNA binding. HMG-like protein. | 8.86 |
| YPL226W | *NEW1* | This gene encodes a protein with an Q/N-rich amino terminal domain that acts as a prion, termed [NU]+. | 8.63 |
| YKR077W | – | Hypothetical ORF. | 8.35 |
| YKL109W | *HAP4* | Transcriptional activator protein of CYC1 (component of HAP2/HAP3 heteromer). Subunit of the heme-activated, glucose-repressed Hap2p/3p/4p/5p CCAAT-binding complex, a transcriptional activator and global regulator of respiratory gene expression; provides the principal activation function of the complex. | 8.22 |
| YLR300W | *EXG1* | Major exo-1,3-beta-glucanase of the cell wall, involved in cell wall beta-glucan assembly; exists as three differentially glycosylated isoenzymes. | 8.12 |
| YJL177W | *RPL17B* | Structural constituent of ribosome: protein component of the large (60S) ribosomal subunit, nearly identical to Rpl17Ap and has similarity to *E. coli* L22 and rat L17 ribosomal proteins. | 8.07 |
| YLR197W | *SIK1* | Component of the small (ribosomal) subunit (SSU) processosome that contains U3 snoRNA; similar to microtubule binding proteins. | 8.04 |
| YKL185W | *ASH1* | Zinc-finger inhibitor of HO transcription; mRNA is localized and translated in the distal tip of anaphase cells, resulting in accumulation of Ash1p in daughter cell nuclei and inhibition of HO expression; potential Cdc28p substrate. | 7.98 |

This list includes the 20 most over expressed genes in the recombinant yeast compared to the wild type strain. The average fold-increase in mRNA levels measured in the three experiments is indicated.

**Table 8**
Genes repressed by SOD expression in stationary phase.

| ID | Gene | Description | Fold-decrease |
|---|---|---|---|
| YCR010C | *ADY2* | Transmembrane protein. Acetate transporter required for normal sporulation. Enables the directed movement of acetate into, out of, within or between cells. | 131.72 |
| YDR384C | *ATO3* | Transporter activity. Transmembrane protein. Plasma membrane protein, regulation pattern suggests a possible role in export of ammonia from the cell; member of the TC 9.B.33 YaaH family of putative transporters. | 125.81 |
| YOR348C | *PUT4* | Proline specific permease (also capable of transporting alanine and glycine). | 92.69 |
| YKL187C | – | Hypothetical ORF | 62.92 |
| YIL057C | – | Hypothetical ORF | 60.39 |
| YIR019C | *MUC1* | GPI-anchored cell surface glycoprotein required for diploid pseudohyphal formation and haploid invasive growth, transcriptionally regulated by the MAPK pathway (via Ste12p and Tec1p) and the cAMP pathway (via Flo8p). Actin. | 42.73 |
| YER015W | *FAA2* | Long-chain-fatty-acid-CoA ligase activity. Long chain fatty acyl-CoA synthetase; accepts a wider range of acyl chain lengths than Faa1p, preferring C9:0-C13:0; involved in the activation of endogenous pools of fatty acids (fatty acid activator 2). | 35.31 |
| YGL205W | *POX1* | Fatty-acyl coenzyme A oxidase, involved in the fatty acid beta-oxidation pathway; localized to the peroxisomal matrix. | 34.00 |
| YDR256C | *CTA1* | Catalase A, breaks down hydrogen peroxide in the peroxisomal matrix formed by acyl-CoA oxidase (Pox1p) during fatty acid beta-oxidation. | 25.42 |
| YGR052W | *FMP48* | Putative protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies. | 23.99 |
| YER179W | *DMC1* | Meiosis-specific protein related to RecA and Rad51p. Dmc1p colocalizes with Rad51p to discrete subnuclear sites in nuclear spreads during mid prophase, briefly colocalizes with Zip1p, and then disappears by pachytene. Meiosis-specific protein required for repair of double-strand breaks and pairing between homologous chromosomes; homolog of Rad51p and the bacterial RecA protein. | 23.83 |
| YLR411W | *CTR3* | Copper uptake transporter activity. High-affinity copper transporter of the plasma membrane, acts as a trimer; gene is disrupted by a Ty2 transposon insertion in many laboratory strains of *S. cerevisiae*. Actin. | 23.65 |
| YHL032C | *GUT1* | Glycerol kinase, converts glycerol to glycerol-3-phosphate; glucose repression of expression is mediated by Adr1p and Ino2p-Ino4p; derepression of expression on non-fermentable carbon sources is mediated by Opi1p and Rsf1p. | 22.46 |
| YOR084W | *LPX1* | Oleic acid-inducible, peroxisomal matrix localized lipase; transcriptionally activated by Yrm1p along with genes involved in multidrug resistance. | 21.49 |
| YFR015C | *GSY1* | Glycogen synthase with similarity to Gsy2p, the more highly expressed yeast homolog; expression induced by glucose limitation, nitrogen starvation, environmental stress, and entry into stationary phase. | 21.15 |
| YPL201C | *YIG1* | Protein that interacts with glycerol 3-phosphatase and plays a role in anaerobic glycerol production; localizes to the nucleus and cytosol. | 21.11 |
| YBR067C | *TIP1* | Major cell wall mannoprotein with possible lipase activity; transcription is induced by heat- and cold-shock; member of the Srp1p/Tip1p family of serine–alanine-rich proteins. | 21.05 |
| YNL194C | – | Hypothetical ORF | 20.52 |
| YKR009C | *FOX2* | Multifunctional enzyme of the peroxisomal fatty acid beta-oxidation pathway; has 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities. | 20.37 |
| YILWTY3-1 | – | Retrotransposon, full length Ty3. TyB Gag-Pol protein; proteolytically processed to make the Gag, RT, PR, and IN proteins that are required for retrotransposition. | 19.12 |

This list includes the 20 most under-expressed genes in the recombinant yeast compared to the wild type strain. The average fold-decrease in mRNA levels measured in the three experiments is indicated.

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evidence of the metabolic burden on the recombinant strain at the genetic level which was also observed in MFA.

A slightly different situation is observed in the pentose phosphate (PPP) and aminoacid synthesis pathways (Fig. 8B (Appendix)). Here a smaller number of genes is underexpressed in P+ compared to P− (51 genes, 64%) and the levels of under and overexpression are very small.

Tables 5 and 6 show the 20 genes most overexpressed and underexpressed in P+ compared to P− when growing on ethanol.

### 3.4. Genes under and overexpressed in the early stationary phase

Fig. 9A and B (Appendix) show the ratios of the gene expression levels (fold change) in the recombinant strain in relation to the non-recombinant or native strain (P+/P−). Fig. 9A (Appendix) corresponds to the central metabolic pathways and Fig. 9B (Appendix) the PPP and biosynthetic pathways. Fig. 9 (Appendix) shows that similarly to the comparison of the two strains (P+/P−) on glucose and ethanol (Figs. 6 and 8(Appendix)) a large number of genes are underexpressed in the P + (recombinant) strain in the central pathways (68 genes, 83%) and in the pentose phosphate and biosynthetic pathways (65%). Similarly to growth on ethanol it can be seen that the levels of underexpression are relatively low and for the genes that show overexpression these values are even smaller and very close to 1.0. Protein synthesis without consideration of SOD was extremely small in both strains but SOD synthesis was very high in P+. Ethanol consumption was ca. 80%.

Compared to the previous two cases (glucose and ethanol) gene *pdc5* was not the one most underexpressed, but was amongst the 5 genes most underexpressed (12 fold). Again, genes involved in the reaction of UDP-GLU to glycogen fall into this category, genes *gsy1*, *glg1* and *gsyz* (22 and 5.4 fold respectively) as well as those involved in the conversion of UDP-GLU to trehaloses: genes tsl1 and *tpsz* (5.4 and 4.4 fold respectively). The gene that was most underexpressed in P+ was *gut1* (22.5 fold), a very important gene in glycerol consumption. This is related to the lowering of the rate of glycerol consumption in the early stationary phase in the study of MFA. Other genes under expressed in P+ are those related to TCA cycle activity: *cit3* (13 fold), *idh2* (9 fold) and *icl2* (9.5 fold).

A slightly different situation is observed in the pentose phosphate (PPP) and in the aminoacid synthesis pathways (Fig. 9B(Appendix)). Here a smaller number of genes is underexpressed in P+ (52 genes, 65%) and the levels of under and overexpression are very small. Tables 7 and 8 show the 20 genes most overexpressed and underexpressed in P+ compared to P− in the early stationary phase.

Regarding recombinant protein synthesis, the system used is constitutive, hence an inducible system which would not show a "recombinant burden" on the cell in the earlier phases of cell growth, would probably be more advantageous in terms of rate and yield of recombinant protein synthesis.

## 4. Conclusions

The results show that it is possible to clearly distinguish between two strains of *S. cerevisiae,* based on gene expression data. The recombinant strain synthesizes a large amount of the recombinant protein (SOD) constitutively. Previously DeRisi et al. (1997) showed the change in gene expression for *S. cerevisiae* during the diauxic shift from fermentative growth to respiration. This paper shows the changes in the recombinant and non-recombinant strains from fermentative growth to respiration and from this to the early stationary phase and compares the behaviour of both strains in these phases.

When comparing the two strains in the 3 growth stages, 4 genes were found to be under or overexpressed in all cases (genes *ctr3, muc1, pdr15* and *pst1*). The products of all of these genes are expressed at the plasma membrane or cell wall of the yeast. Gene *ctr3* is involved in the incorporation of copper ions into the cell and is strongly underexpressed in the P+ strain. The *pdr15* gene is associated with stress response, particularly detoxification of the cell (Wolfger et al., 2004).

Comparing the gene expression for P− (and to a large extent P+) during growth on ethanol to that on glucose (Eth/Gluc), overexpression is related to an increase in consumption of glycerol, activation of the TCA cycle (e.g. genes *cit3, fum1, idh2, mdh2*), degradation of glycogen (*gdb1*) and metabolism of ethanol (*adh1*). Furthermore, in the diauxic shift from glucose to ethanol 97.6% of genes (80 genes) involved in the central metabolic pathway are overexpressed. This is similar to that observed by DeRisi et al. (1997) but different from what has been observed for MFA of P− (and also P+), where the specific growth rate is lowered to ca. 40%, the fluxes in the TCA cycle are reduced to ca. 40% (to 30% in P+), glycolysis is reduced to virtually 0 and protein synthesis to ca. 50% (to 40% in P+). It clearly shows that it is not possible to correlate, in a simple or direct way, quantitative mRNA expression levels with cell function which is shown by the MFA.

Comparing the gene expression for P− (and to some extent P+) during the early stationary phase to the growth on ethanol (Stat/Eth), underexpression is generalized as the biosynthetic machinery of the cell shuts down e.g. genes *icl1*, isocitrate lyase important in the glyoxylate cycle. Again, this is different from the behaviour observed for MFA where ethanol was still consumed at a rate of ca. 30% of that in the exponential phase on ethanol and fluxes in the TCA cycle were somewhat higher than in the ethanol phase and ca. 60% of those observed during exponential growth on glucose both for P− and P+. This shows that the switch in metabolism between ethanol and early stationary phases has an almost instantaneous effect on gene expression but a much more retarded effect on metabolic fluxes and that the "early stationary" phase represents a "late ethanol" phase from the metabolic analysis point of view since ethanol is still present and being consumed although at a much slower rate. MFA does constitute an oversimplification of the metabolism as only 78 reactions are being considered in this model.

Comparing the strains (P+/P−) when growing on glucose or ethanol most of the genes of the central metabolic pathways (53 genes, 65%) are underexpressed in P+. However, the values of underexpression are very much smaller than those observed when different growth phases are compared for each of the two strains. In the early exponential phase a large number of genes are underexpressed in the P+ (recombinant) strain in the central pathways (68 genes, 83%) and in the pentose phosphate and biosynthetic pathways (65%). Similarly to the behaviour on glucose and on ethanol it can be seen that the levels of underexpression are relatively low and for the genes that show overexpression these values are even smaller and very close to 1.0.

Comparing the strains (P+/P−) when growing on glucose, in P+ there is underexpression of genes involved in the step UDP-GLU to glycogen e.g. genes *gsy1, glg1* and *gsy2*. Comparing the strains (P+/P−) when growing on ethanol, in P+ there is underexpression in many genes in the central pathways indicating a decrease in respiratory metabolism compared to P−. When growing on ethanol, the PPP and amino acid biosynthesis pathways show repression of genes important in the synthesis of glutamate, glutamine, proline and glycine. This is evidence that there will be less protein synthesis in P+ compared to P−. Comparing the P+/P− strains in the stationary phase, many genes are repressed in P+ which are the same as in glucose and ethanol phases e.g. those involved in the conversion of UDP-GLU to glycogen and UDP-GLU to trehaloses.

Regarding recombinant protein synthesis, the system used is constitutive, hence an inducible system which would not show a "recombinant burden" on the cell in the earlier phases of cell growth, would probably be more advantageous in terms of rate and yield of recombinant protein synthesis.

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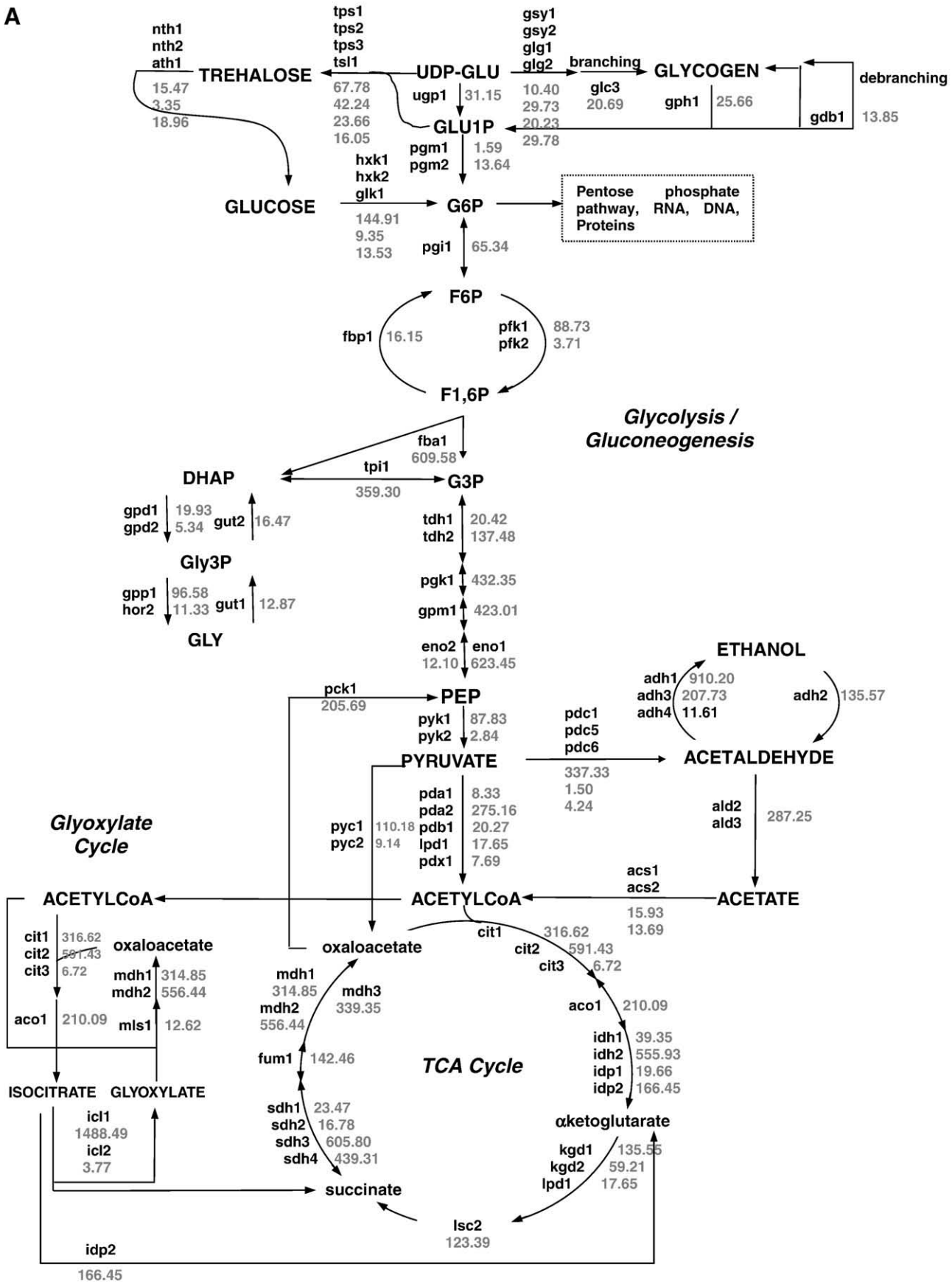
**Appendix A**

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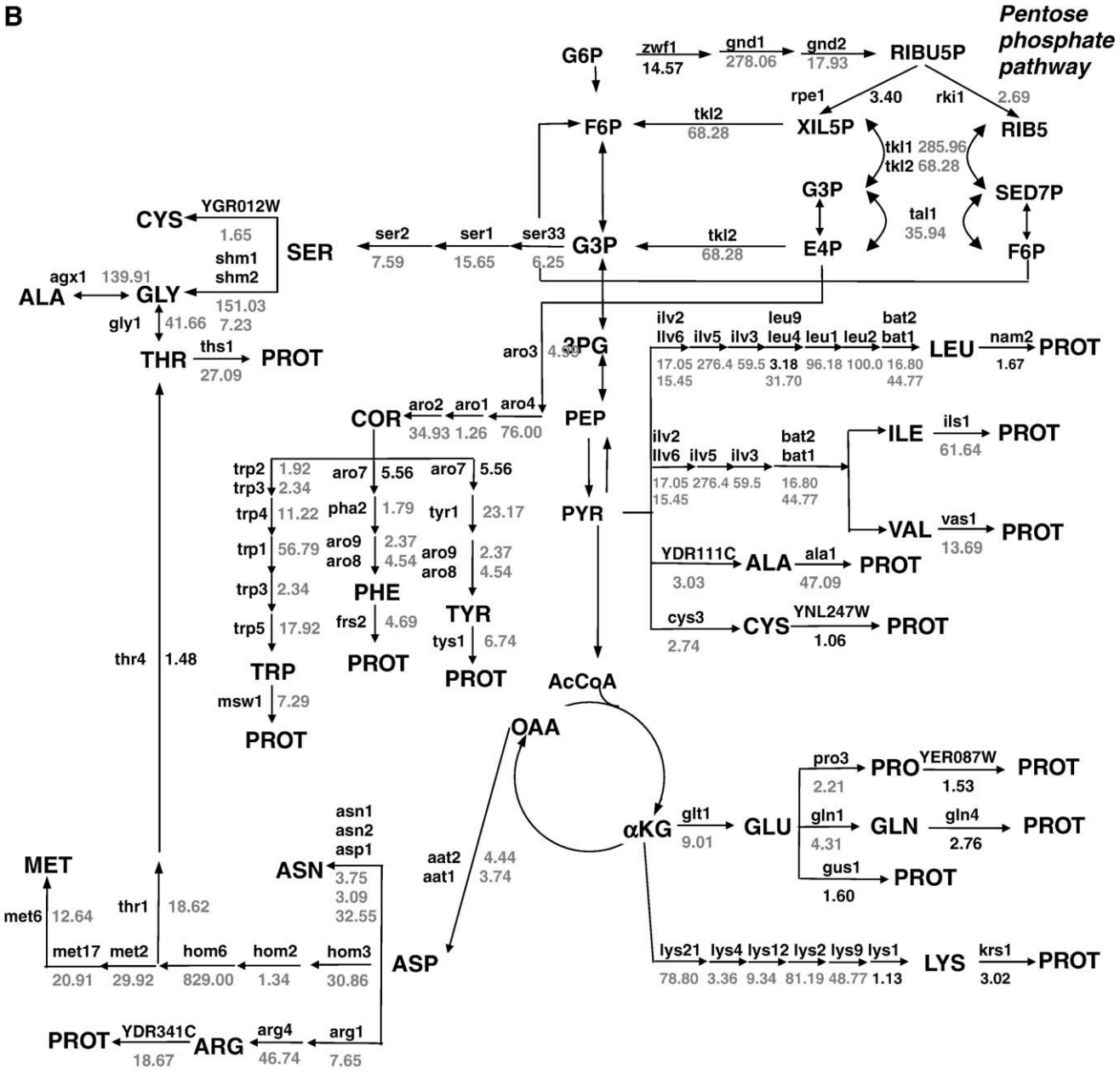


**Fig. 7**. A. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) growth from ethanol to stationary phase ethanol (Sta/Eth), of the central metabolic pathways. Names of the genes are shown on the arrows. The abbreviations correspond to: uridine biphosphoglucose (UDP-GLU), glucose-1-phosphate (GLU1P), glucose-1-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-phosphate (F1,6P), glyceraldehyde-3-phosphate (G3P) and phosphoenolpiruvate (PEP). Bold represents genes whose expression increases in the recombinant yeast when grown on ethanol and grey, genes whose expression is decreased in this phase. B. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) growth from ethanol to stationary phase ethanol (Sta/Eth), of the pentose phosphate pathway and the most important biosynthetic pathways. The abbreviations correspond to: 3-phosphoglycerate (3PG), phosphoenolpyruvate (PEP), pyruvate (PYR), acetyl-CoA (AcCoA), corismate (COR), ribose-5-phosphate (RIB5P), ribulose-5-phosphate (RIBU5P), xilulose-5-phosphate (XIL5P), pseudoheptulose-7-phosphate (SED7P), eritrose-5-phosphate (E4P), oxaloacetate (OAA), α-ketoglutarate (αKG) and proteins (PROT). Symbols of three letters have been used for the amino acids (blue). Other abbreviations as in Fig. 7A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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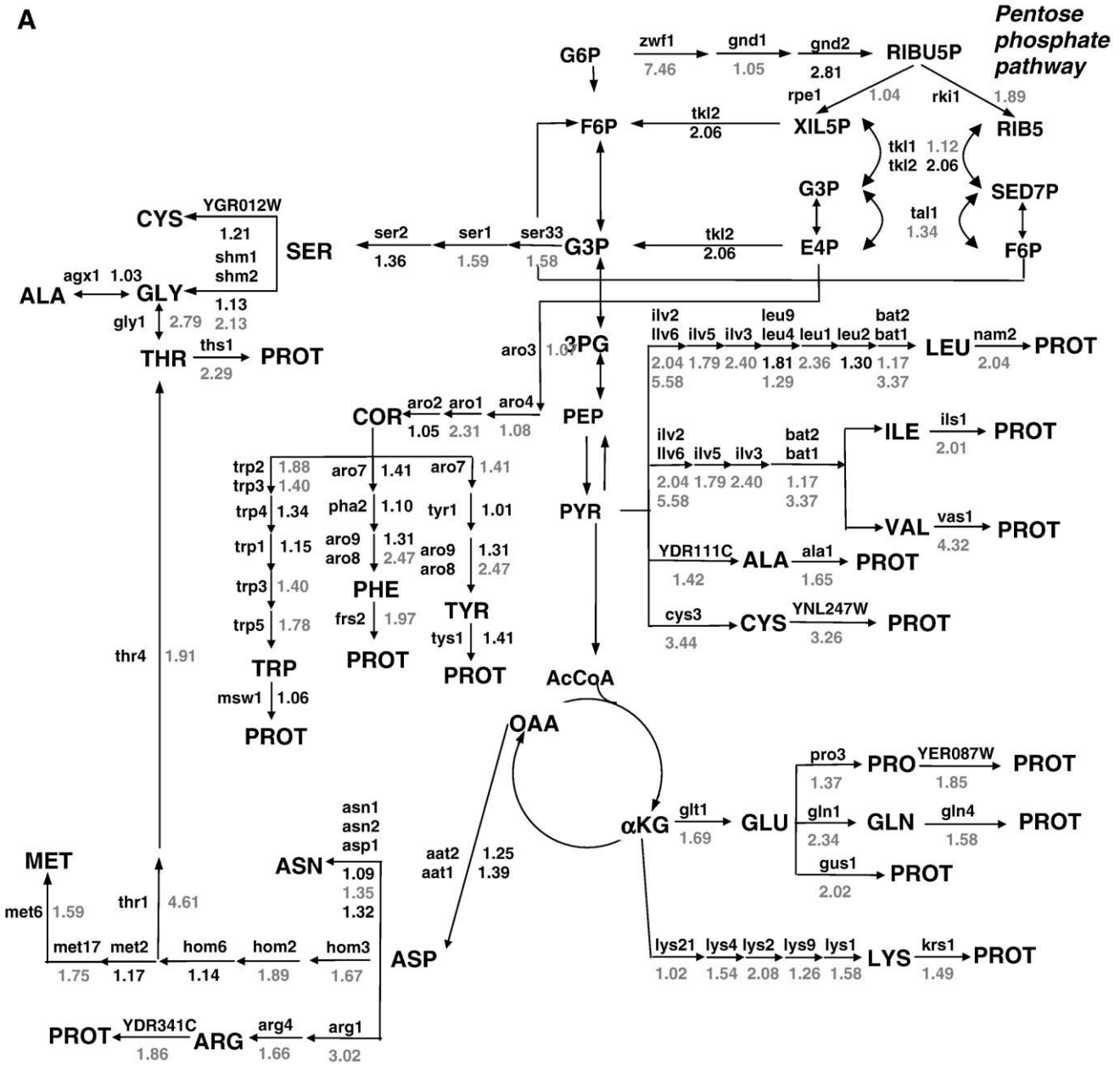


**Fig. 8.** A. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) compared to the wild type strain (P−) growth on glucose (P+/P−), of the pentose phosphate pathway and the most important biosynthetic pathways. Abbreviations as in Fig. 7A and B. B. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) compared to the wild type strain (P−) growth on ethanol (P+/P−), of the pentose phosphate pathway and the most important biosynthetic pathways. Abbreviations as in Fig. 7A.

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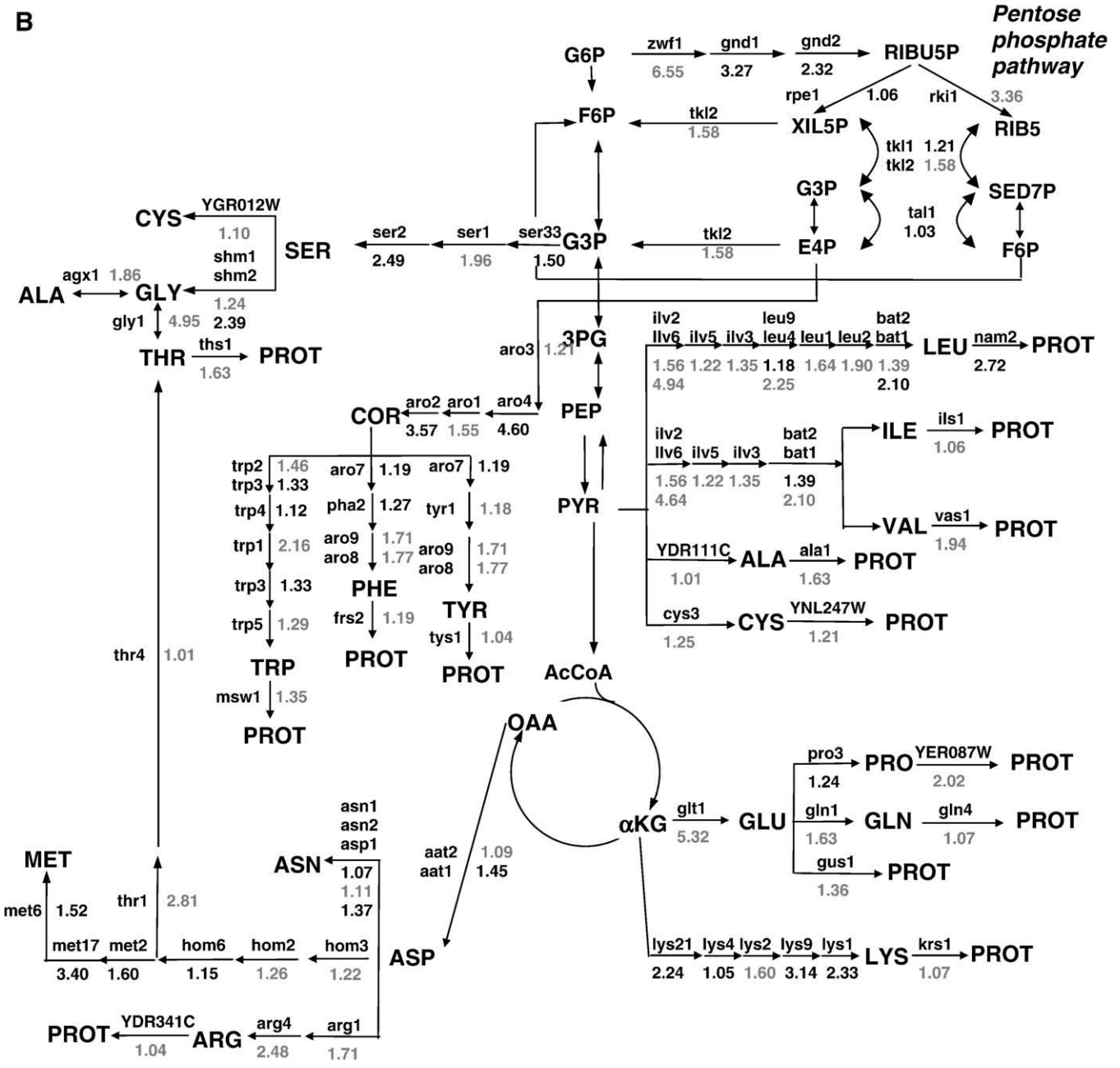


**Fig. 8** (*continued*).

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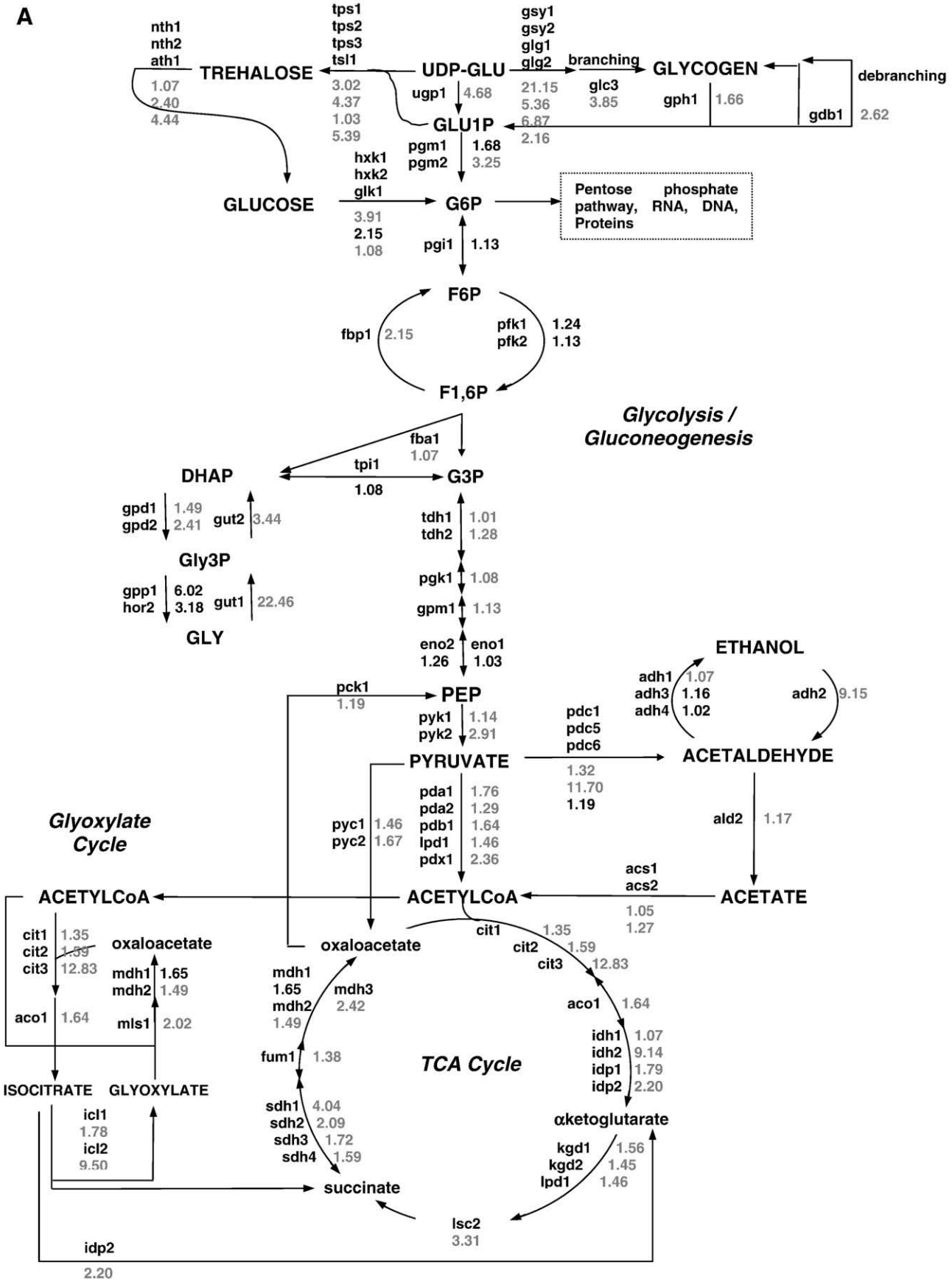


**Fig. 9.** A. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) compared to the wild type strain (P−) growth in stationary phase (P+/P−), of the central metabolic pathways. Abbreviations as in Fig. 7A. B. Ratios of the levels of transcribed (fold change) of the genes of the recombinant yeast (P+) compared to the wild type strain (P−) growth in stationary phase (P+/P−), of the pentose phosphate pathway and the most important biosynthetic pathways. Abbreviations as in Fig. 7A and B.

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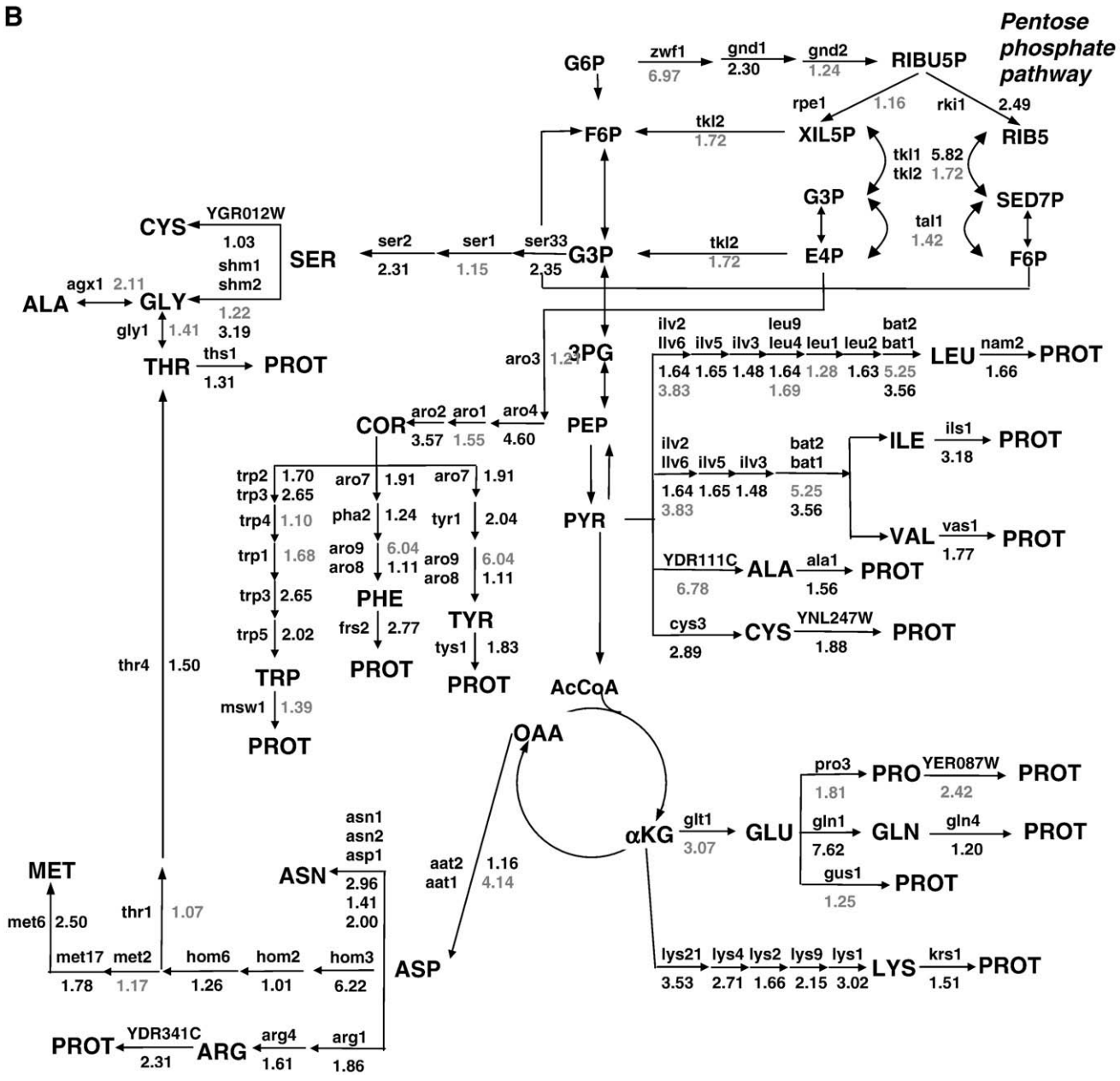


**Fig. 9** (*continued*).

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