Comparative Proteome Analysis of *Saccharomyces cerevisiae* Grown in Chemostat Cultures Limited for Glucose or Ethanol*

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The use of chemostat culturing enables investigation of steady-state physiological characteristics and adaptations to nutrient-limited growth, while all other relevant growth conditions are kept constant. We examined and compared the proteomic response of wild-type *Saccharomyces cerevisiae* CEN.PK113-7D to growth in aerobic chemostat cultures limited for carbon sources being either glucose or ethanol. To obtain a global overview of changes in the proteome, we performed triplicate analyses using two-dimensional gel electrophoresis and identified proteins of interest using MS. Relative quantities of about 400 proteins were obtained and analyzed statistically to determine which protein steady-state expression levels changed significantly under glucose- or ethanol-limited conditions. Interestingly, only enzymes involved in central carbon metabolism showed a significant change in steady-state expression, whereas expression was only detected in one of both carbon source-limiting conditions for 15 of these enzymes. Side effects that were previously reported for batch cultivation conditions, such as responses to continuous variation of specific growth rate, to carbon-catabolite repression, and to accumulation of toxic substrates, were not observed. Moreover, by comparing our proteome data with corresponding mRNA data, we were able to unravel which processes in the central carbon metabolism were regulated at the level of the proteome, and which processes at the level of transcriptome. Importantly, we show here that the combined approach of chemostat cultivation and comprehensive proteome analysis allowed us to study the primary effect of single limiting conditions on the yeast proteome. *Molecular & Cellular Proteomics 4:1–11, 2005.*

Two-dimensional (2D)[1] gel electrophoresis is a powerful tool to visualize hundreds of proteins at a time, which in combination with MS leads to their identification. This technology has been applied to the yeast *Saccharomyces cerevisiae* for the large-scale identification of more than 400 proteins, resulting in yeast reference maps (1–7). Other *S. cerevisiae* studies used 2D gel electrophoresis to obtain an overview of global changes in the yeast proteome as function of stimuli such as cadmium (8), lithium (9), H_2O_2 (10), or sorbic acid (11).

Most of these differential proteome studies on *S. cerevisiae* were performed on cells cultured in batch mode, *i.e.* in shake flasks or in reactors. Batch cultivation makes use of a closed system, in which all nutrients are in excess at the start of the cultivation. In terms of microbial physiology, such batch cultivation is relatively poorly controlled, because the composition of the growth medium and consequently the growth rate changes continuously. The yeast cells take up nutrients from the media while metabolites are excreted in the culture system, and their growth arrests when one of the nutrients is depleted or when too many toxic substrates are accumulated. The high concentration of carbon source in the culture, which is essential for this type of cultures, may lead to carbon-catabolite repression (12). The specific growth rate μ is directly affected by these continuous changes and is only constant during the exponential growth phase. Changes in specific growth rate are known to have a high impact on gene expression in *S. cerevisiae* (13–15) and thus also on the yeast proteome. Although most studies concern batch-cultured yeast cells that are collected from the exponential growth phase, their proteome analyses may not only reveal the primary effect of interest, but may additionally reflect the aforementioned effects.

A few research groups used chemostat cultivation for yeast proteome analysis (see for example Refs. 16 and 17), an approach that avoids growth rate-dependent changes and thus allows investigation of the effect of *e.g.* a single nutrient limitation at a fixed growth rate. A chemostat culture is continuously fed with fresh media at a constant rate, and the volume in the chemostat vessel is kept constant by continuous, constant-rate removal of culture fluid that contains yeast cells, spent media, and metabolites. As a result, the specific growth rate μ of the culture can be fixed and the dilution rate can be controlled accurately. A steady-state condition is

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[1] The abbreviation used is: 2D, two-dimensional.

achieved when the total number of cells and the total volume in the chemostat vessel remain constant (18). In a sugar-limited chemostat culture, the carbon source is almost completely consumed, resulting in very low residual concentrations and therefore avoiding carbon-catabolite repression. Moreover, the very low residual concentrations avoid accumulation of toxic substrates. The growth medium in a chemostat is designed such that only one single nutrient limits the growth, while all other nutrients are present in excess. Because growth of microorganisms like *S. cerevisiae* in their natural environment and also in many industrial applications (*e.g.* industrial production of bakers' yeast) is generally limited by nutrient availability, the effect of nutrient limitation on the yeast proteome can be optimally studied using chemostat cultures. Thus chemostat cultivation enables proteome-wide investigation of the effect of one particular nutrient limitation at a fixed growth rate, while all other growth parameters are kept constant.

The aim of the present study is to investigate the carbon source-dependent response on the proteome of *S. cerevisiae*. Aerobic chemostat cultures were used, which were limited for the carbon sources glucose or ethanol, allowing analysis of the protein expression levels under glycolytic and gluconeogenic conditions, respectively. It has been shown by Piper *et al.* (19) that this culturing approach provides highly reproducible results at the level of transcriptome analyses, not only between independent chemostat cultures at one laboratory, but also between two different laboratories. However, as has been suggested by Daran-Lapujade *et al.* (20), who compared genome-wide transcript levels with *in vivo* fluxes for glucose- and ethanol-limited chemostat cultures, control of the central carbon pathways takes place to a large extent via post-transcriptional mechanisms. Therefore, we performed a comparative proteome analysis of the wild-type *S. cerevisiae* CEN.PK113-7D, using 2D gel electrophoresis followed by MS for protein identification. Triplicate analyses allowed adequate statistical analysis, resulting in appropriate relative quantitative analysis of protein expression of glucose- *versus* ethanol-limited conditions. To investigate which of the genes involved in the central carbon metabolism are regulated at the level of the transcriptome and which at the level of the proteome, we compared the corresponding expression ratios for equal growth conditions. Our results indicate the importance of using chemostat cultures, as we observed significant effects in steady-state expression levels solely of enzymes involved in the central carbon metabolism. By using the combination of chemostat cultivation and comprehensive proteome analysis, we show that we have a unique possibility to study the effect of single limiting conditions on the yeast proteome.

EXPERIMENTAL PROCEDURES

Strain and Growth Conditions—Wild-type *Saccharomyces cerevisiae* strain CEN.PK113-7D (*MAT***a**) (21) was grown at 30 °C in 2-liter chemostats (Applikon, Schiedam, The Netherlands), with a working volume of 1.0 liter as described (22). Cultures were fed with a defined mineral medium that limited growth by glucose or ethanol with all other growth requirements in excess. The dilution rate was set at 0.10 h^{-1}. The pH was measured online and kept constant at 5.0 by the automatic addition of 2 M KOH with the use of an Applikon ADI 1030 biocontroller. Stirrer speed was 800 rpm, and the airflow was 0.5 liters·min^{-1}. Dissolved oxygen tension was measured online with an Ingold model 34-100-3002 probe (Mettler Toledo, Greifensee, Switzerland) and was between 60 and 75% of air saturation. The off-gas was cooled by a condenser connected to a cryostat set at 2 °C and analyzed as previously described (23). Steady-state samples were taken after ~10–14 volume changes to avoid strain adaptation due to long-term cultivation (24). Dry-weight, metabolite, dissolved oxygen, and off-gas profiles had to be constant over at least 5 volume changes prior to sampling for protein extraction. Culture dry weights were determined via filtration as described by Postma *et al.* (25) The defined mineral medium composition was based on that described by Verduyn *et al.* (26). The carbon source was 256 ± 19 mmol of carbon per liter.

Protein Extraction—*S. cerevisiae* protein extracts were prepared for analysis with 2D gel electrophoresis using a combined approach of Boucherie *et al.* (5) and Harder *et al.* (27). In brief, yeast cells were lyophilized prior to protein extraction. Between 65 and 75 mg dry-weight of yeast cells was used as starting material for protein extraction. Glass beads (acid washed, 425–600 μm; Sigma, St. Louis, MO) were added to the lyophilized yeast cells. Cells were disrupted by vortexing six times 60 s. The samples were cooled on ice for 30 s in between the vortex steps. After cell lysis, the yeast cells were resuspended in 650 μl of hot (95 °C) SDS sample buffer (0.1 M Tris-HCl, pH 7.0, 1.0% (w/v) SDS supplemented with protease inhibitors (Complete Protease Inhibitor Mixture Tablets; Roche Diagnostics, Somerville, NJ). The sample was boiled for 10 min and subsequently cooled on ice. Subsequently, 75 μl of a DNase and RNase solution (1% (w/v) DNase I, 0.25% (w/v) RNase A, 50 mM $MgCl_2$, 0.5 M Tris-HCl, pH 7.0) was added and incubated on ice. The sample was diluted by adding 2.0 ml of a solubilization buffer containing 2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 2.5% (w/v) DTT, 2% (v/v) carrier ampholytes, pH 3–10 nonlinear, and protease inhibitors. The samples were shaken for 1 h on a Roller mixer SRT2 (Merck Eurolab B. V., Amsterdam, The Netherlands) at room temperature followed by clearing through centrifugation at 3,000 × *g*. Protein concentration was determined with a Bradford protein assay (Bio-Rad, Hercules, CA) using BSA as a standard. The cleared supernatants were stored in aliquots at −80 °C.

2D Gel Electrophoresis—For the first dimension, an amount of 150 μg of protein was loaded on a 13 cm Immobiline Dry-Strip pH 3–10 NL (Amersham Biosciences, Piscataway, NJ) in 250 μl sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2.5% (w/v) DTT, and 2% (v/v) carrier ampholytes, pH 3–10 nonlinear, and protease inhibitors. Rehydration and isoelectric focusing were carried out using an IPGphor (Amersham Biosciences). Strips were rehydrated for 13–15 h at 30 V, followed by IEF, with the current limited to 100 μA per strip, at 20 °C, for a total of 40–45 kVh (1 h at 500 V, 1 h at 1,500 V followed by 8,000 V for 38–43 kVh). Prior to the second dimension, the IPG strips were incubated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS) containing 1% (w/v) DTT, followed by 15 min incubation in equilibration buffer containing 2.5% (w/v) iodoacetamide. Second-dimension electrophoresis was performed on laboratory-cast 12.5% polyacrylamide gels in a Hoefer SE600 system (Amersham Biosciences). The IPG strips were placed on top of 12.5% polyacrylamide gels and sealed with a solution of 1% (w/v) agarose containing a trace of bromphenol blue. Gels were run at 10 mA per gel for 15 min followed by 20 mA per gel until the bromphenol blue had migrated to the bottom of the gel. Proteins were visualized using silver staining as described by Shevchenko *et al.* (28). The silver-stained gels were scanned using a

GS-710 Calibrated Imaging Densitometer (Bio-Rad).

Experimental Design, Image Analysis, and Statistics—For each growth condition, namely for glucose- and ethanol-limitation, 2D gels were run in triplicate. Additionally, a master 2D gel was prepared, which contained a 1:1 mixture of the protein extract of the ethanol- and glucose-limited yeast cultures. In theory, this master 2D gel should contain all protein spots present on the ethanol- and the glucose-limited 2D gels and was used during image analysis as a master gel. Image analysis was performed using the PDQuest 7.1.0 software package (Bio-Rad). Normalization of spot volumes in a gel was performed using the "total density in valid spots" option. The quantitative and statistical analyses were performed using suitable functions within the PDQuest software and Excel software (Microsoft, Redmond, WA). The normalized intensity of spots on three replicate 2D gels was averaged and the standard deviation was calculated for each condition. The relative change in protein abundance for ethanol-*versus* glucose-limitation (indicated with "fold change E/G") for each protein spot was calculated by dividing the averaged normalized spot quantity from the ethanol gels by the averaged normalized spot quantity from the glucose gels. A two-tailed nonpaired Student's *t*-test was performed to determine if the relative change was statistically significant. The faintest spot that was detected had an intensity of 250 ppm, and if a spot was below this detection level for one growth condition while (far) above 250 ppm in the other condition, this was considered as a significant change in expression. In those situations the ratio "fold change E/G" could not be calculated and was thus indicated with E (*i.e.* only expression under ethanol limitation) or G (*i.e.* only expression under glucose limitation).

In-gel Tryptic Digestion—Protein spots of interest were excised and in-gel digested with trypsin with a slightly modified protocol as described by Wilm *et al.* (29). In brief, the gel pieces were first destained using 30 mM potassium ferricyanide and 100 mM sodium thiosulphate solution, followed by washing and shrinking steps using 50 mM ammonium bicarbonate and ACN, respectively. Proteins were digested overnight at 37 °C by adding trypsin at a concentration of 10 ng/μl.

MALDI-MS—After tryptic digestion, peptides were concentrated and desalted using a ZipTip μ-C18 (Millipore, Bedford, MA). Peptides were eluted directly on the MALDI-target with 1 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% ACN and 0.1% (v/v) TFA. Peptides were analyzed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems) using delayed extraction in positive reflectron mode at 20 kV accelerating voltage.

Nano-LC-MS/MS—Nano-LC-MS/MS was performed by coupling an Ultimate HPLC (LC Packings) to an ESI Q-TOF instrument (Micromass UK Ltd., Manchester, UK), operating in positive ion mode and equipped with a Z-spray nano-ESI source as described before (30). Briefly, peptides were delivered to a trap column (Aqua^{TM} C18RP (Phenomenex, Torrance, CA); 15 mm $\times$ 100 μm inner diameter) at 5 μl/min by using a Famos autosampler (LC Packings, Amsterdam, The Netherlands) (31). After reducing the flow to ~150 nl/min by a splitter, the peptides were transferred to the analytical column (PepMap C18 (LC Packings); 20 cm $\times$ 50 μm inner diameter). The peptides were eluted with a linear gradient from 0–50% buffer B (0.1 M acetic acid in 80% (v/v) ACN) in 30 min. The column eluent was sprayed directly into the ESI source of the mass spectrometer *via* a butt-connected nano-ESI emitter (New Objectives, Woburn, MA). Peptides were fragmented in data-dependent mode.

Protein Identification—Proteins were identified using MASCOT software (www.matrixscience.com), and searches were performed using the Swiss-Prot or NCBInr database. The following search parameters were used: trypsin was used as enzyme, the peptide tolerance window was set to 100 ppm, one missed cleavage was allowed, and carbamidomethyl and oxidized methionine were set as fixed and variable modification, respectively.

RESULTS

To investigate the effect of carbon source limitation at the level of *Saccharomyces cerevisiae* protein expression, a comparative proteome analysis was performed using aerobic chemostat cultures with glucose or ethanol as single growth-limiting nutrient, respectively. The dilution rate in both cultures was equal, namely 0.1 h^{-1}. Under these conditions, the concentration of the carbon sources in the reservoir medium was *ca.* 250 mmol of carbon per liter, whereas their residual concentration in steady-state cultures were below their detection limits (*i.e.* less than 0.5 mM). In the glucose-limited culture, no ethanol was produced, and cells grew with a biomass yield on glucose of 0.5 g·g^{-1}, reflecting complete respiratory catabolism. This is typical for steady-state growth of *S. cerevisiae* strain CEN.PK113-7D under glucose limitation at dilution rates below 0.3 h^{-1} (32). In the ethanol-grown culture, over 95% of the substrate carbon was recovered, as either biomass or carbon dioxide and HPLC analysis of culture supernatants did not reveal the production of any low-molecular-mass metabolites.

Yeast cells from both cultures were harvested and proteins were extracted using the extraction protocol developed by Harder *et al.* (27), which we further optimized for our yeast samples. In Harders' protocol, yeast cells are sonicated in buffer containing SDS to disrupt the cell walls and dissolve the proteins. In order to improve cell disruption and to minimize proteolysis, we performed an additional step. The yeast cells were lyophilized and subsequently vortexed with glass beads as described by Boucherie *et al.* (5), prior to SDS boiling. Furthermore, protease inhibitors were added to both the solubilization buffer and the hot SDS sample buffer for maximal reduction of endogenous proteolytic enzyme activity. More high-molecular-mass proteins (> 75 kDa) were observed on the 2D gels when this optimized protocol was used.

In Fig. 1, typical 2D gel electrophoresis images of the ethanol-limited (Fig. 1*A*) and the glucose-limited (Fig. 1*B*) yeast cultures are shown. An average of ~400 spots was detected on each 2D gel. The analyses were performed in triplicate to allow proper statistical analysis, *i.e.* a Student's *t*-test was used to determine if the relative change in protein expression for glucose- *versus* ethanol-limitation was statistically significant.

Based on this analysis we could sort differences in steady state protein abundances into three categories, *i.e.* proteins that were detected only in either glucose- or ethanol-limited conditions, proteins that were statistically significantly up- or down regulated, and proteins of which the steady state expression levels were unchanged. The first category consists of spots that were exclusively detected in one nutrient limitation group, *e.g.* a spot is observed in the glucose-limited gels but undetectable in the ethanol-limited gels, or vice versa, which is indicated with G or E in Table I, respectively. In total, 15 of these so-called on/off spots were detected on our 2D gels, typical examples of which are shown in Fig. 1*C*. These

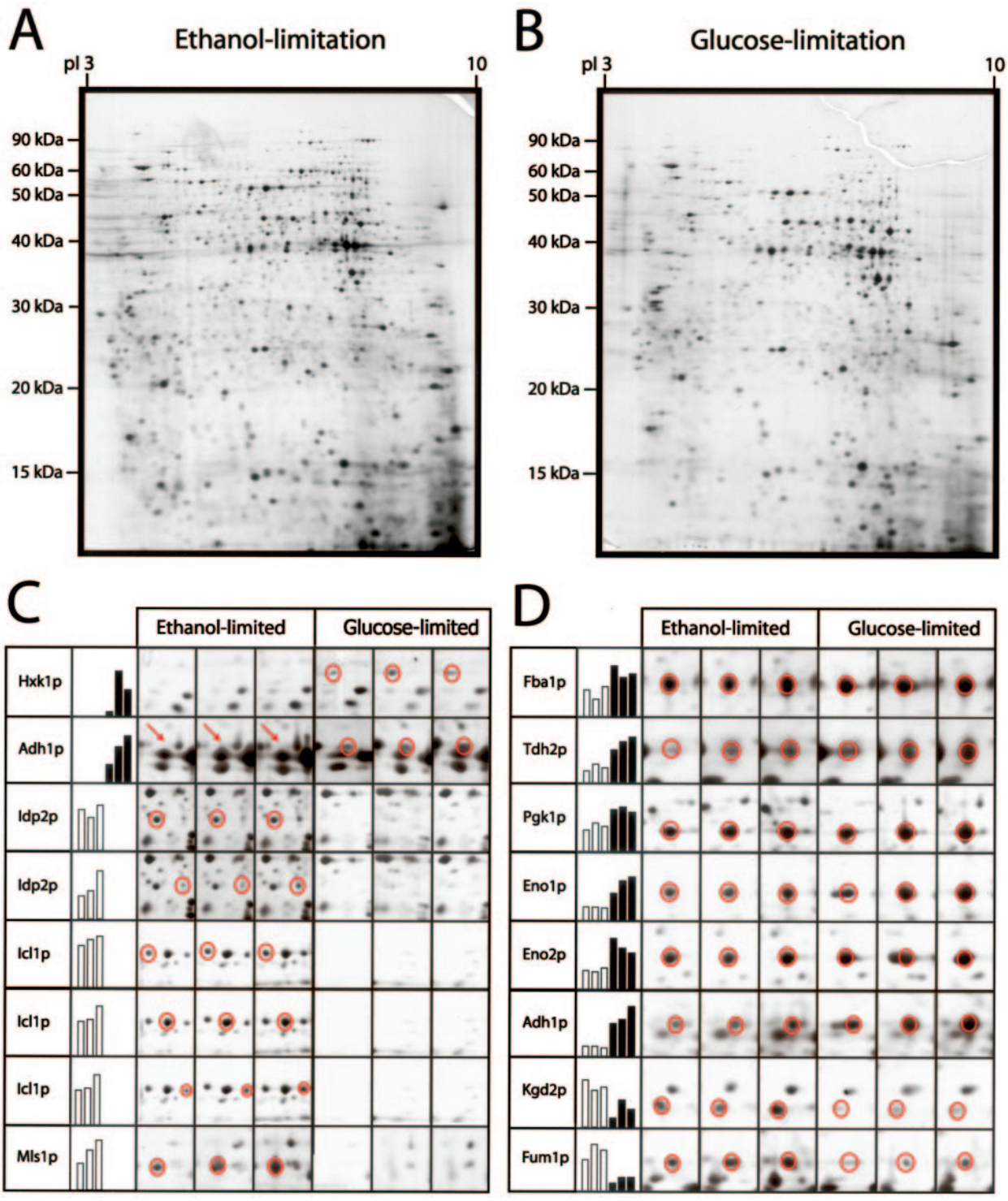

FIG. 1. **Images of the 2D gels of protein extracts from chemostat-grown yeast cells, limited for either ethanol or glucose.** The 2D gels were run of 150 μg of protein extract from yeast cells, which were grown in aerobic chemostat cultures, carbon-limited for ethanol (*A*) or glucose (*B*), respectively. Proteins were visualized by silver staining. In *C* and *D*, magnified regions of triplicate 2D gel images are shown. Protein spots of interest are indicated with a *circle*, with the corresponding protein names pointed out at the *left*. Histograms show the protein abundance, with protein intensity of the 2D gels indicated in black and white, for the glucose- and the ethanol-limited cultures, respectively. *C* shows examples of proteins that were only detected on the 2D gels of one of the nutrient-limited chemostat cultures and were undetectable on the 2D gels of the other nutrient-limited chemostat culture. A number of other statistically significant changes in protein expression levels between the ethanol-limited and glucose-limited yeast chemostat culture are given in *D*.

spots were further analyzed with MS, resulting in identification of 11 unique proteins, namely Hkx1p, Pda1p, Adh1p, Cit1p, Idp2p, Lsc2p, Sdh1p, Mdh2p, Icl1p, Mls1p, and Pck1p, whereas some of these spots were revealed to originate from the same protein (protein isoforms, Table I). Interestingly, all proteins from these on/off spots play a role in the central carbon metabolism. Furthermore, we searched for the other proteins that are known to play a role in the central carbon metabolism, thus we compared our 2D images with yeast proteome maps available on the internet (www.ibgc.-bordeaux2.fr/YPM/, www.expasy.org/cgi-bin/map2/def?Yeast) to spot the location of proteins of interest and confirmed the identities of these spots using MS (examples are presented in Fig. 1*D*). All identified and quantified central carbon metabolism proteins are listed in Table I, and their relative expression changes are depicted in Fig. 2, in which the central carbon metabolism proteins are put into four different categories, namely glycolytic enzymes (gray), enzymes that convert pyruvate to ethanol and acetyl-CoA and *vice versa* (white), enzymes from the TCA cycle (black), and enzymes from gluconeogenesis and glyoxylate cycle (hatched). This figure clearly shows the clustering of the enzymes involved in pathways of the central carbon metabolism, which are expected to change upon different carbon source limitation.

In total, 44 spots of proteins that play a role in the central carbon metabolism were relatively quantified and identified. As can be concluded from Table I, 15 protein spots were exclusively detected in either glucose- or ethanol-limited cultures, seven protein spot volumes changed significantly at the 98% confidence level ($p < 0.02$), seven protein spot volumes changed significantly at the 95% confidence level ($p < 0.05$), while two protein spot volumes showed a relative change in expression significant at the 90% confidence level ($p < 0.1$). From the 2D gel image analysis, we learned that the combined spot intensities of all identified glycolytic enzymes increased from 11 to 22% of the total density in all valid spots when comparing ethanol to glucose limitation, respectively. The combined spot intensities of all the identified enzymes from the TCA cycle show a 2-fold increase when comparing the 2D gel images of the samples from the ethanol- with the glucose-limited cultures. Isocitrate lyase (Icl1p) and malate synthase 1 (Mls1p), which are both key enzymes in of the glyoxylate cycle, were solely detected when ethanol was used as carbon source (Table I and Fig. 1*C*), and their spots represent 2% of the total spot density in the 2D images of the ethanol-limited culture.

A number of proteins were detected in different spots, of approximately the same M_r but with different pI. For example, Hxk1p was detected in two different spots on the 2D gels of the glucose-limited culture but was undetectable on 2D gels of the ethanol-limited culture. As can be seen in Fig. 1*C*, Icl1p was identified in three different spots on the 2D gels. Adh1p could be detected in two different spots, one of which was only detected on the 2D gels of the glucose-limited chemostat culture, and the other Adh1p spot showed a relative change in expression level for ethanol *versus* glucose of 0.24 (Table I and Fig. 1, *C* and *D*).

Finally, we considered the remaining protein spots in the 2D gels that did not significantly change in volume, a number of which were randomly picked and identified, and typical examples are given in the bottom section of Table I. These proteins had functions other than in the central carbon metabolism, such as proteins with a role in amino acid, protein, and nucleotide metabolism, and proteins with antioxidant properties.

DISCUSSION

We analyzed the proteomic response of the yeast *S. cerevisiae* grown at a fixed growth rate in aerobic chemostat cultures limited for glucose or ethanol, respectively. The reproducibility for chemostat culturing is significant as has been shown in previous work (19, 20, 33), for which both intra- and inter-laboratory comparisons were made, resulting in highly reproducible DNA micro-array data. The present study enables the analysis of relative protein expression levels under glycolytic and gluconeogenic conditions. This resulted in many changes in the levels of proteins involved in the central carbon metabolism, as is illustrated in Fig. 3, in which enzymes that are significantly up-regulated in the glucose- or in the ethanol-limited culture are depicted in red and blue, respectively. Below, we discuss in further detail the outcome of our proteome analysis, focusing on the different parts of the central carbon metabolism.

Glycolytic Proteins—As expected, the glycolytic enzymes, which are involved in the conversion of glucose into pyruvate, were significantly more abundant in the glucose-limited yeast chemostat culture (Table I and Fig. 2). In particular, the on/off regulation of Hxk1p is most likely due to the fact that this enzyme catalyzes the first and irreversible step in the glycolysis and is specific for glucose metabolism. Two other enzymes are known to be involved in this first step in glycolysis, *i.e.* the enzymes Hxk2p and Glk1p. Of these, Hxk2p was not detected on the 2D gels, while the expression level of Glk1p did not vary significantly (Table I).

Enzymes Around the Pyruvate Branchpoint—Of the five identified enzymes that play a role around the pyruvate branch point, the aldehyde dehydrogenases were not specifically up- or down-regulated in one of the limited cultures (Table I and Fig. 2). However, the α subunit of pyruvate dehydrogenase (Pda1p), an enzyme that plays a role in the overall conversion of pyruvate to acetyl-CoA and CO_2, could only be detected in the ethanol-limited 2D gel images. In contrast, alcohol dehydrogenase (Adh1p) expression was strongly induced in the glucose-limited culture, although this enzyme is expressed at a lower level than Adh2p (Table I).

Proteins from the TCA Cycle—We observed that proteins with a role in the TCA cycle were strongly up-regulated in the ethanol-limited yeast culture (Table I and Fig. 2). Citrate syn-

TABLE I

Relative changes in protein expression in ethanol- versus glucose-limited cultured yeast

The normalized intensity of spots on three replicate 2D gels was averaged and the standard deviation was calculated for each condition. If a spot was solely detected on 2D gels for the glucose- or solely for the ethanol-limited condition, this is indicated with G or E, respectively. For all other spots, the relative change ethanol versus glucose (E/G) was calculated by dividing the averaged normalized spot quantity from the ethanol gels by the averaged normalized spot quantity from the glucose gels. A Student's *t*-test was performed to determine if the relative change was statistically significant. Relative changes that are significant at the 98%-confidence level ($p < 0.02$) are represented as underlined and bold. Relative changes significant at the 95% confidence level ($p < 0.05$) are represented underlined and in italic, whereas relative changes significant at the 90% confidence level ($p < 0.1$) are represented in italic. Identified proteins involved in the central carbon metabolism pathways are summarized in the top section of the table. The last column contains the corresponding mRNA fold changes, taken from Ref. 20: transcriptome fold changes significant at the 95% confidence level are indicated in bold. A number of identified proteins that are involved in other pathways than the central carbon metabolism are presented in the bottom section of the table. If proteins were identified and quantified in more than one spot on the 2D gels, this is indicated in the table with *a*, *b*, and *c*, respectively. Avg, averaged value from three spot volumes; NP, spot was not present in the 2D gel; *, spot was only detected in two out of three gels.

Protein	Description	ORF		Normalized averaged spot quantity				Fold change E/G	*p*-value	mRNA fold change E/G
				Ethanol		Glucose				
				Avg	SD	Avg	SD			
Proteins involved in the central carbon metabolism pathways										
Glycolysis										
Hxk1p	Hexokinase I	YFR053C	*a*	NP	–	1914	723	**G**	–	**0.17**
			b	NP	–	1513	526*	**G**	–	**0.17**
Glk1p	Glucokinase	YCL040W		1605	477	2006	1412	0.80	0.680	0.59
Fba1p	Fructose 1,6-bisphosphate adolase	YKL060C	*a*	3877	939	7877	529	**0.49**	0.007	1.11
			b	18395	4680	32591	4359	**0.56**	0.019	1.11
			c	5104	1334	7925	2134	0.64	0.138	1.11
Tpi1p	Triosephosphate isomerase	YDR050C		10230	1652	18779	8617	0.54	0.225	1.11
Tdh2p	Glyceraldehyde 3-phosphate dehydrogenase	YJR009C		4657	1006	11815	1899	**0.39**	0.010	1.09
Tdh3p	Glyceraldehyde-3-phosphate dehydrogenase 3	YGR192C		23369	2809	49050	9593	*0.48*	0.035	1.42
Pgk1p	3-phosphoglycerate kinase	YCR012W	*a*	19185	2554	31477	1971	**0.61**	0.003	0.93
			b	2872	436	8145	862	**0.35**	0.003	0.93
Gpm1p	Phosphoglycerate mutase	YKL152C		11416	3776	35854	1690	**0.32**	0.003	0.95
Eno1p	Enolase I	YGR254W		6199	282	16914	3915	*0.37*	0.041	0.96
Eno2p	Enolase	YHR174W	*a*	9070	889	19739	3388	*0.46*	0.026	**0.63**
			b	3311	2117	8176	3092	*0.40*	0.097	**0.63**
Pyruvate branchpoint										
Pda1p	Pyruvate dehydrogenase alpha subunit	YER178W		531	63	NP	–	**E**	–	1.02
Adh1p	Alcohol dehydrogenase	YOL086C	*a*	3529	352	14557	3267	*0.24*	0.027	**0.70**
			b	NP	–	4371	1852	**G**	–	**0.70**
Adh2p	Alcohol dehydrogenase II	YMR303C	*a*	38028	16816	37627	3558	1.01	0.971	1.49
			b	33810	9170	31417	3295	1.08	0.704	1.49
Ald4p	Aldehyde dehydrogenase	YOR374W	*a*	10412	1510	12845	5668	0.81	0.539	1.14
			b	19464	428	31826	6626	*0.61*	0.083	1.14
			c	7127	1538	8314	4200	0.86	0.682	1.14
Ald6p	Aldehyde dehydrogenase	YPL061W		8122	2167	5878	2770	1.38	0.335	1.45
TCA cycle										
Cit1p	Citrate synthase	YNR001C		6498	2346	NP	–	**E**	–	**1.62**
Aco1p	Aconitase	YLR304C	*a*	1316	289	810	637	1.63	0.305	**1.50**
			b	2667	823	911	573	*2.93*	0.045	**1.50**
Idh1p	NAD-dependent isocitrate dehydrogenase 1	YNL037C		5304	287	3051	859	*1.74*	0.035	**1.76**
Idh2p	NAD-dependent isocitrate dehydrogenase	YOR136W		1596	783	690	511*	2.31	0.180	**1.65**
Idp2p	NADP-dependent isocitrate dehydrogenase	YLR174W	*a*	1096	436	NP	–	**E**	–	**1.76**
			b	2920	463	NP	–	**E**	–	**1.76**
Kgd2p	Alpha-ketoglutarate dehydrogenase	YDR148C		3800	482	1638	831	*2.32*	0.026	1.05
Lsc1p	Succinyl-CoA ligase (alpha subunit)	YOR142W		7342	1714	6830	964	1.07	0.681	1.02
Lsc2p	Succinyl-CoA ligase (beta subunit)	YGR244C		815	215	NP	–	**E**	–	1.24
Sdh1p	Succinate dehydrogenase	YKL148C		301	98	NP	–	**E**	–	**2.34**
Fum1p	Fumarase	YPL262W	*a*	5154	1001	1547	432	**3.33**	0.014	**1.90**
			b	2384	750	1643	678	1.45	0.273	**1.90**
Mdh1p	Mitochondrial malate dehydrogenase	YKL085W		11126	1881	9102	672	1.22	0.196	1.14
Mdh2p	Cytosolic malate dehydrogenase	YOL126C		4292	1623	NP	–	**E**	–	**4.64**
Glyoxylate cycle and gluconeogenesis										
Icl1p	Isocitrate lyase	YER065C	*a*	2642	141	NP	–	**E**	–	**4.71**
			b	1879	381	NP	–	**E**	–	**4.71**
			c	7860	773	NP	–	**E**	–	**4.71**
Mls1p	Malate synthase	YNL117W		4275	283	NP	–	**E**	–	**6.62**
Pck1p	Phosphoenolpyruvate carboxylkinase	YKR097W		1283	180	NP	–	**E**	–	**17.64**

TABLE I—*continued*

Protein	Description	ORF	Normalized averaged spot quantity				Fold change E/G	*p*-value
			Ethanol		Glucose			
			Avg	SD	Avg	SD		
Proteins involved in pathways other than the central carbon metabolism								
Amino acid metabolism								
Leu4p	2-isopropylmalate synthase	YNL104C	19464	428	31826	6626	0.61	0.25
Gdh1p	NADP-specific glutamate dehydrogenase 1	YOR375C	2409	433	2560	1338	0.94	0.50
Ilv5p	Ketol-acid reductoisomerase, mitochondrial	YLR355C	2692	152	2493	582	1.08	0.48
Protein metabolism								
Cpr1p	Peptidyl-prolyl cis-trans isomerase	YDR155C	48420	12583	53143	11023	0.86	0.34
Nucleotide metabolism								
Adk1p	Adenylate kinase cytosolic	YDR226W	8271	905	9862	5301	0.84	0.44
Mitochondrial transport								
Omp2p	Outer mitochondrial membrane protein porin 1	YNL055C	16250	6030	11685	858	1.39	0.91
Antioxidant								
Sod2p	Superoxide dismutase	YHR008C	8109	1213	7302	4269	1.11	0.62
Respiration								
Qcr1p	Ubiquinol-cytochrome C reductase complex protein I	YBL045C	2146	429	1569	551	1.37	0.77
Qcr2p	Ubiquinol-cytochrome C reductase complex protein II	YPR191W	5094	897	4173	1176	1.22	0.62
Atp2p	ATP synthase beta chain	YJR121W	11416	3667	7298	2128	1.56	0.95

thase (Cit1p), NADP-dependent isocitrate dehydrogenase (Idp2p), succinate-CoA Ligase (Lsc2p), succinate dehydrogenase flavoprotein subunit (Sdh1p), and cytosolic malate dehydrogenase (Mdh2p), all enzymes with a known function in the TCA cycle, were exclusively expressed in the ethanol-limited yeast culture and could not be detected under glucose-limited conditions (Table I). The TCA cycle is required for the provision of bio-precursors, irrespective of the carbon source used, ethanol or glucose. However, when the yeast cells are grown on the nonfermentable carbon source ethanol, the TCA cycle also serves for the dissimilation of ethanol (this role is fulfilled by the glycolysis when glucose is used as carbon source), which explains the detected expression patterns.

Gluconeogenic and Glyoxylate Cycle Proteins—Isocitrate lyase (Icl1p) and malate synthase 1 (Mls1p), which are both key enzymes in the glyoxylate cycle, were solely detected when ethanol was used as carbon source (Table I and Fig. 1*C*). In the glyoxylate cycle, two molecules of acetyl-CoA are converted into oxaloacetate, thus bypassing reactions of the TCA cycle in which CO_2 is released. It is essential during growth on C2 compounds for the synthesis of all cellular compounds with three or more carbon atoms, for example for the biosynthesis of amino acids and nucleotides (34). As a consequence, it could be expected that this pathway would be up-regulated in the presence of ethanol compared with the presence of glucose. The gluconeogenic protein phosphoenolpyruvate carboxykinase (Pck1p) could only be detected in 2D gel images of ethanol-limited yeast culture. Pck1p decarboxylates and phosphorylates oxaloacetate to phosphoenolpyruvate. It switches the direction of the flow of metabolites toward the essential biosynthetic precursor, glucose-6-phosphate, which is needed among others for the storage of carbohydrates. Because the gluconeogenic pathway is essential for the production of glucose-6-phosphate on ethanol as the sole carbon source, a relatively high abundance of Pck1p in the ethanol-limited culture can be readily explained.

We could not detect all proteins that play a role in the central carbon metabolism on our 2D gels (Fig. 3), such as Fbp1p and Cit2p, two major proteins of the C2 metabolism. These proteins were detected and quantified using [^{35}S]methionine labeling by Haurie *et al.* (35), which is a very sensitive approach. Although we analyzed with MS all spots from the 2D gels in sections surrounding the proposed locations of both proteins, none of these spots could be identified as either Fbp1p or Cit2p. Probably we did not detect these proteins because they have expression levels below the detection limit of our silver-staining method. We identified substantially more spots on the 2D gels than discussed here, and established that these proteins do not play a role in the central carbon metabolism. Importantly, these identified proteins did not respond statistically significantly to a change in carbon source limitation, examples of which are given in Table I.

A number of proteins appeared in multiple spots, *i.e.* Hxk1p, Fba1p, Pgk1p, Eno2p, Adh1p, Adh2p, Ald4p, Aco1p, Idp2p, Fum1p, and Icl1p. The proteins appeared on the gels at approximately the same molecular mass but at a different pI position (Fig. 1, *C* and *D*). This can be explained by differential post-translational protein processing such as processing of signal sequences, acetylation, or phosphorylation, or may be an artifact of amidation, observations that have been reported in a number of other yeast proteome studies (3, 5, 17, 36). For example, Fum1p was detected in two spots, one showed a nonchanged abundance, while the other isoform was statistically significantly up-regulated under ethanol limitation (Table I and Fig. 1*D*). It is known that a single transla-

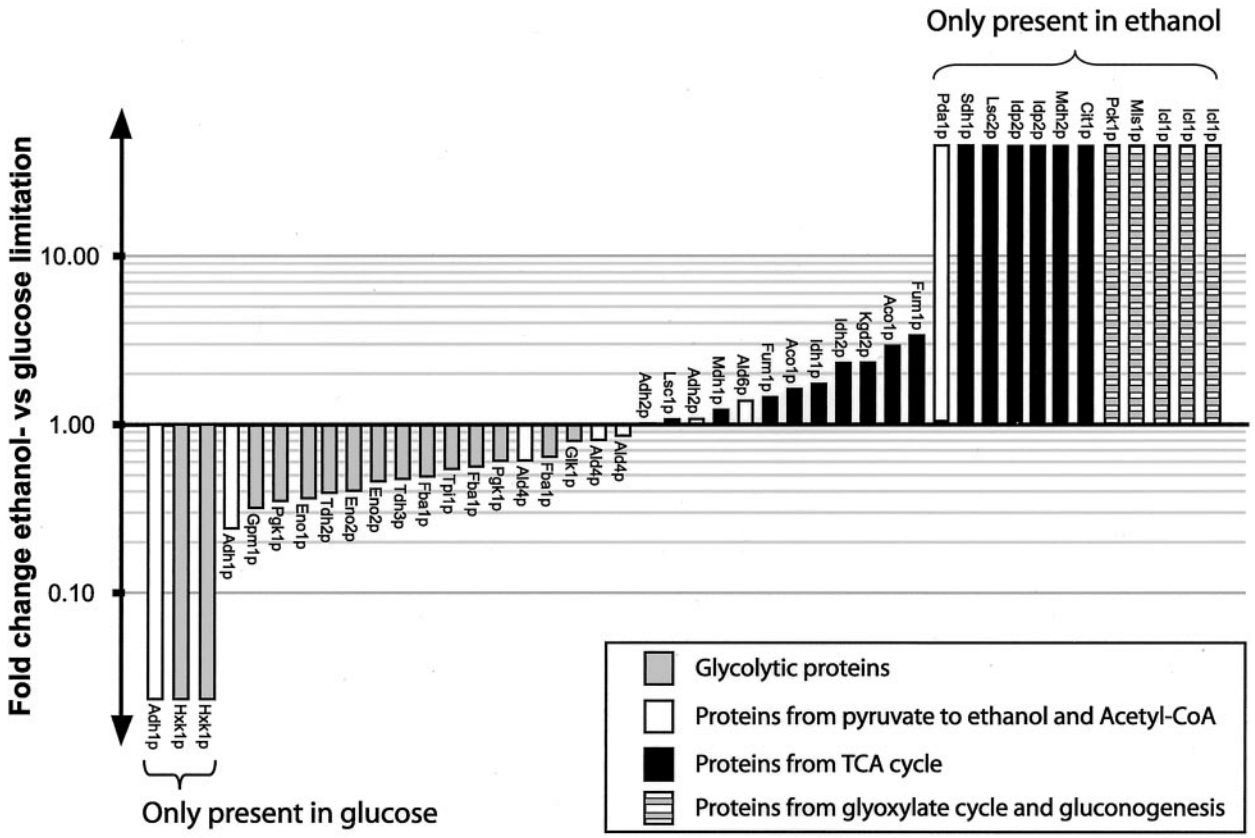

FIG. 2. **Relative protein expression changes of ethanol *versus* glucose (E/G) limitation.** Relative expression changes (ethanol *versus* glucose) of proteins that play a role in the central carbon metabolism are depicted. Proteins are divided into four different categories, namely glycolytic enzymes (*gray*), enzymes that convert pyruvate to ethanol and acetyl-CoA and vice versa (*white*), enzymes from the TCA cycle (*black*), and enzymes form the gluconeogenesis and the glyoxylate cycle (*hatched*).

tional product of the *FUM1* gene, encoding fumarase, is distributed between the cytosol and the mitochondria (37). These two isoenzymes of fumarase are encoded by the same gene, and mature cytosolic and mitochondrial fumarase isoenzymes are identical (38). Nevertheless, we detected two isoforms in the 2D gels, probably indicating that fumarase is post-translationally modified to a further extent. Unfortunately, no data could be extracted from our MALDI-TOF and tandem LC-MS analyses to identify the differences in Fum1p or any of the other enzyme isoforms.

A major advantage of 2D electrophoresis is that it offers the resolution to separate protein isoforms, in contrast to transcriptome analyses, because in the latter approach post-transcriptional steps cannot be detected. In our proteome study, we found isoforms for a number of enzymes, of which the individual regulation was analyzed (Table I), whereas in comparable transcriptome studies (20) there is evidently only one data point per gene. A major advantage of transcriptome analyses is that they are more comprehensive, as nowadays expression levels of all known *S. cerevisiae* genes can be analyzed. In our proteome study, only a subset of proteins could be considered, *i.e.* the proteins that can be separated and visualized on 2D gels. Still in our proteome approach many enzymes of interest, *i.e.* those involved in the central carbon metabolism, could be analyzed, as these proteins are relatively highly abundant.

As can be seen from Table I, we compared for identical *S. cerevisiae* chemostat cultures the proteome data with the corresponding transcriptome fold changes. We combined these datasets to investigate at which levels gene expression is regulated, *i.e.* if regulation primarily takes place at the level of the proteome, by which all post-transcriptional processes are indicated, such as protein degradation and other post-translational modifications, or rather at the level of the transcriptome. Most glycolytic enzymes, except for Hxk1p (Table I), appear to be regulated at the level of the proteome, because these enzymes are significantly up-regulated when *S. cerevisiae* was grown under glucose-limited conditions, in contrast to their corresponding transcripts, which do not show significant changes in abundance. Under glucose-limiting growth conditions, the glycolytic enzyme Hxk1p was significantly up-regulated at both the mRNA and the protein level, suggesting that the first step in the glycolysis is mainly regulated at the transcriptome level. In the pyruvate branch-point, Pda1p is most likely regulated at the level of the proteome, because only the protein fold changes were signifi-

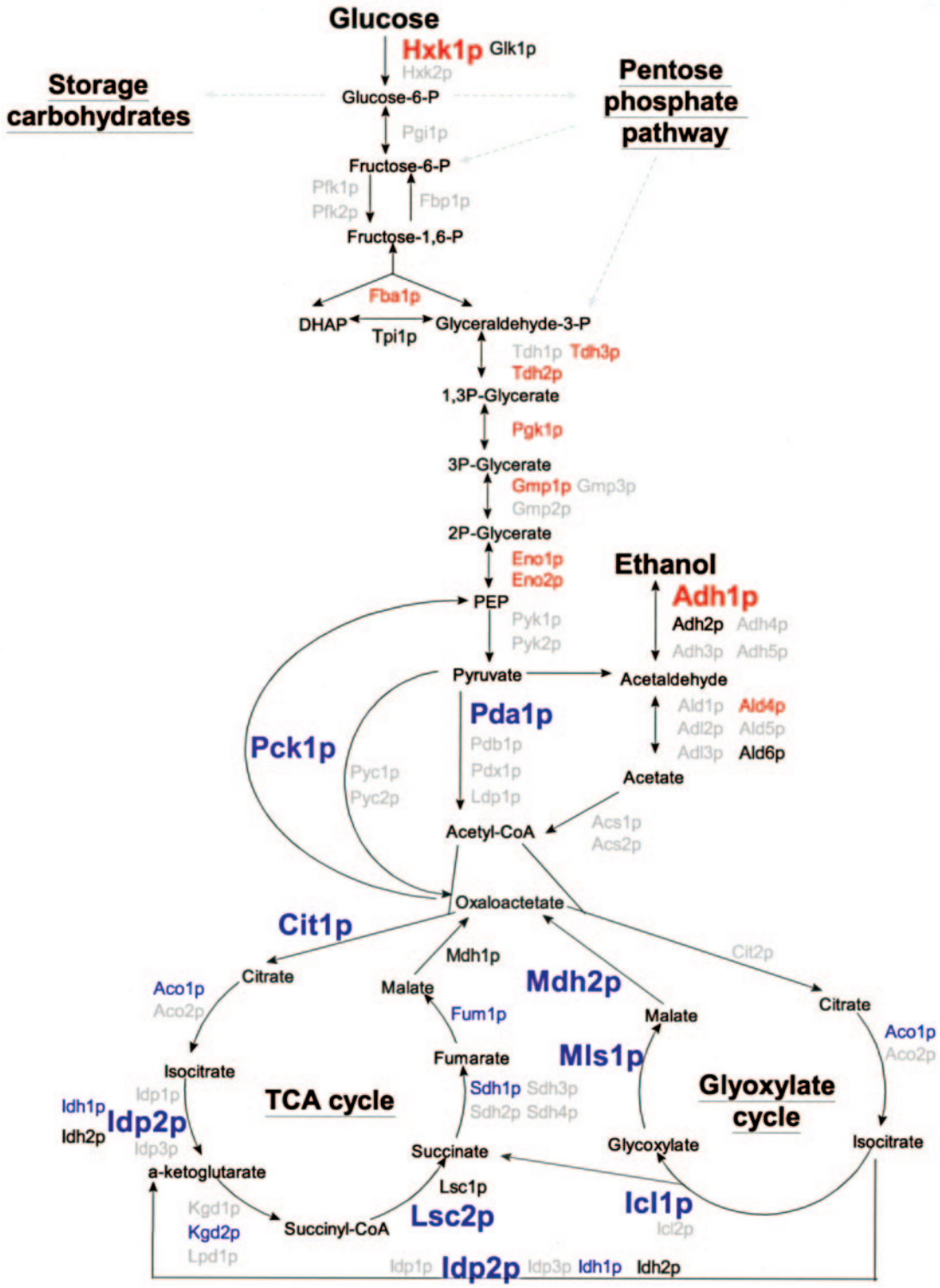

FIG. 3. **Scheme of the central carbon metabolism in *S. cerevisiae* for the utilization of glucose and ethanol.** Proteins, which are detected and identified by MS, are color coded in *red*, *blue*, and *black*. Proteins indicated in red are up-regulated in the glucose-limited chemostat culture, while proteins indicated in blue are up-regulated in the ethanol-limited chemostat culture. Proteins indicated in black did not change significantly when changing the limiting carbon source. If a protein could exclusively be detected in the glucose-limited yeast cultures, it is shown in large red letters, while proteins, which are only expressed in ethanol-limited yeast cultures, are indicated in large blue letters.

cant, whereas Adh1p is regulated transcriptionally, as both mRNA and protein fold changes are significant. Most enzymes involved in the TCA cycle are transcriptionally regulated, because both mRNA and corresponding protein expression levels were increased under ethanol limitation. All detected enzymes involved in the glyoxylate cycle and gluconeogenesis (*i.e.* Icl1p, Mls1p, and Pck1p) appear to be transcriptionally regulated, because both the mRNA and corresponding protein expression levels were up-regulated in the ethanol-limited chemostat cultures. The gluconeogenic and glyoxylate cycle genes are known to be transcriptionally regulated (20).

The influence of different carbon sources on the yeast proteome has been studied by others as well. A related study is that of Futcher *et al.* (39), who studied *S. cerevisiae* growing exponentially in batch cultures that contained either ethanol or glucose as carbon source. Several protein expression changes detected by Futcher *et al.* (39) were consistent with our findings, such as induction of proteins from the glyoxylate cycle by ethanol. However, in contrast to Futcher *et al.* (39), we observed that some enzymes from the TCA cycle and gluconeogenesis were up-regulated in the ethanol-limited culture. Moreover, Futcher *et al.* (39) reported that many heat shock proteins (Hsp60p, Hsp82p, Hsp104p, and Kar2p) showed an increase in relative abundance in the ethanol batch cultures. According to Futcher *et al.* (39) this indicated an increased heat resistance of cells grown in ethanol. Furthermore, Futcher *et al.* (39) established up-regulation of proteins involved in protein synthesis (Eft1p, Rpa0p, and Tif1p) in the glucose culture, which they explained by a higher growth rate of yeast cells grown on glucose. Because we used chemostat cultures, the specific growth rate was equal and constant for both carbon-limiting cultures. Therefore, we did not detect either effects of increased heat resistance nor growth rate-dependent changes. An example to illustrate the influence of glucose repression occurring in batch cultivation on glucose is the observed expression ratios of Hxk1p. We established that Hxk1p protein expression was virtually induced upon glucose limitation, and we assigned this to the specific role of the enzyme at the onset of glycolysis. In contrast, Futcher *et al.* (39) did not find significant differences in expression levels for Hxk1/2p, when comparing ethanol and glucose *S. cerevisiae* cultures, whereas Hxk1p was glucose-repressed in a similar study by Boucherie *et al.* (1). Besides the effect of differences in cultivation techniques, discrepancies between the aforementioned studies of Futcher *et al.* (39), Boucherie *et al.* (1), and our study may additionally be a result of the use of different yeast strain backgrounds (*S. cerevisiae* W303 and FY5, *versus* CEN.PK113-7D, respectively), and of differences in analytical procedures, such as for sample preparation.

Our scope of interest is not only exploration of the differential proteome for typical carbon sources, but in particular the effect of nutrient limitation, such as applied for industrial applications. For example, for the industrial production of bakers' yeast, high biomass yields are needed, and therefore cultivation takes place typically under sugar-limited and aerobic conditions at relatively low specific growth rates. For this purpose, we consider chemostat culturing of *S. cerevisiae* as an improved model when compared with batch culturing, because it allows investigation of the primary effect of nutrient limitation under otherwise carefully controlled growth conditions.

In conclusion, we show that comparative proteome analysis of yeast extracts from aerobic chemostat-cultivated yeast cells limited for either glucose or ethanol revealed only proteome changes in the central carbon metabolism pathways. Mapping the changes in protein expression levels provided us insight in the way the wild-type *S. cerevisiae* CEN.PK113-7D adapts to a single change in growth condition. Moreover, we could establish for many steps in the central carbon metabolism if gene expression was regulated primarily at the level of the proteome or of the transcriptome. In this report, we studied in-depth the adaptation to two different carbon sources. However, this method can be applied to study the proteomic response of a wide range of single nutrient limitations, yeast genome mutation(s), and various *S. cerevisiae* strains, which we are currently further exploring.

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