Double Standards in Quantitative Proteomics

DIRECT COMPARATIVE ASSESSMENT OF DIFFERENCE IN GEL ELECTROPHORESIS AND METABOLIC STABLE ISOTOPE LABELING*

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Quantitative protein expression profiling is a crucial part of proteomics and requires methods that are able to efficiently provide accurate and reproducible differential expression values for proteins in two or more biological samples. In this report we evaluate in a direct comparative assessment two state-of-the-art quantitative proteomic approaches, namely difference in gel electrophoresis (DiGE) and metabolic stable isotope labeling. Therefore, *Saccharomyces cerevisiae* was grown under well defined experimental conditions in chemostats under two single nutrient-limited growth conditions using ^{14}N- or ^{15}N-labeled ammonium sulfate as the single nitrogen source. Following lysis and protein extraction from the two yeast samples, the proteins were fluorescently labeled using different fluorescent CyDyes. Subsequently, the yeast samples were mixed, and the proteins were separated by two-dimensional gel electrophoresis. Following in-gel digestion, the resulting peptides were analyzed by mass spectrometry using a MALDI-TOF mass spectrometer. Relative ratios in protein expression between these two yeast samples were determined using both DiGE and metabolic stable isotope labeling. Focusing on a small, albeit representative, set of proteins covering the whole gel range, including some protein isoforms and ranging from low to high abundance, we observe that the correlation between these two methods of quantification is good with the differential ratios determined following the equation $R_{Met.Lab.} = 0.98R_{DiGE}$ with $r^2 = 0.89$. Although the correlation between DiGE and metabolic stable isotope labeling is exceptionally good, we do observe and discuss (dis)advantages of both methods as well as in relation to other (quantitative) approaches. ***Molecular & Cellular Proteomics 4:255–266, 2005.***

The proteome is generally defined as the total protein complement of a genome present in cells and/or tissue (1). Through splice variation and/or post-translational modifications the proteome is several orders of magnitude more complex than the genome. One extra factor that adds to the relative complexity of the proteome is that protein abundance varies over time, either as a reaction to changes in the environment or during development. To understand these dynamic processes, which may lead to indications why *e.g.* "healthy" cells are different from "diseased" or "stressed" cells or how cells change during differentiation, it is not only important to identify which proteins are involved but also to measure their differential expression levels. It is especially this latter notion that makes quantitative protein profiling an essential part of proteomics, which requires technologies that accurately, reproducibly, and comprehensively quantify the protein content in biological samples (2–4).

Traditionally, and probably still, the most frequently used method to investigate differential protein abundances in large scale proteomic experiments on protein mixtures from cellular extracts or tissue is by two-dimensional (2D)[1] gel electrophoresis (5–9). In such experiments proteins are separated by their pI and molecular weight on a 2D gel and subsequently stained for visualization. The spot density on the gel is used to assess relative quantification through comparison with "matched" protein spots on 2D gels run in parallel. For protein staining many protocols are in use (6), whereby in practice Coomassie Brilliant Blue and silver staining have found most widespread applications. These stains have appeared to be not ideal because of relatively poor detection sensitivity (Coomassie Brilliant Blue) or diminished peptide recovery from in-gel-digested proteins for MS (silver staining). Both Coomassie Brilliant Blue and silver staining also have a rather limited dynamic range as far as quantification is concerned. The accuracy of quantification depends on the intrinsic characteristics of the visualization methods. More recently, a variety of improvements and alternatives that are more reproducible and have an increased linear dynamic range have been introduced. Significant quality improvements have been achieved with the introduction of fluorescent stains like that of the SYPRO family (10) that, in addition to an increase in linear dynamic range, turned out to be satisfactorily compatible with MS analysis.

Another recently introduced novel approach in 2D gel-based quantitative proteomics is the application of fluorescent cyanine dyes (Cy2, Cy3, and Cy5) to label proteins before they are separated on a 2D gel (11–13). These fluorescent labels carry a *N*-hydroxysuccinimidyl ester functionality de-

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[1] The abbreviations used are: 2D, two-dimensional; R, ratio; DiGE, difference in gel electrophoresis; S/N, signal-to-noise.

signed to modify the ε-amino group of lysine residues in proteins. The design results in the introduction of three spectrally resolvable fluorophores that carry a positive charge to compensate for the vanished lysine charge, thereby balancing the pI of the protein. The molecular masses of the CyDyes are ~450 Da and will not significantly affect the protein migration in the second dimension. Taken together, the characteristics of these labels allow the analysis of up to three pools of protein samples simultaneously on a single 2D gel. This approach eliminates to a great extent technical, *i.e.* gel-to-gel, variation, which is the main limitation of 2D gel electrophoresis. In a standard protocol, two of the dyes (typically Cy3 and Cy5) are used to label two different pools of protein samples, while the third label (Cy2) is used to label an internal standard that consists of equal amounts of the two pools. This internal standard allows a correction for further experimental errors, thereby distinguishing biological from experimental variation (14). Since its introduction, this so-called difference in gel electrophoresis (DiGE) approach has found applications in quantitative proteomics (15–21) for instance in comparative quantitative proteomics of primitive hematopoietic cell populations (17).

In recent years also entirely different, mass spectrometry-based, methods to assess protein expression levels have been developed whereby differential quantification is accomplished by labeling peptides/proteins with stable isotope tags. These techniques are quite different from radioactive isotope labeling (with [^{35}S]methionine for instance) (22), which is probably still the most sensitive and accurate method to label/stain proteins but which is rather hazardous. In stable isotope labeling, proteins or peptides in two sets of samples are differentially labeled using different stable isotope tags. These different isotope tags will produce specific mass shifts in the mass spectra of peptides/proteins that may then be used as internal standards in differential analysis. In this way differential quantification by mass spectrometric analysis can be achieved. Many different stable isotope labels have now been developed that may be classified on the basis of *how* and *when* they are introduced into the protein samples. The stable isotope labels may be incorporated by chemical or biological means at different stages of the proteomics experiment, *i.e.* from the start *in vivo* in cells or organisms up to the end by modifying the protein digest with appropriate labels just prior to mass spectrometric analysis (23, 24).

Chemically, the stable isotope label can be incorporated via reactions with isotope-containing reagents at different functional groups in the peptides/proteins such as the lysine side chains or the free N termini etc. (25–28). By using an isotope-coded affinity tag, such as the biotinylated ICAT reagent, which reacts selectively with free cysteines, stable isotope-labeled peptides/proteins can be enriched prior to mass analysis (29–34). Alternatively, generation of C-terminal labeled peptides can be achieved by enzymatic digestion in heavy $H_2{}^{18}O$ water (35–40). Most recently, a novel chemical isotope labeling approach has been introduced, termed iTRAQ, that uses a multiplexed set of isobaric reagents that yield amine-derivatized peptides. The derivatized peptides are indistinguishable in MS but exhibit intense low mass tandem MS marker ions that may be used for relative quantification of proteins originating from up to four different samples (41). A disadvantage of these "chemical" approaches is that the stable isotope label is introduced into the sample only after several stages of sample preparation, such as cell lysis, protein extraction, and/or even proteolysis. When the mixing of the differentially labeled samples occurs only after several of these sample preparation steps, it is of ultimate importance in these approaches that the sample preparation is highly consistent.

Therefore, it is preferred to introduce the stable isotope label very early in the process. In these approaches, the cells or organisms need to be grown in defined media that contain a stable isotope label that can be incorporated during protein synthesis (42–44). In a typical approach, termed metabolic labeling, a growth medium is prepared in which a stable isotope-labeled compound is used, such as ^{15}N-labeled ammonium sulfate, as the sole nitrogen source. Alternatively, stable isotope-labeled amino acids also can be introduced into the medium that will be incorporated (in the case of essential amino acids) during protein synthesis (45–49). So far metabolic labeling has been applied mostly to unicellular organisms, such as yeast (42) and bacteria (43) and to tissue cell cultures (45), which can be easily grown on defined media in the laboratory. Recently, the multicellular organisms *Caenorhabditis elegans* and *Drosophila melanogaster* (50) also have been metabolically labeled, and lately this has even been extended to the isotope labeling of a complete rat (51) and potato plant (52).

In general, all these different quantitative proteomic approaches have their merits and disadvantages, and the method of choice often depends on the particular biological question. However, as far as accuracy and validation of methods in protein quantification is concerned, only a very few reports exist in which different quantification techniques are directly compared (22, 53, 54). For instance, Lopez *et al.* (54) compared the quantification of about 400 protein spots stained by silver and SYPRO Ruby on 2D gels and found an overall correlation of just 0.75 with the largest deviation at lower protein abundances. Fievet *et al.* (22) compared protein quantities from yeast proteins labeled with radioactive ^{35}S or stained with Coomassie Brilliant Blue. They observed a very weak correlation and found the relative ratios determined by these two methods to vary for individual proteins from 0.37 to 1.86. As it is of absolute importance in quantitative proteomics that methods are able to accurately, reproducibly, and comprehensively quantify the protein content in biological samples, we set out to evaluate in a direct comparative assessment two current state-of-the-art quantitative approaches, namely DiGE and metabolic stable isotope labeling.

Therefore, we used as a model system *Saccharomyces cerevisiae*, which was grown under well defined experimental conditions in chemostat cultures under two different single nutrient-limited growth conditions (*i.e.* nitrogen *versus* carbon). One of the two yeast samples was grown in the chemostats using medium containing a stable isotope (*i.e.* ^{15}N), while the other was grown on natural isotope-containing medium with ammonium sulfate being the sole nitrogen source. Throughout this work the term ^{15}N indicates proteins, or peptides thereof, that were extracted from yeast grown on medium containing 98% $(^{15}NH_4)_2SO_4$ as sole nitrogen source, while proteins extracted from yeast grown on medium containing $(NH_4)_2SO_4$ are referred to as the natural isotope. Following lysis and protein extraction, the two samples were fluorescently labeled using two different fluorescent CyDyes prior to mixing. Proteins were separated by 2D gel electrophoresis and after in-gel digestion further analyzed by mass spectrometry. Protein expression levels of these two yeast samples were relatively quantified both using DiGE and metabolic stable isotope labeling. Focusing on a small, but representative, set of protein spots with a wide variety in pI, M_r, and abundance, we observe that, when excluding so-called on-off spots, the correlation between the two methods of quantification is very good with the differential ratios determined following the equation $R_{Met.Lab.} = 0.98R_{DiGE}$ with a correlation coefficient r^2 of 0.89.

EXPERIMENTAL PROCEDURES

Strain and Culture Conditions—Wild-type *S. cerevisiae* strain CEN.PK113–7D (*MAT***a**) (55) was grown at 30 °C in 2-liter chemostats (Applikon) with a working volume of 1.0 liter as described in Ref. 56. Cultures were fed with a defined mineral medium that limited growth by either carbon or nitrogen with all other growth requirements in excess and at a constant residual concentration. The defined mineral medium composition was based on that described by Verduyn *et al.* (57). The medium contained the following components (per liter): carbon-limited, 19 mM $(NH_4)_2SO_4$ and 42 mM glucose; nitrogen-limited, 7.5 mM $(NH_4)_2SO_4$ and 330 mM glucose.

Both cultures were started with $(^{14}NH_4)_2SO_4$ as sole nitrogen source (Merck). In the case where ^{15}N isotope was used, the medium vessel was replaced by a new vessel containing $(^{15}NH_4)_2SO_4$ (Isotec Inc., Miamisburg, OH) after five volume changes. The carbon-limited culture was fed with 98% $(^{15}NH_4)_2SO_4$ as supplied by Isotec Inc. After five additional volume changes, a new steady state was reached, and samples for proteome analysis were taken. Dry weight, metabolite, dissolved oxygen, and gas profiles were constant over at least three volume changes prior to sampling. Samples dedicated to proteome analysis were sampled on ice and immediately centrifuged (5 min at 0 °C), washed twice with ice-cold sterile water, and stored 5× concentrated in water at −80 °C.

Protein Extraction—Protein extracts were prepared as described previously (58). Protein concentration was determined using the Plus One 2D Quant kit (Amersham Biosciences). The protein samples were stored in aliquots at −80 °C.

Labeling of Proteins with CyDyes—Protein samples were prepared and labeled according to the manufacturer's protocol. Briefly 50 μg of protein was precipitated using the Plus One 2D Clean-Up kit (Amersham Biosciences), dissolved in labeling buffer, and labeled at 0 °C in the dark for 30 min with 400 pmol of cyanine dye (Cy2, Cy3, and Cy5; Amersham Biosciences), dissolved in 99.8% *N*,*N*-dimethylformamide (Sigma). The reaction was quenched by the addition of 1 μl of a 10 mM L-lysine solution (Merck) and left on ice for 10 min.

2D Gels—Two-dimensional gels were run as described before (58). Briefly the three 50-μg aliquots of the Cy2-, Cy3-, and Cy5-labeled proteins were mixed and loaded on a 24-cm Immobiline Dry-Strip, pH 3–10 NL (Amersham Biosciences). Isoelectric focusing was carried out using an IPGphor (Amersham Biosciences) to a total of 50–55 kV-h. After equilibration, 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 overnight at a constant power of 2 watts until the bromphenol blue front had migrated to the bottom of the gel.

Image Acquisition and Analysis—Gels were scanned using the Typhoon 9400 Imager (Amersham Biosciences) according to the manufacturer's protocol. Scans were acquired at 100-μm resolution. After cropping and filtering, images were subjected to automated Difference in-gel Analysis (DIA) and Biological Variation Analysis (BVA) using the Batch Processor of DeCyder software, Version 5.01 (Amersham Biosciences).

Poststaining—2D gels were poststained using silver staining as described by Shevchenko (59) with slight modifications. Briefly, after fixing and washing, the gels were sensitized using 0.04% sodium thiosulfate and impregnated with 0.1% silver nitrate at 4 °C for 20 min. Development of the gel was performed using 3% sodium carbonate, 0.05% formalin. Silver-stained gels were scanned using a GS710 calibrated densitometer (Bio-Rad).

In-gel Tryptic Digestion—Protein spots of interest were digested in-gel with trypsin with a slightly modified protocol as that described by Wilm *et al.* (60). The gel pieces were first destained using 30 mM potassium ferricyanide and 100 mM sodium thiosulfate solution followed by washing and shrinking steps using 50 mM ammonium bicarbonate and acetonitrile, respectively. Proteins were digested overnight at 37 °C.

MALDI-MS and Protein Identification—Tryptic digests were desalted and concentrated with μC_{18} ZipTips (Millipore) and analyzed on a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems) using α-cyano-4-hydroxycinnamic acid as matrix. The MALDI-MS resolution for the peptides was typically ~10,000. The raw MALDI-TOF spectra were processed using Data Explorer software (Version 4.0, Applied Biosystems). The following process parameters were used before the final peak list was generated: advanced baseline correction, smoothing, and peak deisotoping. The MALDI-MS spectra were internally calibrated using the singly protonated trypsin autodigestion peaks at *m/z* 2273.159 and 2163.056. The MALDI-MS spectra were searched against the Swiss-Prot data base using a local MASCOT search engine (61). The following settings were used: trypsin was used as enzyme, a maximum of two missed cleavages was allowed, the peptide tolerance was set at 150 ppm, and carbamidomethylcysteine and oxidized methionine were set as a fixed and variable modification, respectively. The MALDI-MS spectra were searched twice against the Swiss-Prot data base both times with the above described parameters and the second time with an extra newly defined fixed modification, *i.e.* assuming that all nitrogen atoms in the amino acids are ^{15}N-labeled. In this way, both the natural abundance ^{14}N-peptides as well as ^{15}N-labeled peptides were identified, significantly increasing the confidence score for identification.

Protein Expression Ratio Determination—Ratios of differentially expressed proteins (R_{DiGE}) were calculated using DeCyder (Version 5.01, Amersham Biosciences) for DiGE and show the -fold change of the expression under nitrogen-limiting conditions *versus* carbon-limiting conditions (N/C). In the DeCyder output, an increase in protein abundance under nitrogen limitation is expressed as a positive value (*e.g.* a 2-fold increase = 2), while a decrease in protein abundance

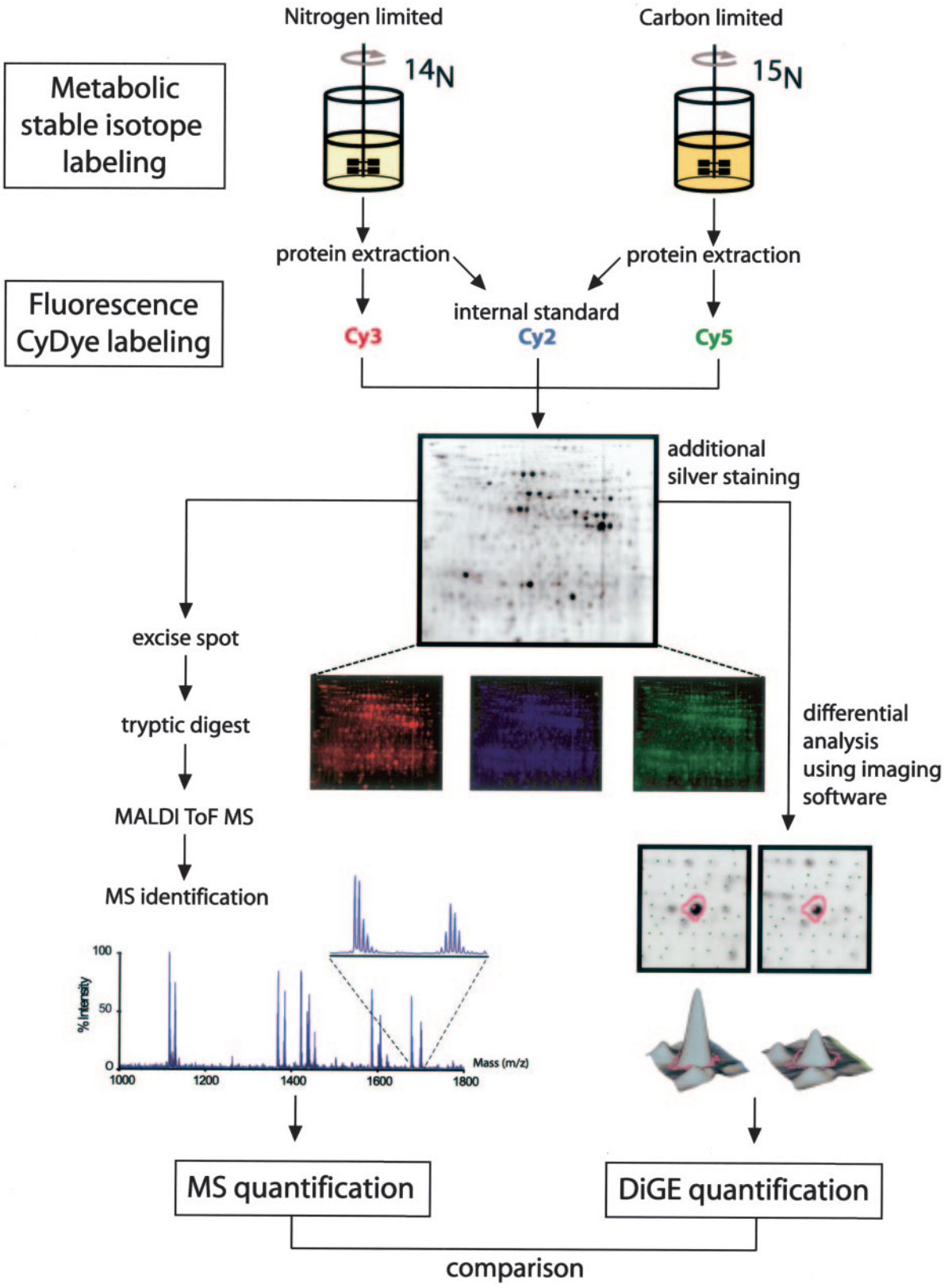

FIG. 1. **Experimental setup for the direct comparative assessment of DiGE and metabolic stable isotope labeling.** *S. cerevisiae* was cultured in nitrogen-limited and carbon-limited chemostats in media containing either "natural" ammonium sulfate or ^{15}N-labeled ammonium sulfate as the sole nitrogen source, respectively. Protein extracts from both cultures were prepared, and proteins were labeled with the CyDyes

TABLE I
Labeling strategy used

Yeast sample composition of the 2D gels used for the direct comparative assessment is shown. Yeast was grown under either nitrogen-limited or carbon-limited conditions in chemostat cultures. The growth media either contained natural isotope or ^{15}N-labeled ammonium sulfate as the sole nitrogen source. Furthermore the CyDye labels, which were used to label the protein samples, are indicated, *i.e.* Cy3 or Cy5.

Gel	Limitation	Nitrogen source	CyDye
1	Nitrogen	Natural isotope	Cy3
	Carbon	^{15}N-labeled	Cy5
2	Nitrogen	Natural isotope	Cy5
	Carbon	^{15}N-labeled	Cy3

under nitrogen limitation is expressed as a negative value (*e.g.* a 2-fold decrease = −2).

For metabolic stable isotope labeling, proteins were relatively quantified as described previously (50). Briefly peaks of all isotopes of the unlabeled peptide were integrated and divided by the integrated peak area of the ^{15}N-labeled peptide. The integration was performed by zooming in on all the isotopes of the peptide of interest, and subsequently the area under all isotopes was calculated in the Data Explorer software (Version 4.0, Applied Biosystems) and was subsequently exported to Excel (Microsoft). The protein expression ratio nitrogen limitation *versus* carbon limitation was calculated for each peptide pair. This was performed for multiple peak pairs in the same MALDI-TOF mass spectrum, and the $R_{\text{Met.Lab.}}$ was calculated as the average ratio of the multiple peak pairs. To enable a direct comparison with the DiGE quantitative data an increase in protein abundance under nitrogen limitation was expressed as a positive value, while a decrease in protein abundance under nitrogen limitation was expressed as a negative value. This representation is used throughout this work.

RESULTS

In this study we used two state-of-the-art relative quantitative proteomic approaches, namely DiGE and metabolic stable isotope labeling, in a direct comparative assessment. To make an appropriate technological comparison, whereby we aimed to limit any side effects, for instance due to experimental variation, the setup of the experiment is of vital importance. In Fig. 1 and Table I, the details of the selected experimental setup are shown. First of all, yeast cells were cultivated in well controlled chemostats limited for either the carbon or nitrogen source. The only nitrogen source in these chemostat cultures was ammonium sulfate ($(NH_4)_2SO_4$). For the yeast cells grown under nitrogen-limited conditions the natural isotope $(NH_4)_2SO_4$ was used, while in the carbon-limited culture ^{15}N-enriched $(NH_4)_2SO_4$ was used as sole nitrogen source. Following cell lysis, proteins from these two chemostat yeast cultures were extracted. Complete incorporation of ^{15}N in yeast proteins was checked using tryptic digestion and mass spectrometry (data not shown). Subsequently, proteins were labeled with the fluorescent cyanine dyes. As described in Table I, we performed two DiGE experiments where in the first experiment the yeast grown under nitrogen limited conditions was labeled with Cy3, and the ^{15}N-labeled yeast grown under carbon-limited conditions was labeled with Cy5. In the second DiGE experiment, we used the same chemostat cultured samples but reversed the two fluorescent dyes. By this so-called dye swap we added an extra internal control for the DiGE experiments. Additionally, for the DiGE experiments an internal standard was used that consisted of equal amounts of protein from both yeast samples that was labeled with Cy2. The DiGE internal standard was used to normalize the data, thereby limiting technical variation (14). Following fluorescent labeling, the samples were mixed, and the proteins were separated on one 2D gel. For the DiGE analysis, 2D gel images were acquired by fluorescence scanning and analyzed using DeCyder software. The DiGE analysis of the two separate fluorescent labeling experiments resulted in two distinct values for the relative -fold change in protein expression between the yeast grown under nitrogen-limited *versus* carbon-limited conditions. In theory these values should be equal, and any variation observed must be due to intrinsic variation in the DiGE methodology.

To allow analysis of the in-gel separated proteins, the gels were poststained with silver. Upon comparison of the individual images, no differences concerning spot position were seen. We observed, however, some differences between the staining methods, resulting in some spots being preferably "stained" by either of the two (data not shown). Comparison of the linear dynamic range of CyDye and silver staining showed DiGE to have a range of at least 4 orders of magnitude while that of silver staining had a maximum of 2 orders of magnitude, in agreement with previously reported data (54).

In this comparative evaluation, we picked a number of spots on the 2D gel (Fig. 2) that ranged over the whole gel (with extensive variation in mass and pI) and varied over a wide range of protein expression levels (as determined by the DeCyder analysis). These protein spots were excised, and after tryptic digestion, the proteins were identified by peptide mass fingerprinting using a MALDI-TOF mass spectrometer. Of the spots analyzed, we selected 20 spots that originated exclusively from a single protein as revealed by mass spectrometric analysis (*i.e.* all peptides observed in the mass spectra originated from that protein), excluding the possibility that the spot intensity as measured by DiGE originated from more than one protein. The measured ratios of protein expression obtained by DiGE of these 20 selected proteins are given in

as described in Table I. A mixture of CyDye-labeled proteins was prepared and run on a single 2D gel. 20 protein spots of interest were excised, digested in-gel with trypsin, and analyzed with MALDI-TOF-MS to identify the protein. Protein expression ratios were determined in two ways: first, via comparing the fluorescence spot intensities (DiGE); and second, via comparison of mass spectrometry peak areas of the unlabeled *versus* ^{15}N-labeled peptide.

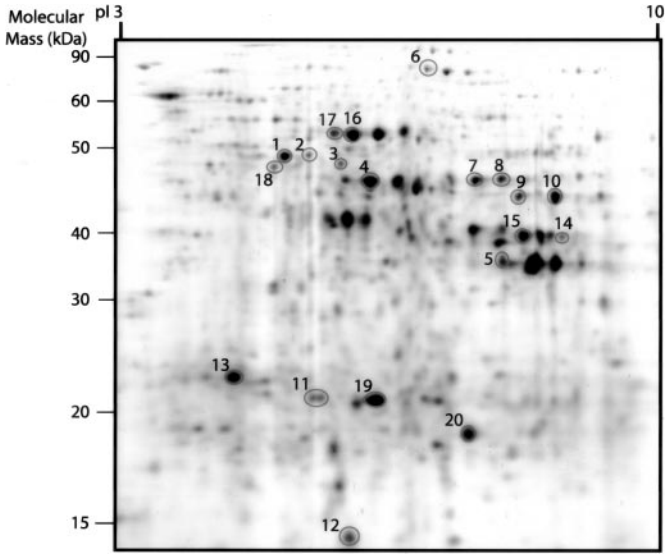

FIG. 2. **Typical example of a 2D gel of a 1:1 mixture of protein extracts from chemostat-grown yeast cells limited for either nitrogen or carbon.** 150 μg of protein extract was separated in the first dimension on a 24-cm immobilized pH gradient strip (pH 3–10 NL) and by 12.5% SDS-PAGE in the second dimension. Protein spots that were excised and analyzed by mass spectrometry are *numbered* and correspond to the numbers in Table II.

Table II. Protein identifications of these 20 protein spots are given in Table II and revealed that the 20 spots corresponded to 12 different proteins with seven proteins appearing in more than one spot on the 2D gel, indicating the presence of protein isoforms and/or post-translational modifications. Peptides carrying a fluorophore modification on a lysine residue were not observed in our MALDI-TOF spectra; this was expected as only a very small percentage (<3%) of the proteins are labeled.

As each selected spot contains proteins from the isotope-labeled and unlabeled cultures, the resulting MALDI peptide fingerprint mass spectra displayed numerous peptide pairs (Fig. 1). Differential quantification was achieved by comparing the peak areas of the natural isotope-containing and ^{15}N-labeled peptides averaged over all peptide pairs available. Thus, using this metabolic stable isotope labeling approach, we determined the relative protein expression levels between the yeast samples grown under nitrogen-limited *versus* carbon-limited conditions in duplicate. The protein expression ratios measured by stable isotope labeling and the number of peptides pairs on which quantification is based, as well as the interprotein quantification standard deviation are also given in Table II.

In Fig. 3 we have zoomed in on a few spot/protein exam-

TABLE II

Protein expression ratios (nitrogen-limited versus carbon-limited) as determined by DiGE (R_{DiGE}) and stable isotope metabolic labeling ($R_{Met.Lab}$)

In total 20 spots were relatively quantified on two 2D gels with both DiGE and stable isotope metabolic labeling. The spot numbering corresponds with the numbering in Fig. 2. The protein identity, Swiss-Prot accession number, the observed pI and approximate molecular mass, and the sequence coverage for each protein spot are indicated. In R_{DiGE} column 1 and 2 the two ratios for the protein expression levels as obtained by the DiGE image analysis of the two separate gels are shown followed by the average of the two values (Av) and the standard deviation. Similarly, in $R_{Met.Lab.}$ column 1 and 2 the two ratios (Av) as well as the interprotein standard deviation and the number of peptide ion peak pairs (No.) on which the relative quantification is based are indicated. In the last two columns the average ratio (Av) and standard deviation for the protein expression levels as obtained by the two metabolic stable isotope labeling experiments are given. A negative ratio indicates a down-regulation of the protein in the nitrogen-limited culture, and a positive value indicates an up-regulation of the protein in the nitrogen-limited culture.

Spot	Protein	Swiss-Prot	pI	Molecular mass	Sequence coverage	R_{DiGE}				$R_{Met.Lab.}$							
						1	2	Av	SD	1			2			Av	SD
										Av	SD	No.	Av	SD	No.		
				kDa	%												
1	Gdh1p	P07262	5.56	49.5	30	1.87	1.73	1.8	0.1	1.29	0.13	4	1.22	0.09	4	1.26	0.05
2	Gdh1p	P07262	5.58	49.5	38	1.27	1.3	1.29	0.02	1.27	0.13	2	1.51	0.00	2	1.39	0.17
3	Gdh1p	P07262	5.62	49.5	40	1.94	1.52	1.73	0.3	1.25	0.13	4	1.26	0.10	4	1.26	0.01
4	Eno2p	P00925	5.67	46.8	39	1.62	1.49	1.56	0.09	1.07	0.34	4	1.4	0.27	4	1.24	0.23
5	Tdh3p	P00359	6.49	35.5	35	1.26	1.6	1.43	0.24	1.11	0.06	4	1.22	0.07	4	1.17	0.08
6	Met6p	P05694	6.07	85.8	26	1.97	1.8	1.89	0.12	1.83	0.15	2	1.3	0.04	2	1.57	0.37
7	Eno1p	P00924	6.17	46.5	54	−1.27	−1.22	−1.25	0.04	−1.58	0.27	3	−3.57	0.39	3	−2.58	1.41
8	Eno1p	P00924	6.45	46.5	31	−1.8	−1.73	−1.77	0.05	−1.83	0.21	3	−2.44	0.24	3	−2.14	0.43
9	Pgk1p	P00560	7.09	44.6	53	1.01	1.07	1.04	0.04	1.33	0.26	3	1.16	0.45	3	1.25	0.12
10	Pgk1p	P00560	7.58	44.6	39	1.17	1.33	1.25	0.11	1.02	0.03	4	1.01	0.05	4	1.01	0.01
11	Tpi1p	P00942	5.75	26.6	33	1.6	1.46	1.53	0.1	1.85	0.35	4	1.5	0.19	4	1.68	0.25
12	Sod1p	P00445	5.63	15.5	71	1.01	1.06	1.04	0.04	1.05	0.05	4	1.08	0.52	4	1.07	0.02
13	Hsp26p	P15992	5.31	24	43	−5.99	−6.86	−6.43	0.62	−12.98	46.7	5	−34.44	12.5	5	−23.7	15.20
14	Adh2p	P00331	6.26	36.6	27	−9.83	−13.3	−11.6	2.44	−9.65	52.3	3	−25.17	42.8	3	−17.4	11.00
15	Adh2p	P00331	7.15	36.6	17	−14.4	−41.4	−27.9	19.2	−23.86	16.7	3	−37.97	12.5	3	−30.9	9.98
16	Pdc1p	P06169	5.8	61.4	35	2.75	2.45	2.6	0.21	4.23	3.23	4	4.99	1.40	4	4.61	0.54
17	Pdc1p	P06169	5.6	61.4	34	3.46	2.77	3.12	0.49	10.30	5.30	4	20.36	20.14	4	15.3	7.11
18	Arg1p	P22768	5.53	47	28	1.4	1.22	1.31	0.13	1.30	0.17	4	1.44	0.24	3	1.37	0.10
19	Tpi1p	P00942	5.75	26.6	28	1.75	1.76	1.75	0.02	1.51	0.21	5	1.54	0.16	5	1.53	0.04
20	Tdh3p	P00359	6.49	18	24	−1.07	−1.12	−1.09	0.05	−1.22	0.15	2	−1.31	0.10	2	−1.26	0.05

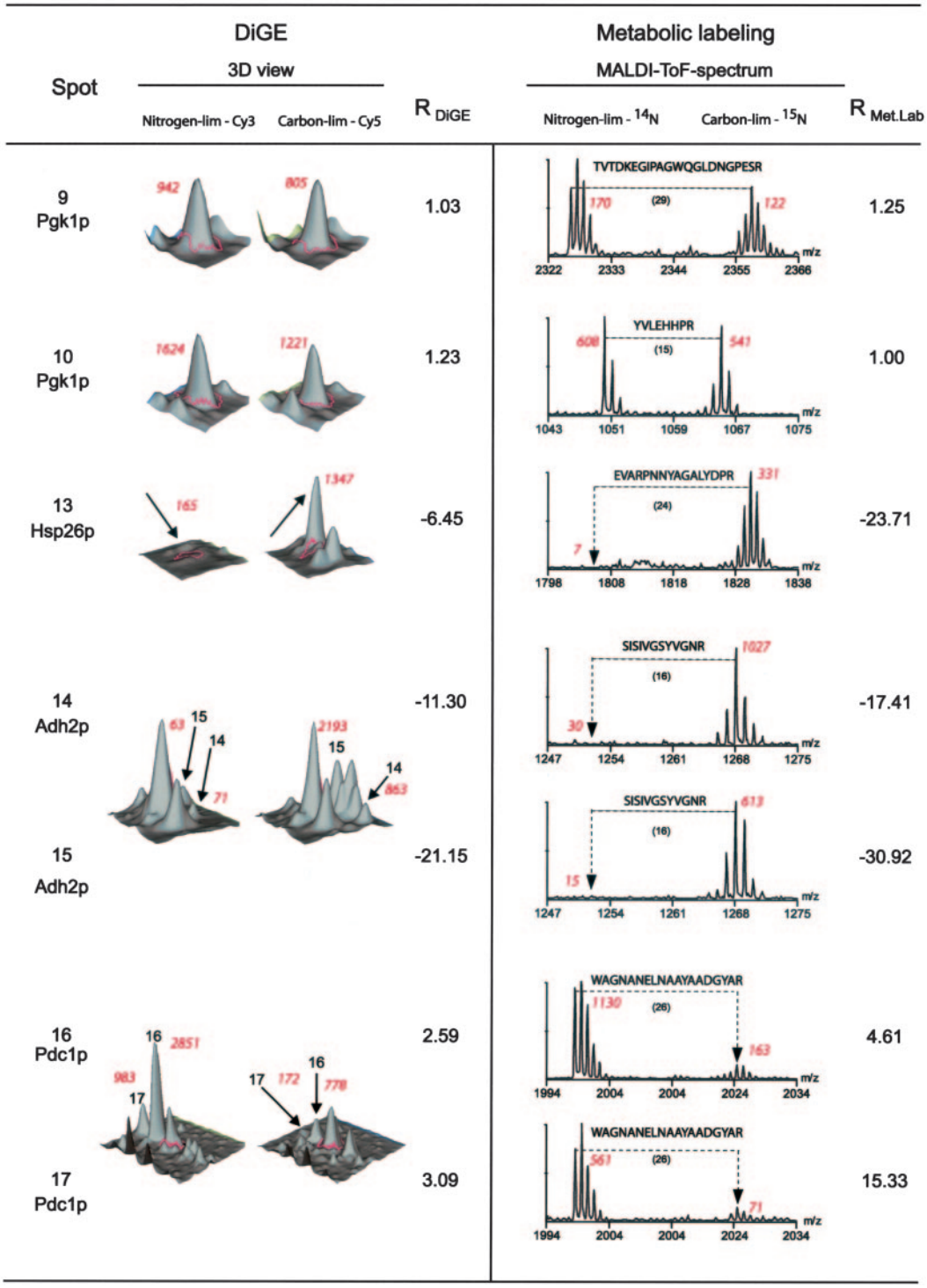

FIG. 3. **Typical examples of relative protein quantification by DiGE and metabolic stable isotope labeling.** On the *left*, next to the *column* with the *spot number* and *protein name*, three-dimensional (*3D*) views of the fluorescent intensities of the spots are shown. In the given example, protein spot intensities in the nitrogen-limited (*lim*) chemostat culture were visualized using Cy3 fluorescence, and protein spot intensities in the carbon-limited chemostat culture were visualized using Cy5 fluorescence. On the *right*, a typical tryptic peptide ion pair of the same protein spot measured by MALDI-TOF-MS is shown. The sequence of this peptide is indicated, and in *parentheses* the number of nitrogen atoms of that particular peptide is indicated. Protein ratios, as defined under "Experimental Procedures," obtained by both methods (R_{DiGE} and $R_{Met.Lab.}$) are indicated next to the three-dimensional views and the peptide ion pairs, respectively. For both quantification methods, the signal-to-noise ratio for the individual protein signals (DiGE) and peptide signals (metabolic stable isotope labeling) are given in *red italic script* revealing that for proteins present at low concentration the S/N ratios are generally smaller in the mass spectrometric approach.

ples, showing that both with DiGE and stable isotope labeling a wide range of differential expression ratios can be determined. Three-dimensional views of the fluorescent abundance of a protein spot in yeast grown under nitrogen limitation (Cy3) and carbon limitation (Cy5), next to a typical peptide ion peak pair measured in the MALDI-TOF spectra of the same protein spot are given. We also depict in Fig. 3 the results for two proteins that appeared in multiple spots on the gel, *i.e.* Adh2p and Pdc1p.

DISCUSSION

High throughput proteome analyses, in which thousands of proteins are analyzed in a single experiment, call for design standards and guidelines that enable proper validation of the quality of the proteome data set and the conclusions drawn from the results. Recently, two reports appeared that address these issues and suggest parameters to judge the quality both of peptide and protein identification data (62) and of 2D gel-based proteomic analyses (63). These are important steps in the direction of quality control and validation. A next step that is equally essential is the cross-comparison and validation of different quantification techniques that are in use in proteomics. The aim of this report is to contribute to this process, and therefore we assessed in a direct comparative approach two currently popular quantification methods. The results of this comparison are comprehensively given in Table II, with some illustrative detailed experimental results depicted in Fig. 3.

Comparison of DiGE Versus Metabolic Stable Isotope Labeling—A more direct comparison of both DiGE and stable isotope labeling methods for quantification of proteins is shown in Fig. 4. Performing both the DiGE and metabolic stable isotope labeling quantification in duplicate provides a measure for the experimental standard deviation in the quantification by both methods. For both DiGE and the metabolic stable isotope labeling experiments, the average ratio of the two separate measurements was determined and is given in Table II as well. These average ratios were used for a comparison between the DiGE- and stable isotope labeling-based quantification. Therefore, we divided the average ratio determined by metabolic stable isotope labeling by the ratio determined by DiGE for all 20 proteins. Theoretically, when both methods would provide accurate quantitative results, these values should be 1 for all individual protein spots. These divided ratios of the 20 spots are plotted in Fig. 4*A* sorted by descending -fold change values. Inspecting Fig. 4*A*, it is clear that the ratio between the -fold changes observed by DiGE and metabolic stable isotope labeling are indeed close to 1 in particular when the ratio in protein expression between yeast grown under nitrogen-limited *versus* carbon-limited conditions is between −3 and 3. When we plot the average ratio determined by DiGE *versus* the one measured by stable isotope labeling, as shown in Fig. 4*B*, we find for this limited set of data (taking 15 of the 20 spots) a good correlation. The data could be fitted with a linear relationship between the two determined ratios following the equation $R_{\text{Met.Lab.}} = 0.98R_{\text{DiGE}}$ with a r^2 value of 0.89 where $R_{\text{Met.Lab.}}$ is the average ratio determined by metabolic stable isotope labeling, and R_{DiGE} is the average ratio determined by DiGE. The obtained coefficient of 1 and the correlation r^2 value of 0.89 indicate that the differential quantification by metabolic stable isotope labeling and DiGE are within the margin of error equivalent.

The five spots outside the limited range described above are the "on-off" spots that show "extreme" -fold changes in protein expression. For these particular spots we observe that the calculated *R* is primarily determined by the low signal-to-noise (S/N) values of the "off" spot of either the fluorescent intensities on the DiGE gels or the peptide ion signals. Our data indicate that especially for the proteins present at low concentration the S/N values in the mass spectrometric approach are generally smaller than those in the DiGE approach (see Fig. 3), and therefore the ratio *R* is more difficult to accurately determine in the mass spectrometric approach. These smaller S/N values also lead to larger standard deviations for the calculated protein ratios *R* in the metabolic stable isotope labeling approach compared with fluorescent labeling (see Table II). Overall this demonstrates that DiGE may be better suited to determine changes of proteins present at low concentration and proteins that show extreme changes in expression.

Stable isotope labeling has experienced a dramatic increase in popularity in recent years in quantitative proteomic applications (43, 44, 50, 64–68), to some extent replacing the conventional 2D gel-based approaches. This is probably due to the fact that at present stable isotope labeling is considered as one of the most accurate ways to relatively quantify protein expression levels, and additionally stable isotope labeling can be used in combination with (multidimensional) LC tandem MS approaches. As described in the Introduction, in stable isotope labeling there are quite a few alternative approaches, both by chemical introduction of the isotope label (*e.g.* ICAT (29) and iTRAQ (41)) and biological introduction of the label (^{15}N or ^{13}C metabolic labeling (50) and stable isotope labeling by amino acids in cell culture (SILAC) (45, 47)). The advantages and disadvantages of the different stable isotope labeling approaches have been discussed in detail in several reviews (23, 44, 66). Here we just reiterate that some of the major advantages of the metabolic stable isotope labeling approach chosen here are that the label used for quantification is introduced very early on in the procedure (during cell growth), thereby decreasing the potential effect of differential losses in subsequent steps during sample preparation, and additionally all proteins, and even all peptides, are uniformly labeled, increasing the probability that proteins may be quantitated by a larger set of peptide pairs, which is essential for accurate quantification. The latter is at present a major limitation in the stable isotope labeling approach whereby in most reported experiments so far the quantification of proteins is often only based on a single or just a few peptides per protein,

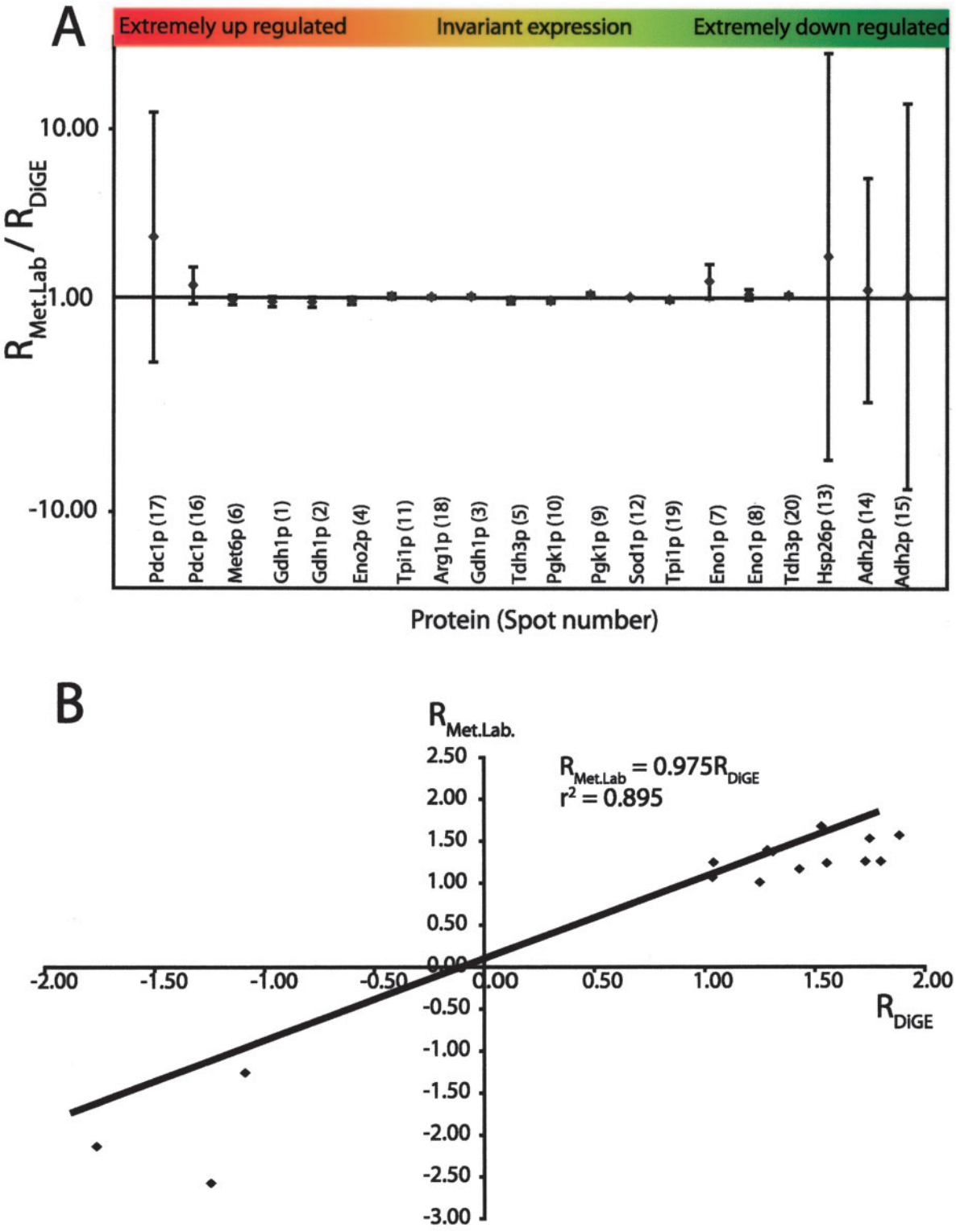

FIG. 4. **Correlation between the average ratios obtained by DiGE (R_{DiGE}) and metabolic labeling ($R_{Met.Lab.}$).** *A*, the ratios obtained by metabolic stable isotope labeling are divided by the ratios obtained from the DiGE analysis ($R_{Met.Lab.}/R_{DiGE}$) and centered around 1, the value expected for a optimal correlation. *B*, correlation plot of the ratios obtained by DiGE (*x axis*) against the ratios obtained via metabolic stable isotope labeling (*y axis*). Extreme ratios (on-off spots), which show large standard deviations, were excluded from this graph, leaving only ratios between −3 and 3.

hampering a meaningful error analysis in the quantification.

This study, and other DiGE experiments, reveal that with the implementation of preseparation fluorescent dyes for protein labeling, an alternative method capable of determining both small and large changes in protein expression has been added to the quantitative proteomic toolbox, producing accurate differential expression data. Compared with more conventional staining methods used in 2D gel electrophoresis, DiGE has a large dynamic range, allowing both the differential analysis of abundant proteins and proteins present at low concentration. In the differential analysis of individual proteins DiGE is probably even better than the stable isotope labeling approach whereby the S/N level in the latter is largely dependent on the sensitivity and accuracy of the mass spectrometer used and the complexity of the sample analyzed. Concerning the sensitivity and limits of stable isotope labeling and DiGE, we observed that protein spots with low concentration that still could be detected and quantified using DiGE could not be detected and thus also not quantified using the mass spectrometry-based approach.

Another clear advantage of DiGE over metabolic stable isotope labeling is the general applicability: all protein samples irrespective of their origin (*e.g.* clinical samples) can be labeled as long as they contain lysine residues. In that sense metabolic stable isotope labeling is limited to more simple uni- and multicellular organisms. In that respect chemical introduction of stable isotopes such as in ICAT and iTRAQ are not hampered by this limitation.

A disadvantage of the DiGE technology is that proteins quantified on the gel still need to be identified and therefore that subsequent mass spectrometric analysis still is required. Additionally, as with every 2D gel-based technology, only subsequent analysis of protein spots, by for instance mass spectrometry, can reveal whether the spot of interest is "pure," *i.e.* originating from just one protein. If two or more proteins do co-migrate on the gel relative quantification is impossible. However, in general this report shows that the quantifications by metabolic stable isotope labeling and DiGE are in very good agreement. Interestingly, the combined approach of stable isotope labeling and DiGE has, in addition to the achieved 2-fold quantification/validation, some other unique advantages particularly in that some of the disadvantages of each of the methods are compensated by the other.

The illustrative examples in Fig. 3 not only show that both methods provide similar results in up- and down-regulation but also directly point out some intrinsic advantages of the combined 2D gel and stable isotope labeling approach used here. For instance, spots 14 and 15, both identified as Adh2p, differ only in pI, thereby indicating that these proteins are most likely isoforms and/or post-translationally modified. Both forms are extremely up-regulated under carbon-limiting conditions. Also spots 16 and 17 are identified as "identical" proteins, *i.e.* Pdc1p, and differ only in pI. Interestingly, although both these Pdc1p isoforms are significantly down-regulated under carbon-limiting conditions, both DiGE and stable isotope labeling indicate that spot 17 is more down-regulated than spot 16 (Table II and Fig. 3). In particular these data reveal an advantage of using 2D gel approaches instead of the direct analysis of total cell lysate digests by, for instance, a combination of stable isotope labeling and multidimensional LC. In this latter approach the WAGNANELNAAYAADGYAR peptide used for quantification of spots 16 and 17 (see Fig. 3) would be analyzed only once, and a single ratio averaged over the different protein isoforms would be determined, leading to erroneous quantification.

Another strong advantage of the combined approach of stable isotope labeling and DiGE is that on the one hand the mass spectrometric analysis can be used to ensure that the spot on the gel originates from only one protein, excluding co-migrating proteins in the analysis. On the other hand digestion of the gel-separated proteins directly provides multiple peptide pairs originating from the same protein isoform, facilitating quantitative analysis.

Comparison of Gel-based Versus Non-gel-based Technologies—Although the combined stable isotope labeling and DiGE approach has advantages, certain disadvantages linked to both methods remain. The classical 2D gel electrophoresis protein separation method is labor-intensive, hard to automate, and a technical variation-sensitive approach. Additionally, despite the high resolution separation capabilities of the 2D gel approach, certain classes of proteins (hydrophobic proteins or those with high molecular weights and/or extreme pI values) are normally underrepresented in these analyses, and moreover the risk of overlapping proteins is introduced, hampering quantification of the individual proteins.

Alternative separation approaches based on liquid chromatographic separation of peptides resulting from proteolytically digested proteins from complete lysates, such as for example in multidimensional protein identification technology (MUDPIT) (69, 70), were originally thought to replace 2D gel-based approaches as they generally lead to higher throughput and wider coverage of the full proteome. However, the direct LC-based approaches also have their own intrinsic disadvantages as they are more difficult to use for quantitative proteomics and for the analysis of protein isoforms. For instance, identification and quantification of post-translationally modified proteins or protein isoforms is, in direct LC-based approaches, only possible when the actual modified peptide is detected, significantly reducing the chance of quantification of the different protein forms. With all these pros and cons of the gel- and non-gel-based approaches it is becoming increasingly clear that both LC- and gel-based technologies are more or less complementary, not only in protein identification, but particularly in protein quantification.

In summary, both gel-based and liquid chromatography-based methods have their advantages and remaining challenges in quantitative proteomics. The metabolic stable isotope labeling and DiGE approach, comparatively assessed here, are

both able to provide efficiently accurate and reproducible differential expression values for proteins in two or more biological samples and may therefore find wide applications in proteomic research. Combining the two methods not only allowed a direct validation of the two methods but also revealed unique strong features particularly in that some of the disadvantages of each of the methods could be compensated by the other.

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