Gel-free and Gel-based Proteomics in *Bacillus subtilis*

A COMPARATIVE STUDY水

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The proteome of exponentially growing *Bacillus subtilis* cells was dissected by the implementation of shotgun proteomics and a semigel-based approach for a particular exploration of membrane proteins. The current number of 745 protein identifications that was gained by the use of two-dimensional gel electrophoresis could be increased by 473 additional proteins. Therefore, almost 50% of the 2500 genes expressed in growing *B. subtilis* cells have been demonstrated at the protein level. In terms of exploring cellular physiology and adaptation to environmental changes or stress, proteins showing an alteration in expression level are of primary interest. The large number of vegetative proteins identified by gel-based and gel-free approaches is a good starting point for comparative physiological investigations. For this reason a gel-free quantitation with the recently introduced iTRAQ™ (isobaric tagging for relative and absolute quantitation) reagent technique was performed to investigate the heat shock response in *B. subtilis*. A comparison with gel-based data showed that both techniques revealed a similar level of up-regulation for proteins belonging to well studied heat hock regulons (SigB, HrcA, and CtsR). However, additional datasets have been obtained by the gel-free approach indicating a strong heat sensitivity of specific enzymes involved in amino acid synthesis. *Molecular & Cellular Proteomics* 5:1183–1192, 2006.

Two-dimensional polyacylamide gel electrophoresis invented by O’Farrell (1) and Klose (2) is undoubtedly still the gold standard to separate complex protein mixtures. This technique allows the mass spectrometric identification of hundreds of proteins after their separation on a two-dimensional (2D) gel, thereby covering an essential portion of “low complexity” proteomes such as those of bacteria. However, there are limitations to 2D PAGE that make certain classes of proteins inaccessible. Gel-critical properties include extremes in pI and molecular mass, but the most significant shortcoming is certainly the poor separation of proteins showing a pronounced hydrophobicity. The dynamic range in protein concentration that can be covered by 2D PAGE regarding non-radioactive staining methods spans 3–4 orders of magnitude, whereas the protein concentration in human blood serum extends at least 9 orders (3). For this reason a simple 2D gel approach is insufficient to analyze entire proteomes, including very low abundance proteins. The challenges inherent to a gel-based approach point to a demand for alternative techniques.

Although the characterization and quantitation of stained protein spots on 2D gels was introduced decades ago and further developed to date (4–6), attempts of protein identification and concurrent quantitation exclusively based on mass spectrometry have emerged over the last years (7). Initially the combination of multidimensional chromatography and tandem mass spectrometry that became known as shotgun proteomics was used to identify hundreds of proteins out of highly complex peptide mixtures (8–12). Very soon the first gel-free methods arose allowing a relative quantitation of proteins from different samples. A common technique is the use of stable isotope labeling of proteins or peptides, mostly realized by chemical linking of tag and biomolecule. Here one of the sample sets is provided with a “light” tag, whereas the others are linked to heavy isotope-enriched variants of the tag. Although almost all of the previously established methods (13–15) make use of a quantitation procedure based on ion signal intensity observed at the MS level, the recently introduced isobaric tagging for relative and absolute quantitation (iTRAQ™) supports a quantitation based on reporter ion signals observed at the MS/MS level that is linked with several advantages (16). First of all, the differential labeling of peptides does not challenge scan rates of mass spectrometers because the complexity at the MS level is not increased. Second, the detection of peptides originating from low abundance proteins is facilitated by the addition of ion currents of equal but differentially labeled peptides in MS spectra. On the one hand the requirement of MS/MS experiments only allows a quantitation of peptide signals exceeding a given threshold, but on the other hand the unambiguous identification of a peptide becomes more likely because it is not only based on...
the determination of its peptide mass fingerprint. Moreover, iTRAQ reagent technology surpasses other gel-free quantitation methods with the capability of performing multiplex experiments in which up to four different conditions can be compared.

In this study we used shotgun proteomics to investigate the cytosolic proteome and a semigel-based approach to identify membrane proteins of vegetative *Bacillus subtilis* cells. Specifically, iTRAQ reagents were used to analyze the heat shock response in *B. subtilis* as a model. Because heat shock is the best characterized stress of this organism and is associated with a substantial change in proteome signatures (17–19), it was chosen for the initial application of iTRAQ reagent technology. Resultant quantitative datasets were verified by a simultaneous protein quantitation based on 2D gels.

**EXPERIMENTAL PROCEDURES**

Sample Preparation for a Gel-free Identification of Proteins—*B. subtilis* 168 wild type (20) was grown aerobically at 37 °C in a synthetic medium (21). Cells were harvested in exponential growth phase at an *A*₅₀₀ of 0.5. After centrifugation (8000 × *g* for 10 min at 4 °C) cell pellets were washed twice with water and again centrifuged. Cell lysis was performed in a French press (minicell, SLM Aminco, Rochester, NY). The lysate was centrifuged (20,000 × *g* for 30 min at 4 °C), and protein concentration of the supernatant was determined using Roti-Nanoquant (Roth, Karlsruhe, Germany). For the removal of impurities, proteins were extracted with phenol, precipitated by the addition of ice-cold acetone, and incubated overnight at −20 °C. Proteins were pelleted by centrifugation (20,000 × *g* for 30 min at 4 °C), air-dried, resuspended in 30 mM NH₄HCO₃, and digested with trypsin (Promega, Madison, WI), which was reconstituted prior to use as suggested by the manufacturer. After digestion for 16 h at 37 °C, peptides were subjected to ultracentrifugation (100,000 × *g* for 16 h at 4 °C). Aliquots of the supernatant were stored at −20 °C.

Preparation of Membrane Proteins with Subsequent 1D SDS-PAGE Separation—The purification of membrane proteins, their separation via 1D SDS-PAGE, and their in-gel digestion was carried out as described by Eymann et al. (22). Peptides were separated via reverse phase chromatography in a 3-h gradient and analyzed by an LTQ FTICR mass spectrometer (see “2D LC” and “MS/MS Analysis”).

Sample Preparation with Subsequent iTRAQ Reagent Labeling—*B. subtilis* cells grown in the synthetic medium to an *A*₅₀₀ of 0.5 were stressed by a sudden temperature shift to 52 °C. Cell harvest occurred shortly before (control) and at 10, 30, and 60 min after continuous heat shock. Cell pellets were washed twice with ice-cold 10 mM monium bicarbonate) was accomplished by drying the peptide solution via vacuum centrifugation. An amount of 3 μg of peptides was resuspended in SCX running buffer (25% (v/v) ACN, 0.1% formic acid) prior to loading onto a SCX column (μ-Precolumn™ cartridge, BioRad/SCX, 500-μm inner diameter × 15 mm, LC Packings, Amsterdam, Netherlands) using the Ettan™MDLC system (GE Healthcare). Elution of peptides off the SCX column was achieved by the injection of salt plugs of the following concentrations: 7.5, 12.5, 17.5, 25, 37.5, 50, 75, 150, 300, and 500 mM NH₄Cl. With a flow rate of 20 μl/min, collection time per salt fraction was set to 4 min. The SCX fractionation of iTRAQ reagent-labeled peptides had to be adjusted due to a different elution performance of peptides carrying a label. For this reason concentrations of NH₄Cl salt plugs were changed to 50, 100, 150, 200, 300, 400, 500, and 750 mM. The SCX fractions of which each contained 200–300 ng of peptides were subjected to a reverse phase separation carried out on an EttanMDLC system. Prior to MS analysis peptides were loaded onto a trap column (nano-Precolumn™, PepMap™, C₁₈, 300-μm inner diameter × 5 mm, LC Packings) that was washed for 15 min with buffer A (0.05% (v/v) acetic acid). Elution onto the analytical column (PepMap, C₁₈, 75-μm inner diameter × 15 cm, LC Packings) was achieved by formation of a binary gradient (3 h) of buffer A and buffer B (90% (v/v) acetonitrile, 0.05% (v/v) acetic acid) with a flow rate of 250 nl/min.

For MALDI MS/MS analyses buffer A contained 0.1% (v/v) TFA. Buffer B consisted of 90% (v/v) acetonitrile and 0.1% (v/v) TFA. Here the binary gradient was shortened to 70 min.

**MS/MS Analysis**—LC-ESI MS experiments were performed using the Qstar® Pulsar system (Applied Biosystems MDS Sciex) and an LTQ (linear ion trap) FTICR mass spectrometer (Thermo Electron Corp., San Jose, CA).

The Qstar system was used to carry out a survey scan in the mass range of *m/z* 230–2000 in the first step. Exercising dynamic exclusion, up to four precursor ions exceeding a total ion current of 10 counts were selected for a fragmentation in MS/MS experiments. Product ions were detected in the range of *m/z* 70–2000. The LTQ FTICR mass spectrometer was used to acquire a full FT survey scan in the range of *m/z* 300–2000. Subsequently MS/MS experiments of the three most abundant precursor ions were carried out in the LTQ instrument. Meanwhile the masses of the precursor ions were determined with high accuracy via single ion mode scans in the ICR cell of the mass spectrometer.

For MALDI-TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems MDS Sciex) mass spectrometry a Probot™ microfraction collector (LC Packings) was used to spot LC-separated peptides onto a MALDI target with a rate of 20 s/spot. The LC flow of 250 nl/min was mixed with matrix consisting of 2 mg/ml α-cyano-4-hydroxycinnamic acid in 70% (v/v) acetonitrile and 0.1% (v/v) TFA in a ratio of 1:5. The 4700 Proteomics Analyzer acquired MS spectra in a window of *m/z* 900–3700. The three most abundant precursor ions having a signal-to-noise ratio higher than 150 were chosen for MS/MS fragmentation, which was performed using medium collision energy.

**Data Analysis**—For an identification of proteins from LTQ FTICR data, the SEQUEST algorithm Version 27.12 (Thermo Electron Corp. (23)) was used to perform database searches against a *B. subtilis* database extracted from SubtiList (genolist.pasteur.fr/SubtiList/). A mass deviation of 0.01 Da for precursor ions as well as for fragment ions and one missed cleavage site of trypsin were allowed. The

**TABLE I**

Allocation of iTRAQ reagents

| Unit mass of iTRAQ reagent reporter ion in Da | Sample  |
|--------------------------------------------|---------|
| 114                                        | Control |
| 115                                        | 10 min, 52 °C |
| 116                                        | 30 min, 52 °C |
| 117                                        | 60 min, 52 °C |
oxidation of methionine was considered during the search. The search result was filtered using BioWorks 3.2 (Thermo Electron Corp.). A multiple threshold filter applied at the peptide level consisted of the following criteria: (a) peptide sequence length: 7–30 amino acids; (b) RSp ≤ 4; (c) percentage of ions: 70 (70% of all theoretical b- and y-ions of a peptide are experimentally found); (d) Xcorr versus charge state: 1.90 for singly charged ions, 2.20 for doubly charged ions, and 3.75 for triply charged ions. If the same MS/MS spectrum from the same scan was matched to different sequences, identification was assigned only to one protein that gave the better hit. Different forms of a peptide (charge state and modification) were counted as a single peptide hit. At the time this work was performed, a quantitation of iTRAQ reagent-labeled samples using the LTQ FTICR mass spectrometer was not yet possible. Therefore, only data for the purpose of protein identification were obtained.

iTRAQ reagent experiments on the Qstar system were evaluated with the software packages Pro QUANT 1.0 and Pro GROUP 1.0.2 (Applied Biosystems MDS Sciex). A mass deviation for precursor and fragment ions of 0.2 Da was permitted during the search against the B. subtilis database. Only top hit peptides within a confidence interval (C.I.) of 95% and a quantitation error factor below 2 were taken into account. Thereby the error factor defines the quantitative 95% C.I. of a ratio, which is the range within which the true protein ratio is 95% likely to fall. The 95% C.I. for quantitation is calculated as follows.

\[
\text{lower C.I.} = \frac{\text{protein ratio} - 1.96 \times \text{standard deviation}}{\sqrt{2}}
\]

\[
\text{upper C.I.} = \frac{\text{protein ratio} + 1.96 \times \text{standard deviation}}{\sqrt{2}}
\]

For proteins quantitated with one peptide in the Qstar system analyses, error factor calculation was based on the occurrence of this peptide in different experiments.

The processing of spectra obtained with the 4700 Proteomics Analyzer was carried out with the software GPS ExplorerTM Version 3.5 (Applied Biosystems). The database search was mediated by the Mascot® Version 2.0 search engine (Matrix Science Ltd., London, UK). Mass errors of 150 ppm for precursor ions and 0.2 Da for fragment ions were allowed. Only proteins that had been identified and quantified with at least two different top hit peptides within a C.I. of 95% were used for further statistical filtering. To achieve uniform error estimation with Qstar system results, standard deviations given by GPS Explorer were converted to the quantitative 95% C.I. using the formula

\[
95\%\ C.I. = (\mu - 2\sigma, \mu + 2\sigma)
\]

where \(\mu\) = experimental protein ratio and \(\sigma\) = standard deviation.

Proteins whose 95% C.I. showed an error factor larger than 2 were omitted. In database searches of 4700 Proteomics Analyzer data and also of Qstar system results, one missed cleavage site of trypsin, the oxidation of methionine, and a possible iTRAQ reagent labeling of tyrosine residues were considered. Samples of two independent heat shock experiments were measured in 2D LC-ESI Q-TOF as well as 2D LC-MALDI-TOF/TOF analyses. For protein quantitation the arithmetic mean of technical and biological replicates was calculated. Pro QUANT 1.0 as well as GPS Explorer Version 3.5 only determined ratios if all four iTRAQ reporter ions could be detected and if the total peak area exceeded a value of 40 counts.

2D Gel Electrophoresis—2D gel electrophoresis was carried out as described by Büttner et al. (24). To prevent a saturation of spots of high abundant proteins in the gel-based quantitation, 100 \(\mu\)g of protein were loaded onto IPG strips (pH range 4–7, Amersham Biosciences) in the first dimension. Gels were stained with colloidal Coomassie Brilliant Blue G-250 (Amersham Biosciences). For the processing of gel images and gel-based relative quantitation of protein spots the software Delta2D Version 3.3 (Decodon, Greifswald, Germany) was used. 2D PAGE was carried out for two independent heat shock experiments running a replicate for each sample. Quantitative data of this work are the arithmetic mean of technical and biological replicates.

RESULTS

Identification of Proteins Using 2D LC-MS/MS and 1D Gel-LC-MS/MS in Comparison with Standard 2D PAGE-MS/MS—The cytosolic protein fraction of exponentially growing B. subtilis cells was analyzed using a non-gel-based approach. Two-dimensional chromatography coupled to tandem mass spectrometry resulted in the identification of 814 proteins of which 535 were identified with two or more different peptides (see Supplemental Table 3, A and B, for the list of proteins and their physical properties and their proof of identification, respectively).

Because the probability to assign false positive protein hits is much higher for protein identifications based on one peptide, the false positive rate was estimated by performing a reverse database search. Applying the aforementioned filter criteria this search revealed 50 proteins (Supplemental Table 3C), all of them identified by one peptide, leading to an error rate of 6.1%. Furthermore if an identification by two or more peptides was required a reverse database search gave no result. In conclusion the 535 proteins based on at least two peptide identifications represent highly reliable hits. Thirty-nine of the one-peptide identifications could be verified by additional peptides found in 1D gel-LC-MS/MS experiments of the membrane proteome fraction of growing B. subtilis cells. The remaining 240 one-hit wonders detected by 2D LC-MS/MS will require further analyses to ensure their certain identification.

The 1D gel-LC-MS/MS analysis of membrane proteins as a
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### Table II

Proteins altered in amount after heat shock and their relative quantities obtained by iTRAQ and 2D PAGE

Proteins increased in amount were assigned to the known classes of heat shock proteins. The ones with quantity decline were allocated to functional groups and transcriptional units if possible. Protein names in boldface are subject to quantitative changes of a factor of 4 or higher. Protein names in italic have not been identified via 2D PAGE until now. Function of the proteins is according to the SubtiList database. For proteins decreased in amount a trend of transcriptional regulation is given in the column “Array” (19) with the following symbols: −−/− for repression/induction by a factor of 3 or higher; −/− for repression/induction by a factor of at least 2; −/+ means no significant change in gene expression.

| Protein | iTRAQ Gel | Number of modified forms | Charge | Mass | Function | Array |
|---------|-----------|--------------------------|--------|------|----------|-------|
|         | 10 min    | 30 min  | 60 min | 10 min | 30 min  | 60 min |
| HrcA-controlled genes |
| DnaK    | 1.62      | 1.98   | 1.84   | 1.58   | 1.85    | 2.30   | 2 2  | Class I heat shock protein (chaperonin) |
| GroEL   | 2.14      | 2.85   | 3.04   | 3.07   | 4.32    | 5.20   | 2 4  | Class I heat shock protein (chaperonin) |
| GroES   | 2.33      | 3.38   | 3.29   |        |         |        | 2 0  | Class I heat shock protein (chaperonin) |
| SigmaB-dependent genes |
| Ctc     | 4.31      | 5.54   | 6.23   | 3.01   | 2.87    | 3.00   | 1 1  | General stress protein |
| GsiB    | 3.39      | 5.90   | 5.27   | 11.93  | 16.57   | 17.40  | 0 0  | General stress protein |
| GspA    | 4.62      | 4.44   | 3.92   | 7.93   | 11.54   | 7.61   | 1 0  | General stress protein |
| RsbV    | 2.39      | 2.66   | 3.00   |        |         |        | 1 0  | Positive regulator of SigmaB activity (anti-anti-sigma factor) |
| RsbW    | 2.14      | 2.05   | 2.28   | 2.06   | 2.22    | 1.09   | 0 0  | Negative regulator of SigmaB activity |
| SodA    | 1.37      | 1.59   | 1.85   | 1.48   | 1.54    | 1.97   | 1 0  | Superoxide dismutase |
| TrxA    | 1.60      | 1.87   | 2.04   |        |         |        | 0 0  | Thioredoxin |
| Xpf     | 1.25      | 1.40   | 1.60   |        |         |        |      | RNA polymerase PBSX sigma factor-like |
| YdaG    | 4.06      | 5.71   | 7.11   |        |         |        | 0 0  | Unknown; similar to general stress protein |
| YifT    | 4.36      | 5.31   | 4.74   |        |         |        | 0 0  | Unknown; similar to general stress protein |
| YkzA    | 2.67      | 3.73   | 4.51   | 4.54   | 8.84    | 7.56   | 0 0  | Unknown; similar to general stress protein |
| YraA    | 1.41      | 1.49   | 2.42   | 2.12   | 3.50    | 3.90   | 0 0  | Unknown; similar to general stress protein |
| YtxH    | 1.85      | 2.43   | 2.14   | 1.62   | 5.81    | 3.85   | 0 0  | Unknown; similar to general stress protein |
| YvgN    | 1.44      | 1.79   | 2.25   | 1.37   | 1.71    | 2.29   | 0 0  | Unknown; similar dehydrogenase |
| YvxD    | 3.29      | 4.05   | 3.85   | 5.59   | 7.43    | 7.51   | 0 0  | Unknown; similar to ribosomal protein S30AE family |
| YwdJ    | 1.23      | 1.23   | 1.55   |        |         |        |      | Unknown; similar to unknown proteins |
| ClpC*   | 2.23      | 2.84   | 3.06   | 3.48   | 6.26    | 4.03   | 3 0  | Class III stress response-related ATPase |
| ClpE    | 3.72      | 4.76   | 1.59   | 23.31  | 2.32    | 3.41   | 1 0  | ATP-dependent Clp protease-like |
| ClpPp   | 1.83      | 3.09   | 4.25   | 2.79   | 6.11    | 7.40   | 1 1  | ATP-dependent Clp protease proteolytic subunit |
| Other mechanisms |
| AhpC    | 1.25      | 1.42   | 1.52   | 1.68   | 1.87    | 1.86   | 2 2  | Alkyl hydroperoxide reductase (small subunit) |
| CopZ (YvgY) | 1.51 | 2.48 | 2.81 | | | | |
| HtpG    | 1.65      | 2.24   | 2.73   | 1.24   | 0.79    | 3.66   | 2 0  | Heat shock protein (chaperonin) |
| IoIS    | 1.32      | 1.65   | 1.96   | 1.33   | 1.83    | 2.23   | 0 0  | myo-inositol catabolism |
| YitW    | 1.44      | 1.61   | 1.67   |        |         |        |      | Unknown; similar to unknown proteins |
| YjoA    | 1.26      | 1.66   | 2.32   |        |         |        | 0 0  | Involved in coat protein formation, similar to RsfA (42) |
| YlbO    | 1.33      | 1.71   | 1.51   |        |         |        |      | Unknown; similar to NADH-dependent butanol dehydrogenase |
| YtpP    | 1.20      | 1.38   | 1.56   |        |         |        |      | Thioredoxin-like protein, under Spx control (43) |
| YugJ    | 1.28      | 1.50   | 1.71   |        |         |        | 0 0  | Unknown; similar to NADH-dependent butanol dehydrogenase |
A gel-free and gel-based proteomics approach was used to analyze the proteome of Bacillus subtilis. A semigel-based technique resulted in 453 protein identifications, 265 of which were based on two or more peptides. A reverse database search of the datasets from 1D gel-LC-MS/MS experiments led to 16 protein hits, leaving the 453 protein identifications with a false discovery rate of 5%. Table II—continued

| Protein | iTRAQ Gel | Gel |
|---------|-----------|-----|
|         | 10 min | 30 min | 60 min | 10 min | 30 min | 60 min | Charge | Mass |
| Proteins decreased in quantity |
| Branched-chain amino acid synthesis |
| \(ilvB\) operon |
| LeuA | 0.76 | 0.50 | 0.43 | 0.61 | 0.37 | 1 | 1 | 2-Isopropylmalate synthase |
| LeuC | 0.40 | 0.14 | 0.12 | 0.50 | 0.13 | 0.08 | 1 | 0 | 3-Isopropylmalate dehydratase (large subunit) |
| LeuD | 0.49 | 0.22 | 0.19 | 0.55 | 0.12 | 0.08 | 0 | 0 | 3-Isopropylmalate dehydratase (small subunit) |
| IlvA | 0.89 | 0.65 | 0.50 | 0 | 0 | 0 | 0 | 0 | Threonine dehydratase |
| IlvE (YwaA) | 0.62 | 0.18 | 0.17 | 0.70 | 0.48 | 0.38 | 1 | 0 | Unknown, similar to branched-chain amino acid aminotransferase |
| YkwC | 0.64 | 0.32 | 0.30 | 0 | 0 | 0 | 0 | 0 | Unknown; similar to 3-hydroxyisobutyrate dehydrogenase |
| S box regulon |
| cysH operon |
| CysC | 0.85 | 0.53 | 0.40 | 0 | 0 | 0 | 0 | 0 | Probable adenylylsulfate kinase |
| Sat | 1.04 | 0.79 | 0.35 | 0.78 | 0.08 | 0.12 | 1 | 0 | Probable sulfate adenylyltransferase |
| mtnK operon |
| MtnK (YkrT) | 0.50 | 0.19 | 0.17 | 0.52 | 0.30 | 0.17 | 1 | 0 | Methylthioribose kinase (37) |
| MtnA (YkrS) | 0.80 | 0.47 | 0.42 | 1 | 1 | 1 | 0 | 0 | Methylthioribose-1-phosphate isomerase (37) |
| MetC (YjcJ) | 0.93 | 0.62 | 0.42 | 0 | 1 | 0 | 0 | 0 | Cystathionine \(\beta\)-lyase (44) |
| MetE | 0.32 | 0.14 | 0.11 | 0.20 | 0.05 | 0.03 | 5 | 3 | Cobalamin-independent methionine synthase |
| MtnD (YkrZ) |
| ArgG | 0.85 | 0.52 | 0.41 | 0.89 | 0.41 | 0.21 | 1 | 0 | Argininosuccinate synthase |
| ArgJ | 0.92 | 0.64 | 0.43 | 0 | 0 | 0 | 0 | 0 | Omithine acetyltransferase/aminoo-acid acetyltransferase |
| Other |
| CspC | 0.43 | 0.18 | 0.11 | 0 | 0 | 0 | 0 | 0 | Cold shock protein |
| DivIVA | 0.74 | 0.46 | 0.39 | 1.18 | 0.43 | 0.21 | 1 | 0 | Cell-division initiation protein (septum placement) |
| Efp | 0.82 | 0.57 | 0.47 | 0.78 | 0.31 | 0.17 | 1 | 0 | Elongation factor P |
| FusA | 0.69 | 0.31 | 0.32 | 0.70 | 0.56 | 0.40 | 2 | 2 | Elongation factor G |
| PpiB | 0.81 | 0.38 | 0.26 | 1.59 | 0.37 | 0.37 | 1 | 0 | Peptidyl-prolyl isomerase |
| RplM | 0.48 | 0.69 | 1.33 | 0 | 0 | 0 | 0 | 0 | Ribosomal protein L13 |
| YciC | 0.34 | 0.13 | 0.12 | 0.49 | 0.10 | 0.11 | 1 | 1 | Unknown; similar to unknown proteins |
| YjD | 0.61 | 0.65 | 0.49 | 0.51 | 0.11 | 0.25 | 3 | 0 | Unknown; similar to NADH dehydrogenase |
| CspB | 0.88 | 0.59 | 0.35 | 0 | 0 | 0 | 0 | 0 | Major cold shock protein |
| PdxT (YaaE) | 0.82 | 0.47 | 0.37 | 1 | 0 | 0 | 0 | 0 | Glutamine amidotransferase subunit |

\(a\) iTRAQ ratios showed an error factor larger than 2 due to high signal-to-noise values caused by strong alterations in quantity. The 95% C.I. of all iTRAQ datasets is given in Supplemental Table 4A.

\(b\) Also under control of SigmaB.

\(c\) Gel-based data with a relative standard deviation higher than 30%.

A reverse database search of the datasets from 1D gel-LC-MS/MS experiments led to 16 protein hits, leaving the 453 protein identifications with a false discovery rate of 5%.
positive rate of 3.5% (Supplemental Table 4C). A fraction of 51.2% of the 453 proteins possessed transmembrane domains (25) indicating that not all cytosolic proteins could be removed in the course of the isolation procedure of membrane proteins. Still this semigel-based study revealed 204 proteins that were not accessible by standard 2D PAGE-MS/MS or 2D LC-MS/MS. Combining current protein identifications of all three approaches gave rise to a number of 1218 proteins. Fig. 1 shows the allocation of protein identifications to the single techniques as well as the overlap between them.

Proteins Altered in Quantity after Heat Shock—Having the capability of identifying hundreds of proteins from complex peptide mixtures provides the basis to carry out global quantitative proteome studies, which is one major concern in terms of unraveling cellular physiology. The recently introduced iTRAQ reagent technique was used to investigate the cytosolic proteome fraction in the heat shock response of *B. subtilis* as a well characterized physiological model (17–19).

Most of the proteins showing increases in quantity in response to heat shock are members of well characterized classes of heat shock proteins (Table II) and are therefore not extensively discussed at this point. They are either under control of the transcriptional repressor HrcA (26–28), belong to the SigmaB regulon (29–33), are repressed by CtsR (34, 35), or are subject to another, still unknown mechanism of regulation. Although the number of up-regulated proteins reported here only represents a fraction of the entirety of all heat-induced proteins (18), the fact that the iTRAQ approach assessed their increased concentration verifies the reliability of the gel-free method. The non-gel-based technique further justified its potential by the identification of six heat-induced proteins (CopZ, Xpf, YitW,
YlpO, YtpP, and YwdJ that to our knowledge have not been accessible by 2D PAGE to date. Xpf and YwdJ are known to be under SigB control, but the heat induction mechanisms of the remaining four proteins are still unknown.

Nearly all of the proteins that were found to be degraded after heat shock assume "housekeeping" functions in the cell (Table II). In addition to enzymes associated with nucleotide metabolism, ribosomal proteins, and elongation factors, the number of proteins involved in the biosynthesis of important amino acids is most striking. These enzymes could be assigned to three major groups, the S box regulon (36, 37), arginine biosynthesis, and synthesis of branched-chain amino acids. Their quantitation profiles are given in Fig. 2, A–C, respectively.

**iTRAQ Reagent-based Quantitation of Proteins Confirmed by 2D PAGE**—To evaluate iTRAQ reagent-obtained data, a comparison with the well-established quantitation via 2D PAGE was drawn. For this purpose, extracts originating from the same heat shock experiments were subjected to separation on 2D gels, and protein spots were subsequently quantitated using the software Delta2D. Applying qualitative and statistical filters 2D LC-MS/MS analyses of the iTRAQ reagent-labeled samples resulted in the reliable quantitation of 292 proteins (see Supplemental Table 5, A and B, for relative protein ratios and their proof of identification, respectively).

Only proteins that were subject to an alteration in their amount by a ratio outside of 0.5–1.5 at one of the time points determined by iTRAQ technology will be discussed below. Regarding proteins with no change in quantity, datasets of both approaches were in good correspondence (data not shown). The criterion applied to 63 proteins, revealing either an increment or reduction in their amount after heat shock (Table II). Thirty-two of the proteins could be localized on 2D gels acquired in parallel. Overall protein quantities determined at the peptide level via iTRAQ reagents correlated very well with gel-based quantitation at the protein level (Fig. 3). Proteins showing discrepancies in quantity need to be looked at in greater detail, for instance at their appearance on the 2D gel. In the following a few representative examples will be given that demonstrate the sound agreement of the two different quantitation methods but also the pitfalls of which one has to be aware.

The gel-based quantitation of proteins showing only one distinct spot on the gel agreed mostly with the data obtained by the iTRAQ reagent method. YvgN (unknown; possible dehydrogenase; SigmaB-controlled (29)) with an increased amount and NadA (quinolinate synthetase) with a reduced amount are members of this group of proteins. In contrast, the chaperone GroEL and elongation factor G (FusA) scattered over more than one spot on the gel posing a challenge to the gel-based quantitation. Proteins with a change in concentration by more than a factor of 4 were categorized as "on/off" proteins (38). At one of the time points they appeared only with a very low signal-to-noise ratio on the 2D gel. The consequence was a distorted gel-based quantitation result leading to protein ratios that were much higher ("on" for protein GsIB (general stress protein)) or lower ("off" for protein MetE (cobalamin-independent methionine synthase)) than gel-free derived data. Comparative quantity profiles for the exemplary proteins are given in Fig. 4, A–C.

**DISCUSSION**

The proteome of exponentially growing *B. subtilis* cells was extensively investigated by Eymann et al. (22). By the use of the conventional 2D PAGE these authors were able to identify 745 proteins. The present study describes the expansion of the comprehensive vegetative proteome map of *B. subtilis* by
473 proteins. A gel-free analysis of cytosolic proteins and a semi-gel-based technique for the dissection of the membrane proteome enabled the identification of proteins obviously not accessible by 2D PAGE. With an assumption of 2500 genes being transcribed under exponential growth conditions (22) half of the B. subtilis vegetative proteome has been covered now and is therefore ready for physiological exploration.

iTRAQ reagent technology was used to carry out quantitative studies on the heat shock response in B. subtilis. A simultaneously performed 2D gel quantitation as well as earlier published data (18) confirmed the correctness of the results obtained by the gel-free approach. Through the direct comparison of 2D gel and iTRAQ reagent quantitation, the strength and weaknesses of both approaches were disclosed. The iTRAQ reagent technique is most suitable to determine the sum of all subspecies of a protein. Consequently the problem of multiple protein spots or comigration of proteins inherent to a 2D gel quantitation is avoided. Aiming at a quantitation of single variants of a protein, 2D PAGE is currently the appropriate technique because modifications that result in a change of pl or molecular mass come to light on a 2D gel. A major concern in the relative quantitation of proteins is the question of dynamic range of the technique used. An exceedingly narrow range will cause difficulties in the study of proteins showing massively altered quantities. Although we found the iTRAQ reagent technique to have a wider dynamic range than a colloidal Coomassie-stained 2D gel, the problem of an incorrect quantitation due to very low signal-to-noise ratios for one of the samples remains a challenge of both the gel-based and the gel-free approach.

In addition to the expected up-regulation of the well known classes of heat shock proteins, we could provide new physiological insights on the heat shock response of B. subtilis. Despite intensive gel-based studies, the heat-induced large scale degradation of enzymes involved in amino acid anabolism has not been reported for this organism yet. Biran et al. (39) found MetA (homoserine transsuccinylase) of Escherichia coli to be inactivated upon heat shock and interpreted the

![Graphs showing quantitation results for YvgN, NadA, GroEL, FusA, GsiB, and MetE](Fig. 4).

Fig. 4. A, gel-based and non-gel-based quantitation of YvgN (unknown; similar dehydrogenase) and NadA (quinolinate synthetase). Both proteins appear as single spots on a 2D gel. B, correlation of iTRAQ reagent quantitation and relative protein amounts obtained by 2D PAGE for the proteins GroEL (chaperone) and FusA (elongation factor G), which form multiple spots on a 2D gel. C, gel-based and gel-free quantitation of the general stress protein GsiB and the cobalamin-independent methionine synthase MetE, both of which showed an extreme alteration in amount after heat shock.
heat sensitivity of this important enzyme in methionine bio-
chemistry as a control element of growth rate during heat
stress. The same purpose can be presumed regarding the
heat susceptibility of essential enzymes in amino acid synthe-
sis in B. subtilis.

Most interesting is a comparison between the protein quan-
titation of this survey and results from mRNA profiling exper-
iments by Helmann et al. (19). Although the concentration of
many proteins involved in methionine, arginine, and
branched-chain amino acid synthesis was decreasing, Hel-
mann et al. (19) determined several of the corresponding
genes to be induced after heat shock, some of them even in
a dramatic fashion (Table II). One has to note that expression
of these genes is controlled by a feedback mechanism in
which the metabolic product represents the molecular effec-
tor. Therefore, a decreasing quantity of the above mentioned
enzymes with the resulting deprivation of certain amino acids
most likely caused an increased transcription of the corre-
sponding genes after heat shock. The comparison drawn
between array data and relative protein amounts proved once
more that mRNA profiling alone is not sufficient to unravel
cellular and physiological adaptation mechanisms upon envi-
ronmental changes (40).

There remains the question about the fate of proteins found
to be reduced in quantity. Most likely those proteins are
denatured upon heat shock and are either prone to proteoly-
sis or to sedimentation during ultracentrifugation after cell
lysis. To answer this question a comparable experiment with
a mutant deficient in ClpP, the major cytosolic protease in B.
subtilis, was set up, and a qualitative analysis of the ultra-
centrifugation sediment was carried out (data not shown). Many
proteins reduced in quantity in the wild type but stable in a
clpP mutant after heat shock could be assigned as ClpP
substrates under chosen conditions. Several of the proteins
whose amounts decreased in the wild type as well as in the
clpP mutant at 52 °C could be identified from the ultra-
centrifugation sediment, indicating their heat-induced denaturation and subsequent sedimentation.

Overall results of this work demonstrated that the gel-based
approach in which 745 proteins were identified, the gel-free
studies revealing 814 proteins, and the 1D gel-LC-MS/MS
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