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FG Proteomik

Differential proteome analysis of selected lactic acid bacteria, stress response and database construction

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Contents

Abbreviations:

2D	two-dimensional
AA	acrylamide
APS	ammoniumpersulfate
Bis	bisacrylamide
%C	percentage of crosslinker in acrylamide solution
CAI	codon adaptation index
CBB	Coomassie Brilliant Blue
CID	Collision-induced Dissociation
CIRCE	controlled inverted repeat of chaperone expression
Clp	caseinolytic protease
CSP	cold shock protein
Cy	cyanine
Cys	cysteine
Da	Dalton
DIGE	difference gel electrophoresis
DMAA	dimethyl-acrylamide
DNA	desoxyribonucleic acid
DTT	dithiothreitol
ESI	electropspray ionization
e	expectation value (in MALDI-TOF MS analysis)
GRAVY	grand average of hydropathicity
h	hour
HSP	heat shock protein
HTML	hypertext markup language
IEF	isoelectric focusing
IPG	immobilized pH gradient
IPG-DALT	two-dimensional electrophoresis with immobilized pH gradient
IPS	internal pooled standard
LC	liquid chromatography
Lys	lysine
mA	milliampere
MALDI	matrix assisted laser desorption ionization

Met	methionine
MOPS	3-[N-Morpholino]propanesulfonic acid
MPa	megapascal
Mr	relative molecular mass
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NEPHGE	non equilibrium pH gradient gel electrophoresis
NHS	N-hydroxy succinimidyl
NR	non redundant
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PHP	PreHypertextProcessor
pI	isoelectric point
PIP	pressure-induced protein
PMF	peptide mass fingerprint
PRPP	5-phosphoribosyl-1-pyrophosphate
Q-TOF	quadrupole-time of flight
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
SQL	structured query language
%T	percentage of acrylamide in total
TEMED	N,N,N',N'-tetramethylethylendiamine
TOF	time of flight
Tris	tris(hydroxymethyl)-aminomethane
V	volt
v/v	volume per volume
W	watt
w/v	weight per volume

1 Introduction

On the first Siena 2D electrophoresis meeting in 1994, M. Wilkins publicly introduced the term proteome, by which he described the protein complement of the genome. Since the genome of an organism is almost completely static, while the proteome is highly dynamic, the analogy of genome and proteome is only superficial. The proteome varies in the way that i) not all proteins are expressed at the same time, ii) proteins are expressed in different amounts, iii) several forms of one protein occur due to post translational modifications, and iv) different cell types in multi-cellular organisms express specialized sets of proteins. The dynamics of protein expression is again highly dependent on the cellular state and the environment. Exactly these characteristics enable by analysis of the proteome molecular insights into cellular processes like stress response or adaptation.

Heat shock response in bacteria is a commonly accepted mechanism, by which they adapt to the extreme condition and cope with deleterious effects such as protein denaturation [1]. Although general schemes in heat shock response are observed, regulation and scope of such a response can be different. *E. coli* and *B. subtilis* serve as model organisms for Gram-negative and –positive bacteria, respectively. Much is known about the heat shock response of these model bacteria, but knowledge of the response for example in lactic acid bacteria is rather limited to the general scheme (reviewed in [2-4]). The influence of heat treatment on lactic acid bacteria is not less interesting, since these bacteria encounter heat treatments in food processing on a regular basis. *Lactococcus lactis* gained model status among the lactic acid bacteria and thus, is probably the best investigated example of them with respect to heat shock [3, 5-7]. Though, besides prominent heat shock proteins the scope of heat shock response in *L. lactis* is unknown, not to mention the regulation. For example, no counterpart for the alternate sigma factor σ^B, which regulates class two stress genes in the model organism *B. subtilis*, was identified in the genome of *L. lactis* [8]. Analyses of the heat shock response of *L. lactis* with current tools in proteomics provide a more comprehensive view of the scope of this response and promise in combination with the availability of a fully sequenced genome high identification rates.

High hydrostatic pressure is used in food processing as non thermal preservation technique [9-11]. Advantages of this technique are that flavor and

vitamins are less affected by high pressure than by heat treatment [10, 12]. Microorganisms are generally inactivated by high pressure treatment, though the pressure level for successful inactivation varies largely among different bacteria [13, 14]. In addition, certain environmental factors as well as physiological conditions increase the pressure resistance of bacteria [15-18]. The elucidation of deleterious effects of high pressure to bacteria, their adaptation and the potential to acquire resistance has just begun. Reports of unexpected pressure resistance in certain species demonstrate the great need in comprehension of such mechanisms to ensure safe food quality [19, 20]. Analysis of high pressure effects on bacteria at proteome level is highly potential to identify cellular mechanisms, which are activated in response to pressure stress. Few analyses at proteome level of high pressure response in bacteria have been published. Altered protein levels of heat or cold shock proteins have been observed, which indicate similar responses as under heat or cold shock [16, 21]. Indeed, high pressure causes similar effects as, for instance, heat shock causes protein denaturation and thus might explain an analog cell response [22]. Thus, the first analyses show promising results and encourage further investigations of high pressure response at proteome level.

1.1 Aim of the study

The analysis of heat shock response in *L. lactis* at proteome level was so far analyzed by radioactive pulse labeling [5-7] and in these cases, protein identification from 2D gels was moderately successful (reviewed in [23]). In the meantime, access to the complete genomic sequence of *L. lactis* [8] in combination with higher sensitivity in mass spectrometry improves the success rate in spot identification of 2D gels in *L. lactis* [24]. Furthermore, improved technologies in 2D electrophoresis are available, such as 2D electrophoresis with immobilized pH gradients (IPG-Dalt) and difference gel electrophoresis (DIGE) (reviewed in [25]). Therefore, one aim of the study was the analysis of the heat shock response of *L. lactis* with advanced proteomic tools to identify hitherto undetected and unidentified proteins involved in heat shock response. In particular, total protein expression was analyzed after heat shock with the DIGE technique as well as temporal protein expression under heat shock conditions analyzed with radioactive pulse labeling. DIGE is a relatively new technique in the field of proteomics. Therefore, the suitability of this technique for

application in stress analysis was investigated by comparison to results produced in parallel by pulse labeling and previous publications.

Analysis of the stress response of *Lactococcus lactis* and *Lactobacillus sanfranciscensis* to high hydrostatic pressure was another aim of the study to identify proteins, which are influenced in their abundance, isoelectric point or molecular mass due to the treatment. Thus, cellular systems, which are sensible to high pressure, and the way in which the bacteria cope with deleterious pressure effects might become apparent. Both organisms are lactic acid bacteria and were selected, because of their importance in the food industry. In case of *L. lactis*, DIGE analysis was again complemented by radioactive pulse labeling as in the analysis of heat shock. Here, pulse labeling was intended to present in particular proteins, which are increasingly expressed immediately after decompression. Thus, proteins are probably detectable, which are needed for the recovery of the bacteria due to deleterious pressure effects.

Previous proteomic studies of *L. lactis* were concentrated on the separation of proteins in the pH range from 4 to 7 (reviewed in [2, 23]). Based upon predicted isoelectric points, they probably neglected approximately 39% of the lactococcal proteome (see section 3.4.1, p. 89). Therefore, standardized protocols for the separation of alkaline proteins of *L. lactis* needed to be established, in order to pave the way for future analyses in this pH range. As another aim of the study, the optimized protocols were used to set up reference maps for alkaline proteins. These reference maps should be publicly available. Hence, final aim of the study was to initiate a proteome database for *L. lactis*.

1.2 Proteome analysis

Proteome analysis is dedicated to the survey of all proteins in a sample at a certain condition [26]. In consequence of the nature of the proteome, a method for proteome analysis needs to be capable of visualizing several thousand proteins, in order to provide a most complete overview. Two-dimensional electrophoresis meets this criterion and thus, seems to be highly potential for proteome analysis. Classical 2D electrophoresis was established by O'Farrel who used carrier ampholytes to separate proteins in the 1st dimension according to their isoelectric point (pI) and SDS PAGE in the 2nd dimension for separation according to the molecular weight (Mr) [27]. However, the application of carrier ampholytes limited the potential of the method, because the pH gradient was not stable and thus, protein separation was time dependent. In particular drifting of the alkaline carrier ampholytes towards the cathodic side and simultaneously formation of a plateau in the middle of the pH gradient were observed. Therefore, the method of non equilibrium pH gradient gel electrophoresis (NEPHGE) was proposed [28]. Though, the comparability of 2D patterns between different laboratories remained unsatisfying.

In current times, proteomics includes much more methods than the original proteome analysis by 2D electrophoresis. Nearly the complete range of methods in protein biochemistry as well as specialized applications downstream or upstream of 2D electrophoresis like protein fractionation or mass spectrometry joined the term proteomics (e.g. see [29]). Current methods in 2D electrophoresis as well as special analytical techniques applied in this thesis are briefly explained in the following sections.

1.2.1 2D electrophoresis with immobilized pH gradients (IPG-DALT)

2D electrophoresis according to Görg *et al.* [30] is one of the core technologies in proteomics and utilizes immobilines for establishing a pH gradient in the 1st dimension [31]. In contrast to the application of carrier ampholytes, the pH gradient is established while casting the gel and subsequently fixed by cross-linking to the acrylamide matrix. The immobilized pH gradient (IPG) gel can even be dried and easily distributed [32]. Nowadays, pre-cast IPG strips enable the access to 2D

electrophoresis for a broader scientific community. However, IPGs are not calculated, cast and applied from sketch. Buffer capacities in IPGs, solvents for proteins and customized protocols for sample entry or steady state focusing are just a few of many critical factors in isoelectric focusing (IEF) and needed optimization. In this field, Gorg *et al.* established many protocols for casting IPG gels, sample preparation and IEF (e.g. [30, 32-38]). By application of optimized protocols and IPGs, it was demonstrated that highly reproducible 2D patterns were obtained in different laboratories [39, 40].

One major advantage of utilizing immobilines is the possibility to zoom into overcrowded areas of protein patterns by the application of narrow pH gradients, which cover in extreme cases only 0.4 pH units over a distance of 24 cm [41]. With this method, up to several thousand proteins of *Saccharomyces cerevisiae* have been resolved [42]. Limitations of protein loading onto such narrow gradients have recently been overcome by prefractionation of the sample with Sephadex as matrix [41]. Finally, the resolving power of narrow IPGs successfully meets the demand in proteomics to cover thousands of proteins for the analysis of complete proteomes.

1.2.2 Focus on alkaline proteins in proteomics

Major improvements were also achieved for alkaline proteins, which provided severe problems in 2D electrophoresis utilizing carrier ampholytes (see section 1.2). Especially in the alkaline range, IPGs deliver the reproducibility needed for reference maps and comparative proteome analyses. Several IPG strips for the separation of alkaline proteins are available, either commercially or customized, such as IPG 6-12 for an overview of the alkaline range or IPGs 9-12 and 10-12 as zoom-in gradients for higher resolution [43, 44]. The need for zoom-in gradients was effectively demonstrated for example in yeast [42] or in the set-up of the reference map for *Lactococcus lactis* in the pH range from 4 to 7 [24]. In several recent publications, isoelectric focusing of proteins in the alkaline pH range is still described as nonstandard procedure [23, 45, 46]. This is mainly due to water transport to the anode, which is also commonly known as reverse electro-endosmosis, and migration of reducing agents, such as DTT. Among the suggestions for increasing the performance of alkaline IEF were for example application of N,N-dimethylacrylamide,

addition of isopropanol to the rehydration solution [43], replacement of reducing agent by tributylphosphine or hydroxyethyl disulfide [47, 48] and performing IEF at high voltage [44, 49]. Despite these novelties, optimization and standardization of IEF conditions are a necessity.

1.2.3 Advanced protein detection and quantification with fluorescent dyes

Since the concentration of individual proteins in a single cell differs largely, staining of the proteins with a method, which correctly represents their abundance, is a major challenge in proteomics. Furthermore, the occurrence of proteins with low copy number in a cell demands high sensitivity to protein staining. Advanced staining techniques with fluorescent dyes provide higher sensitivity and improved linearity in protein detection than silver or Coomassie Blue staining.

Two major approaches to fluorescent detection of proteins on 2D gels are currently practiced. These are i) covalent derivatization of proteins with fluorophores before IEF, and ii) post-electrophoretic protein staining by intercalation of fluorophores into the SDS micelles coating the proteins, or by direct electrostatic interaction with the proteins (reviewed in [25]). Typical examples for pre-electrophoretic stains are cyanine-based dyes (Cy dyes) that react with epsilon amino residues of lysine (see also 1.2.4). The most prominent post-electrophoretic stain is SYPRO RUBY^{TM}. The detection limit is approximately 1–2 ng protein per spot and the dynamic range for linear protein quantification reaches up to four orders of magnitude [50-53].

1.2.4 Difference gel electrophoresis (DIGE)

In case of DIGE, the attachment of two fluorescent labels (Cy3 and Cy5) to proteins obtained from different samples enables the comparison of these two samples in one gel [54]. Up to three Cy dyes are currently available, which enable the co-detection of as many as three samples in one gel. Protein patterns of fluorescent labeled samples are scanned by laser densitometry after consecutive excitation of the Cy dyes with different wavelengths (absorption maxima Cy2: 491 nm, Cy3: 553 nm and Cy5: 645 nm). Due to the co-migration of samples, the process of registering and matching in image analysis is simplified.

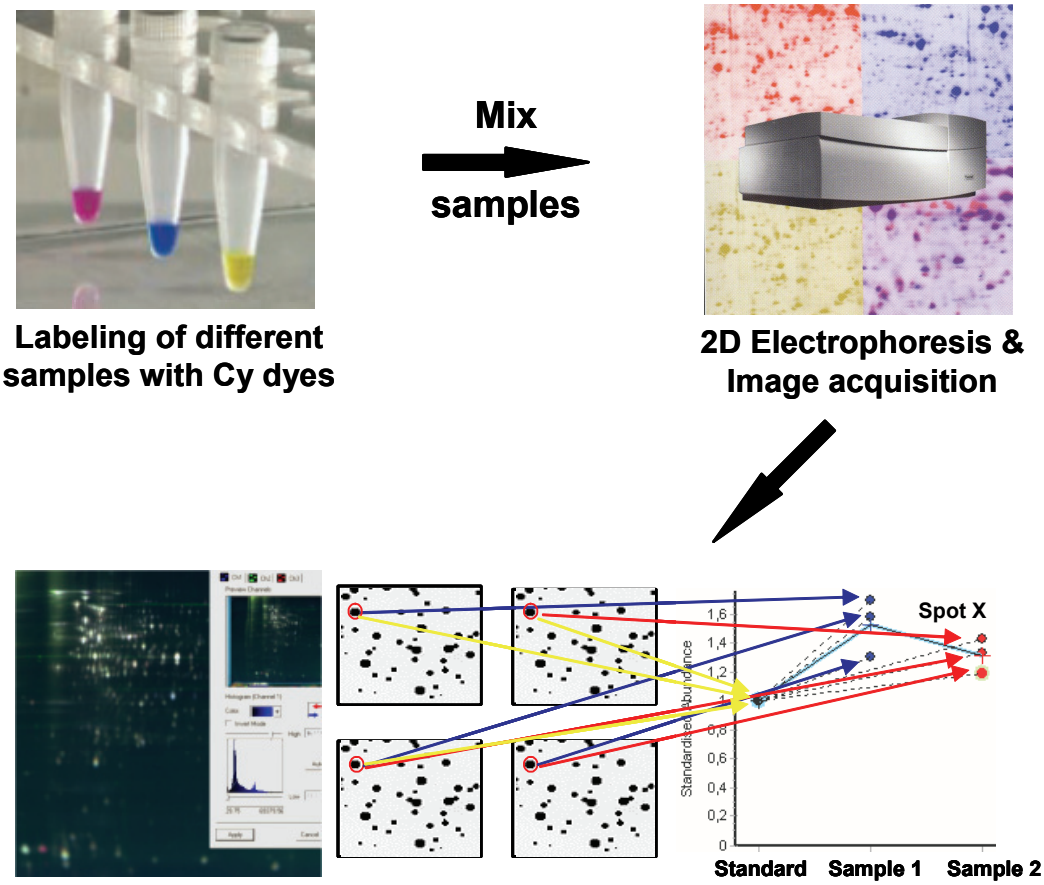

Fig. 1: Workflow in difference gel electrophoresis (DIGE)

Different methodical outlines for DIGE were evaluated including comparison of two samples in single gels or interconnection of gels, in which two samples were compared (reviewed in [25]). In the latter case, spot matching and gel to gel variation were still limiting and only 8 of 138 differences were observed in the comparison of control to toxin-treated mouse liver homogenates and using a set of six gels [55]. Recently, improved spot matching was demonstrated by introduction of an internal pooled standard (IPS) to each gel [56]. The IPS is a mixture of even parts of all protein extracts, which are compared in one set of gels. This mixture is Cy2 labeled and added to each gel of a comparative analysis. In the image analysis, spots are additionally normalized by comparison to the IPS. Since every gel contains an IPS, gel to gel variations are automatically considered by the normalization in proportion of

the IPS. Thus, highest significance for spot ratios in differential analyses can be achieved [57].

1.2.5 Radioactive labeling of proteins for analyses with 2D electrophoresis

Proteins can be labeled with radioactive isotopes such as ^{32}P, ^{14}C, or ^{35}S by the addition of radioactive metabolites such as ^{35}S-methionine to the medium. In general, this requires a chemically defined medium to adjust precisely the incorporation of labels. A special experimental approach to detect differences under certain conditions presents pulse labeling (Fig. 2). In this case, the radioactive label is added precisely at the time of interest, e.g. stress exposition, and labeling is stopped directly after a defined timeframe. By comparison of induced and reference conditions, alterations in protein expression can be detected, which are not detectable at total protein level (discussed in section 4.2, p. 109).

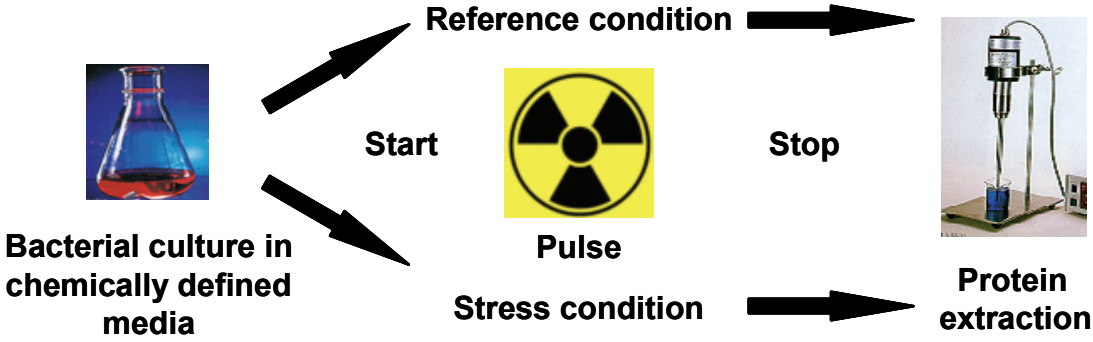

Fig. 2: Experimental outline for radioactive pulse labeling. The samples are labeled only during stress exposition and are compared to samples grown in parallel under reference condition.

Following 2D electrophoresis, radioactively labeled samples can be detected either by exposition to autoradiographic films or by phosphor imaging. Since the time for exposition is much shorter in phosphor imaging (days compared to weeks), this method is predominantly used. Furthermore, the dynamic range for protein quantification is linear up to five orders of magnitude and sensitivity is comparable to, or even better than, silver staining [58]. In phosphor imaging, dried gels are exposed to storage phosphor screens, which contain europium salts in a thin crystalline layer. In case of e.g. ^{35}S, beta particles excite electrons in the crystals and a latent image is

formed on the screen. The screen is then excited with red laser beams (e.g. 633 nm), which results in the emission of blue luminescence proportional to the original amount of radiation. Similar to fluorescent detection, the emission signal is intensified and quantified by a photomultiplier and detector unit.

1.2.6 Mass spectrometry in proteomics

Major advancements in proteome analysis at the level of protein characterization were provided by the development of electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) in mass spectrometry [59-62]. For their pioneering work in the development of these two ionization techniques, J.B. Fenn and K. Tanaka were awarded in 2002 with the Nobel Prize for Chemistry.

In combination with 2D electrophoresis, MALDI MS and ESI MS are in particular applied as downstream analytical methods for protein identification. In the present study, peptide mass fingerprinting with MALDI-TOF MS and determination of protein sequence fragments with LC-MS/MS were performed.

Peptide mass fingerprinting by MALDI-TOF MS provides a high throughput method for protein identification. Peptide mass fingerprints (PMF) are produced by chemical or enzymatic digestion of individual proteins and subsequent MS analysis of the obtained peptide fragments. The PMF is characteristic for each protein and thus, can be used for protein identification by comparison with predicted peptide masses [63-65]. As a result, the analysis of PMFs requires known protein sequences and is most successful for organisms with completely sequenced genomes. PMF is commonly performed with MALDI-TOF MS, which utilizes time of flight (TOF) mass analyzers. The TOF of ions in the mass analyzer is proportional to their molecular weight. High mass accuracies are achieved by delayed ion-extraction technique in combination with so-called reflectrons, which reflect ions in the mass analyzer by an ion mirror [66]. This high performance makes it possible to identify proteins with fewer peptide masses than before and thus, was used in the present thesis.

If protein or genomic sequences are not available for an organism, proteins can be identified by sequencing and comparison to sequence databases. The advantage of sequencing by liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) is that several sequence fragments are obtained and thus, N-terminal blockage presents not a problem as in Edman chemistry. Similar to PMF, individual proteins subjected to LC-MS/MS are first digested and a fragment spectrum is

produced. Then, fragments are automatically or manually selected (precursor ion) for further fragmentation induced by collision gas. Collision-induced Dissociation (CID) of precursor ions produces specific fragments, for which Roepstorff *et al.* proposed a common nomenclature (Fig. 3) [67]. Interpretation of CID MS/MS spectra provides for each precursor ion the protein sequence (reviewed in [68]). Finally, the deduced sequences are compared with protein or genome sequence databases to identify the protein by homology [69-71].

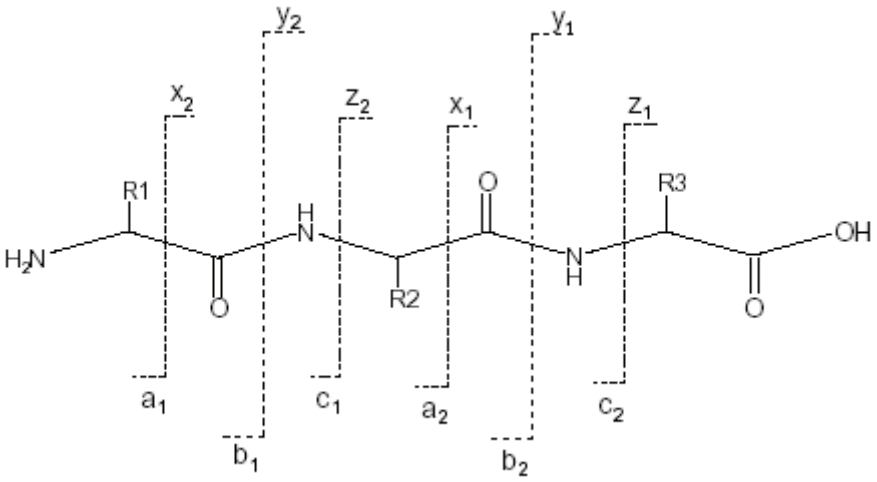

Fig. 3: Nomenclature for sequence ions in mass spectrometry [67]

1.2.7 Databases dedicated to 2D electrophoresis

The analysis of several thousand proteins at the same time in one experiment presents an immense task in 2D electrophoresis. For this reason, several software tools for the analysis and presentation of scanned 2D gels were developed. Gel analysis is covered by programs such as the ImageMaster 2D, Melanie or the recently developed DeCyder, which was especially designed for analyzing multiple samples in one gel/DIGE experiments [56, 72, 73]. These software tools comprise complex algorithms for spot detection and quantification.

The problem of presentation of 2D gels in the manner of reference gels is solved by the construction of databases, in which protein information is connected to marked spots in gels. Online solutions range from databases, which cover multiple species such as the SWISS-2DPAGE database [74], to organism or even subproteome specific databases such as the database of the alkaline proteome of *S. cerevisiae* [49]. The majority of existing databases for 2D electrophoresis is enlisted

in the “World-2DPAGE” at http://www.expasy.org/ch2d/2d-index.html. The structure of some of these databases is based on static Internet pages programmed in Hypertext Markup Language (HTML), which provide clickable reference gels and brief information about the identified spots. Each spot is linked to one HTML page, which contains the spot information. In general, these pages do not support search functions, and in case of extending the spot information, each HTML page must be edited. Dynamic online solutions of relational database systems connect spot maps of reference gels to database fields and generate web pages containing the spot information in real time by accessing the relevant fields. In case of a database focused on pathogenic microorganisms for example, the database fields provide protein information such as predicted pI and Mr, several protein identifiers, etc. [75]. Relational database systems are extensible in batch, by the addition of new database fields and transferring of the new information for all proteins to the fields in one step. This option is comparable to the addition of a new column in a table, which contains a protein list in rows, and copying protein information for all proteins in one step to the new column. Despite the fact that dynamic database solutions enable extensive search functions, to our knowledge no complex search logic is currently implemented in online proteome databases. If a search function is implemented, it is generally limited to a full text search. Thus, the request, e.g. for all identified proteins in a particular pH or molecular weight range, is not supported.

Another aspect, which has not been taken into account, is the connection of the theoretical and experimental proteome. In general, the content of current 2D gel databases in the Internet is limited to protein information of identified spots. On the other hand databases, which provide complete theoretical proteomes such as the Proteome Analysis Database (PAD) [76] or tools, which calculate theoretical proteome maps [77] do not include experimental data. If newly identified spots are added later to the database, the complete protein entry needs to be created. The necessary repetitive input of data is a disadvantage of these database solutions, since it is time consuming and error prone. Furthermore, for scientists interested in not already identified proteins, a database limited to identified proteins is not practical. If such a database supports no search algorithm, a lot of time is lost before it becomes apparent that the protein of interest is not included in the database.

1.3 *Lactococcus lactis* and *Lactobacillus sanfranciscensis*: two lactic acid bacteria

In consideration of high pressure as a relevant method in food processing, both organisms were selected because of their importance in the food industry. It was reported that cheese ripening can be selectively arrested by high pressure treatment and lactococcal enzymes involved in cheese ripening can be activated or inactivated dependent on the pressure level [78, 79]. Thus, high pressure for selective food preservation, for instance, is conceivable in the context of lactic acid bacteria as starter cultures.

Originally named *Bacterium lactis* and later *Streptococcus lactis*, the bacterium known nowadays under the name *Lactococcus lactis* is probably the best characterized lactic acid bacteria. The separation of mesophilic lactic streptococci from the genus *Streptococcus* and generation of the genus *Lactococcus* proposed by Schleifer *et al.* was based on nucleic acid hybridization studies and immunological relationships of superoxide dismutase [80]. Referring to the importance in dairy industry, an estimation of Teuber points out that approximately 100 million tons of milk are annually inoculated with *L. lactis*, which probably results in a microbial biomass of about 500 000 tons after fermentation [81]. After finishing the genomic sequencing project of *L. lactis*, the coccoid bacterium has become at the latest a model organism among lactic acid bacteria [8]. The stress response of *L. lactis* has been intensely investigated (reviewed in [2, 3]), but is still far away from being as well understood as in *E. coli* or *B. subtilis*. A detailed introduction to the heat shock response of *L. lactis* is given in section 1.4.1, p. 14.

Lactobacillus sanfranciscensis is a key lactic acid bacterium in sourdough fermentation [82, 83]. The rod-shaped bacterium received its name from the city where it was first isolated from sourdough and was originally named *Lactobacillus sanfrancisco* [84]. Due to the international code of nomenclature of bacteria also this organism was renamed [85]. In contrast to *L. lactis*, *Lactobacillus sanfranciscensis* is obligately heterofermentative, which indicates that sugars such as glucose are fermented to lactate, ethanol and CO_2. In presence of electron acceptors such as fructose or citrate, acetate is formed instead of ethanol, which contributes to flavor and shelf life of bread [86, 87]. Before this study started, no analyses on stress response of sourdough lactobacilli have been published to our knowledge. In 2001,

the first analysis on stress response was dedicated to acid stress and probably presented also the first 2D gels of *Lactobacillus sanfranciscensis* [88]. In this study, the levels of 63 proteins were determined to be relevant in context of acid stress and adaptation. One of them was identified by immunological detection as GrpE. N-terminal sequencing was successful for two proteins of which one showed 60% identity to the N-terminus of YhaH, which is an uncharacterized protein of *Bacillus subtilis*.

1.3.1 *Lactococcus lactis* in proteomics

Increasingly, *L. lactis* has been the subject of proteomic studies in recent years (reviewed in [2, 23]). In comparison to genomic studies, investigations at the proteome level provide insight for example into protein abundance or posttranslational modifications. Method of choice for the previously mentioned proteomic studies on *L. lactis* was 2D electrophoresis. Mapping of proteins is a first approach in 2D electrophoresis to open up the access for subsequent studies at the protein expression level. To our knowledge, up to now two reference maps of *Lactococcus lactis* were published [24, 89]. Both cover the pH range from 4 to 7, but while in the first map about 17 proteins were identified, the second includes more than 230 identified proteins. Most of the proteins in the latter study were identified by peptide mass fingerprinting. Since peptide mass fingerprinting is based on available protein sequences, this demonstrates the enormous impact of a sequenced genome on 2-DE, because at time of the first study sequencing of the genome of *Lactococcus lactis* was still underway.

After the analysis of the genome of *Lactococcus lactis,* 2266 putative proteins were annotated in the database (NCBI accession: NC_002662). Considering posttranslational modifications, even more proteins can be expected in *Lactococcus lactis*. Predicting the isoelectric point of all proteins included in the database reveals that 882 (40%) are located outside the pH gradient from 4 to 7. Seven (1%) have a predicted pI below 4 while the remaining 39% have pIs above 7, possibly leaving a large amount of proteins not investigated up to now in the alkaline pH range. Similar distributions of isoelectric points throughout the proteome can be found in other sequenced microorganisms using the tool JVirGel [77]. The alkaline part of the

proteome has already been successfully studied in some of these organisms, such as *Helicobacter pylori*, *Caulobacter crescentus* or S*accharomyces cerevisiae* [45, 46, 49]. This enforces the assumption that a great number of lactococcal proteins were not taken into consideration in proteome studies of this bacteria up to now.

1.4 Stress response of microorganisms

The response of microorganisms to major environmental changes like temperature or pH shifts is well investigated and known as stress response. Changes of transcription rates, translation products and/or metabolism are some of the mechanisms how cells react to what is called stimulus. Comprehension of such mechanisms has become interesting for the food industry because of the need of defined and stable starter cultures, and the effect of resistance formation and microbial adaptation during food preservation.

1.4.1 Heat shock response with focus on *Lactococcus lactis*

Heat shock response has proven to exist over a wide area of eukaryotes and prokaryotes and is characterized by the induction of a set of heat shock proteins. In *Lactococcus lactis*, the model organism for lactic acid bacteria, the heat shock response is characterized by strong induction of DnaK, GroEL and GroES [5, 6]. These proteins are prominent chaperones, which support folding and maturation of nascent or denatured proteins [90]. Several more proteins were reported to be induced and even more repressed by heat shock, but these have not been identified [5-7]. Thus, the scope of the heat stress response in *L. lactis* is not entirely solved. The expression level of HSPs varies differently during heat shock. Proteins such as DnaK or GroEL are quickly induced after the increase of temperature, while others are slowly induced [7]. It has been tried to derive the regulation of those proteins in *L. lactis* by comparison to *B. subtilis*, the model for Gram-positive bacteria.

For *B. subtilis*, four classes of heat inducible genes were defined according to their regulation. The first class is controlled by binding of the repressor HrcA to CIRCE elements (controlled inverted repeat of chaperone expression)[91, 92]. The second is regulated by the alternate sigma factor σ^B [93, 94] and the third by

interaction of the repressor CtsR (class three stress gene repressor) with the CtsR-box [95]. The regulation of the last group is unknown. In *L. lactis*, putative CIRCE elements are found upstream of *dnaJ*, *groES-groEL* and *hrcA-grpE-dnaK* [96-98]. Further analysis demonstrated that the CIRCE element upstream of *dnaJ* appeared to be necessary for thermal regulation [96]. On the other hand, unlike the dependency of HrcA activity on GroELS in *B. subtilis* [99] it was suggested that DnaK possibly has an influence on the heat shock protein expression by involvement in the maturation of HrcA [100]. Recently, it was demonstrated that *hrcA* of *L. lactis* cannot replace *hrcA* of *B. subtilis* [101]. This further stresses the difference between the two orthologues.

A comparable regulatory element such as the alternate sigma factor σ^B, which has a major role in regulation of stress response in *B. subtilis*, has not yet been discovered and only two similar factors (ComX and SigX) compared to 18 in *B. subtilis* were found after genome sequencing of *L. lactis* was completed [8].

Sequences homologous to the CtsR box were found in the promoter regions of all clp genes (*clpB*, *clpC*, *clpE* and *clpP*) of *L. lactis* except for *clpX* and a *ctsR* ortholog is located upstream of *clpC* as in *B. subtilis* [95]. ClpP (Clp: caseinolytic protease) is a serine protease [102], which degrades peptides less than seven amino acids long [103]. In association with Clp ATPases, specific substrates determined by the Clp ATPase subunit are degraded [104]. For some Clp ATPases, chaperone function was demonstrated [105, 106]. A *ctsR* mutant showed increased transcription rates of *clpC*, *clpP*, *clpB* and *clpE* mRNAs [107]. One further regulation of the Clp-dependent proteolysis activity was suggested to exist by TrmA [108].

Besides HrcA and CtsR, heat shock regulation was also proposed for RecA by way of FtsH [109]. In that study, a *recA* mutant was thermosensitive and had diminished levels of DnaK, GroEL and GrpE, whereas FtsH was increased. FtsH (also called HflB) is a heat inducible metalloprotease and involved in the regulation of σ^{32}, a sigma factor involved in the control of heat shock response in *E. coli* [1]. In *L. lactis*, a mutant expressing C-terminal truncated FtsH was heat sensitive [110]. Screening for thermoresistant *recA* double mutants by insertional mutagenesis revealed that insertion in only seven genes conferred stress resistance [111]. Besides one uncharacterized gene (*trmA*), these genes were implicated in purine metabolism (*deoB*, *guaA* and *tktA*), phosphate uptake (*pstB* and *pstS*) and mRNA

stability (*pnpA*). Therefore, guanine and phosphate metabolic pathways were proposed to be implicated in stress response. This suggestion was supported by phosphate and guanine dependent abolishment of acid resistance in *pstS* and *guaA* mutants, respectively. Recently, the induction of *hdiR* by heat shock was demonstrated [112]. The *hdiR* gene encodes for a protein with LexA-like features and its induction by heat was eliminated in the *recA* mutant and reduced in the absence of *clpP*. This furthermore indicates the interconnection of *recA* and the heat shock response. In conclusion, several differences such as absence of the σ^B factor are recognizable between heat shock responses in *L. lactis* and *B. subtilis*, which indicate that regulatory stress systems might have evolved differently from the Gram-positive model.

1.4.2 High pressure effects on microorganisms

A recently rediscovered method for food preservation is the application of high hydrostatic pressure [9-11], which means severe stress to bacteria in food but reduces undesirable chemical changes of food components [12, 113]. Although the use of high pressure for preservation has already been described by Certes in 1884, only a few investigations with regard to protein expression under high pressure stress have been performed in comparison to other stresses, such as heat or cold shock [114, 115]. Previous investigations were mainly focused on inactivation kinetics [13, 116, 117], single proteins [22, 118, 119] or membranes [15, 120, 121] rather than on stress response/protein expression [114, 122, 123].

Several hundred MPa are necessary for inactivation of Gram-positive or –negative bacteria such as *Enterococcus facialis* or *Escherichia coli* [14]. Inactivation of bacteria at high pressure is, besides pressure level and duration, dependent on several factors like temperature, pH, osmolarity, sporulation, etc. [18, 124, 125]. Below 200 MPa, cells are able to survive and up to 50 MPa even capable to grow [126-129]. Some remarkable organisms known as piezophiles or piozotolerant show optimal growth rates at up to 94 MPa, but these were in general isolated from deep-sea environments [129, 130].

High pressure has various effects on cells and their biological systems, such as volume reduction in chemical reactions, protein denaturation or perturbations in membranes [22, 121, 131]. Therefore, microbial inactivation, adaptation and

immediate response to high pressure were analyzed at various levels. Morphological analyses showed that the cell envelope of *L. lactis* is damaged at high pressures and resulted in increased cell lysis and release of intracellular proteins within 24 h after pressure release [132]. In *E. coli*, the damage of cell envelope at high pressure was in particular described for exponentially growing cells, followed by release of protein and RNA to the extracellular medium [133]. Thus, damaging effects of high pressure on cell envelopes were suggested to be responsible for inactivation of the cells. Furthermore, the results emphasized the dependence of pressure resistance on the physiological state of the cells.

Other effects of high pressure seem to be more or less reversible after pressure release. At atmospheric pressure, *L. lactis* maintains a transmembrane pH gradient, which is disrupted at high pressure [134]. The reversibility of this disruption is dependent on pressure level and duration. In addition, the data correlated with cell inactivation by high pressure treatment. Pressure induced damages to macromolecules like proteins are in general reversible after pressure treatments up to 200 MPa (reviewed in [135]). This might explain the ability of bacteria to survive pressures up 200 MPa. Recent analysis of cell division in *E. coli* after high pressure treatment demonstrated that FtsZ polymerization and ring formation is reduced, and correlates with filament formation at high pressure [136, 137]. After releasing the cells from high pressure, reassembly of FtsZ rings and fragmentation of filaments indicated once more the reversibility of this pressure effects. On the other hand, the absence of FtsZ rings was also observed in *L. lactis* after pressure treatment, but not their reassembling [128].

1.4.3 Pressure resistant phenotypes

Reports of mutants and strains with higher pressure resistance compared to their counterparts shifted at the latest the focus of analyses from pure inactivation kinetics to the molecular mechanism by which bacteria respond and adapt to high pressure. For example *E. coli* is generally piezosensitive, but mutants and selected strains of this bacterium were isolated, which exhibited increased resistance to pressure inactivation [19, 138]. In case of the pressure resistant strains, the activity of stationary-phase-inducible sigma factor RpoS correlated with the level of pressure

resistance [139]. The different activity of RpoS was explained by heterogeneity in *rpoS* alleles. Piezotolerant mutants of *Listeria monocytogenes* lacked flagella, were elongated and indicated slower growth rates than the wildtype. Furthermore, they were multi-stress resistant to heat, acid and hydrogen peroxide treatment [20]. Later, the resistant phenotype was related to a single glycine codon deletion in the *ctsR* gene [140]. CtsR is a repressor of class three stress genes including *clp* genes and *ctsR* itself (see also section 1.4.1, p. 14). Since ClpC, ClpP as well as CtsR levels were increased in mutants compared to the wildtype and their level was not further inducible by heat shock, it was suggested that the *ctsR* mutants have lost their repressor activity.

1.4.4 High pressure stress analyses with 2D electrophoresis

Previous approaches with 2D-PAGE of organisms treated with high hydrostatic pressure are rare and hardly comparable, because of the different choice of organism and experimental conditions. To our knowledge the first study was conducted with the Archae bacterium *Methanococcus thermolithotrophicus* by Jaenicke *et. al.* [141]. In this study, they observed a decrease in the total protein expression and the additional appearance of several alkaline proteins after a pressure shift to 50 MPa for 10h growing in a defined medium, and by using ^{14}C-labeling and 2D-PAGE with carrier ampholytes in the first dimension. Welch *et al.* [21] also showed by [^{3}H]leucine incorporation that the protein synthesis in *E. coli* is nearly one order of magnitude reduced after a pressure shift to 55 MPa over a period of 6h. Furthermore, they presented the most intense response of 55 pressure-induced proteins (PIPs) of which some were heat shock proteins (HSPs) and others cold shock protein (CSPs). The bacteria were grown on defined medium (until steady state), but in their case pulse labeling for 30 min with TRAN-^{35}S has been carried out. The utilization of pulse labeling enables the display of proteins which are expressed just at the time of addition, e.g. stress induction, but it demands well manageable experimental conditions like a chemically defined medium (see also section 1.2.5, p. 8), which was not available for *Lactobacillus sanfranciscensis*. Regarding the decreased protein expression at high pressure, it was noted that the presented PIPs could include proteins whose synthesis was decreased to a lesser extent. The pressure response

of *E. coli* grown on minimal medium was also analyzed with silver staining for visualization of protein patterns [142]. By this approach the induction of four proteins upon appliance of 40 MPa to cells in the log phase were reported, while only two of these were induced in stationary phase cells. Referring to the positions on the gel, 2D-PAGE with carrier ampholytes for the first dimension was used here as well, they are part of the 55 PIPs detected by Welch *et al.* [21]. *Rhodosporidium sphaerocarpum*, a marine yeast, was also investigated in their study. This eukaryote showed no change at 20 MPa, one additional protein at 40 MPa and four induced proteins at 45 MPa pressure treatment.

Pressurization of *L. monocytogenes* at 200 MPa resulted in the increase of two cold shock proteins [16]. Furthermore, cross protection by adaptation to 10°C before high pressure treatment was observed and once more emphasizes the dependence of the physiological state on pressure resistance (see also section 1.4.2, p. 16). Investigations of the high pressure response of human chondrocytic cells with 2D-PAGE revealed the incidence of increased levels of the heat shock proteins HSP70 and Grp78 after 12h at 30 MPa [123]. Therefore, the occurrence of higher levels of heat and cold shock proteins at elevated pressure seems to represent an essential process. Protein denaturation proceeds at high pressure analog to heat denaturation [22, 143] and thus may induce heat shock response. However, in case of HSP70, mRNA stabilization seemed to be responsible for higher levels of that protein at 30 MPa [144].

Compared to the proteome analysis by 2D electrophoresis, similar extensive analyses of the transcriptome can be achieved by microarray hybridization. With this technique, the expression of 6200 genes after high pressure treatment of *S. cerevisiae* was recently investigated [145]. 274 genes were differentially expressed after 30 min at 200 MPa. Most of the upregulated genes were implicated in energy metabolism, stress response and 45% were uncharacterized genes. These results are unexpected, since transcription tended to stalling at pressures up to 180 MPa [146].

2 Materials and methods

2.1 Bacterial strains, growth conditions and media

2.1.1 Growth media

Media were prepared as indicated in Tab. 1-Tab. 3 and sterilized by autoclaving at 121°C for 15min. Sugars have been autoclaved separately and added to the media afterwards. Heat sensible components, such as amino acids were filter-sterilized (0.2 μm filter). The chemically defined SA medium was completely filter-sterilized. In case of casting agar plates in Petri dishes, 1.5 % agar agar was added before autoclaving the medium. All chemicals used for the media were of biochemical, per analysis or ACS grade (Merck, Darmstadt, Germany; Serva, Heidelberg, Germany; Sigma, St. Louis, MO, USA).

Tab. 1: Modified Homohiochii medium according to Kitahara

Components	**[g/l]**
Peptone from casein pancreatically digested, granulated	10
Extract of meat dry, granulated	2
Yeast extract, granulated	7
D(+)-Glucose H_2O	7
Na Gluconat	2
Maltose H_2O	7
D(-)-Fructose	7
Na acetate $3H_2O$	5
KH_2PO_4	5
$[NH_4]2H$ citrate	5
MgSO4 $7H_2O$	0.2
$Mn(II)SO_4$ H_2O	0.1
$FeSO_4$ $7H_2O$	0.05
L-Cysteine HCL H_2O	0.5
Tween 80	1ml
pH	**5.4**

Tab. 2: M17 medium according to Terzaghi and Sandine [147]

Components	**[g/l]**
Peptone from casein pancreatically digested	5
Extract of meat	5
Soy peptone	5

Components	**[g/l]**
Yeast extract	2.5
D(+)-Glucose H_2O	10
Ascorbic acid	0.5
$MgSO_4$ $7H_2O$	0.25
Disodium-β-glycerophosphate	19
pH	**7.2**

Tab. 3: Chemically defined SA medium according to Jensen *et. al.* [148]

Amino acids	**mM**	**1x [mg/l]**	**10x [g/l]**
L-Alanine	3.4	302	3.02
L-Arginine	1.1	191	1.91
L-Asparagine H_2O	0.8	120	1.2
L-Cyteine H_20 HCl	0.8	140	1.4
L-Glutamate	2.1	308	3.08
L-Glutamine	0.7	102	1.02
Glycine	2.7	202	2.02
L-Histidine	0.3	46	0.46
L-Isoleucine	0.8	104	1.04
L-Leucine	0.8	104	1.04
L-Lysine Cl	1.4	255	2.55
L-Methionine	0.7	104	1.04
L-Phenylalanine	1.2	198	1.98
L-Proline	2.6	299	2.99
L-Serine	2.9	304	3.04
L-Threonine	1.7	202	2.02
L-Tryptophan	0.5	102	1.02
L-Tyrosine	0.3	54	0.54
L-Valine	0.9	105	1.05
Buffer	**mM**	**1x [mg/l]**	**10x [g/l]**
NH_4Cl	9.5	508	5.08
K_2SO_4	0.28	48	0.48
KH_2PO_4	1.3	176	1.76
Na-acetate $3H_2O$	15	2041	20.41
D(+)-Glucose H_2O	50	9908	99.08
MOPS (3-[N-Morpholino]propanesulfonic acid)	40	8370	83.7
Tricine	4	716	7.16
Salts	**mM**	**1x [mg/l]**	**10x [mg/l]**
$CaCl_2$ $2H_2O$	0.0005		0.7
$MgCl_2$ $6H_2O$	0.52	106	1057.2
$Fe(II)SO_4$ $7H_2O$	0.01	3	27.8
NaCl	50	2922	29220
Vitamins	**mM**		**100x [mg/l]**
Biotin	0.0004		9.8
Pyridoxal HCl	0.01		203.6
Folic acid	0.0023		101.5
Riboflavin	0.0026		97.9
Niacinamide	0.008		97.7
Thiamine HCl	0.003		101.2
Pantothenate 1/2 Ca	0.002		47.7

	mM	1000x [mg/l]
$(NH_4)6Mo_7O_{24}$	0.000003	3.7
H_3BO_3	0.0004	24.7
$CoCl_2$ $6H_2O$	0.00003	7.1
$CuSO_4$ $5H_2O$	0.00001	2.5
$MnCl_2$ $4H_2O$	0.00008	15.8
$ZnSO_4$ $7H_2O$	0.00001	2.9

2.1.2 Bacterial strains and growth conditions

For sustaining growth of the sequenced strain *Lactococcus lactis* IL 1403 [8], M17 medium supplied with 1% glucose was used. Before stress treatment, *Lactococcus lactis* was grown over night in 10 ml at 30°C in the chemically defined SA medium [148]. Then, an appropriate volume of SA medium necessary for stress experiments was inoculated with the overnight culture to a final OD_{450} 0.05.

For ^{35}S-methionine/cysteine labeling of proteins, cells were grown in SA medium with reduced methionine (5 µg/ml) and cysteine (2.6 µg/ml) concentrations. Methionine concentration was used as described in [7] and cysteine concentration was adjusted to maintain the same Met/Cys ratio during pulse labeling with Promix (Met/Cys ratio = 70:30; Amersham Biosciences, Uppsala, Sweden) as before the pulse.

Since no chemically defined growth medium for *Lactobacillus sanfranciscensis* was published at the time of the study, the strain DSM20451 was grown on modified Homohiochii medium (Kitahara). Before stress experiments, lactobacilli were cultured as described above at 30°C and always on the same batch of medium. Due to their slow growth compared to lactococcal growth, before stress experiments, the medium was inoculated to a final OD_{578} of 0.1 with the overnight culture.

2.2 Stress treatments

2.2.1 Heat treatment

Cultures of *Lactococcus lactis* subjected to heat shock were split at OD_{450} 0.4 and harvested by centrifugation (10.000xg, 3min). Then, the bacteria were

suspended in fresh medium, which was prewarmed at either 30°C (reference) or 43°C (heat shock). This was performed to ensure an immediate temperature shift, which is independent on the volume of the culture. Depending on the experimental outline, the cultures were kept at continuous temperature in water baths at 30 or 43°C for 20 to 45 min. 43°C was chosen according to previous heat shock experiments with *L. lactis* published in [5-7] Finally, bacteria were again harvested (10.000xg, 3min) and extracted as described in 2.3. All samples were produced at least in triplicate.

2.2.2 High pressure treatment

In case of *Lactobacillus sanfranciscensis*, cells were harvested in the logarithmic period of growth at OD_{578} 0.5 by centrifugation (3000xg, 10 min) and suspended in fresh, prewarmed medium (30°C). Fresh medium was mainly added to prevent acid accumulation and thus pH stress under pressure. The SA medium of *L. lactis* is more stable by buffering it with MOPS/Tricine and showed almost no pH shift in the logarithmic growth phase of *L. lactis*. Therefore, the medium was not changed before pressure stress and cultures were subjected directly to high pressure treatment at OD_{450} 0.5. The pH shift due to compression was negligible. Just before the pressure treatment, cultures were split and either used as reference cultures or subjected to high pressure.

For high pressure treatment cell suspensions were transferred to test tubes, which were subsequently sealed with rubber plugs and parafilm without trapping air in them. Pressurization was performed in thermostatic regulated autoclaves at 30°C for 30 min. The bacterial cultures were slowly compressed and decompressed at approx. 1.25 MPa/s, in order to maintain 30°C and avoid cell disruption. In case of *Lactobacillus sanfranciscensis*, pressures ranging from 25 up to 200 MPa were applied for typically one hour and the ramp for compression and decompression was randomized. This was done to avoid uniform heating or cooling of the samples while compression and decompression in the individual experiments. Thus, correlation of pressure level and heating or cooling is prevented in linked experiments of different pressures. However, the temperature increased only 2 to 3 °C due to compression

(200 MPa) in comparison to 13°C used for heat shock analysis, and thus was negligible.

L. lactis was pressurized at 60 or 90 MPa for 30 min. The two pressure levels were chosen, because cell growth of *L. lactis* ceased at 60 MPa and protein synthesis was reported to cease *in vivo* between 83 and 110 MPa (measured by ^{35}S-methionine incorporation in *E. coli* [21]). Following decompression *L. lactis* was harvested at 10.000xg for 3min and suspended in SA medium with low Met/Cys content for immediate radioactive pulse labeling. Samples subjected to DIGE were prepared in parallel to ensure highest comparability.

Steps from decompression to protein extraction were speeded up and kept simple to reassure reproducible sample processing upstream of 2D electrophoresis. Additionally, the optical density, the microscopic appearance and the pH were measured before and after pressure treatments and compared. Finally, proteins were directly extracted as described in paragraph 2.3. All samples were produced at least in triplicate.

2.3 Protein extraction

In general, the bacteria were harvested in the logarithmic period of growth by centrifugation at 10.000xg for 3min. In case of the lactococcal strains the bacteria were harvested at $OD_{450} \approx 0.5$ and pH ≈ 7.2, and in case of *Lactobacillus sanfranciscensis* at $OD_{578} \approx 0.6$ and pH ≈ 5.2. Then, bacterial cultures were washed once with prewarmed PBS (30°C) and again centrifuged. The pH of the PBS was adjusted to the corresponding pH of the medium at the time of harvesting (pH 7.2 or 5.2 depending on the bacteria). Next, the bacteria pellet was suspended in 200 µl SDS-buffer (100mM Tris/HCl pH 9.5, 1% SDS (w/v)).

Cell disruption was performed with an ultrasonic homogenizer (two times 30 pulses, 4 mm probe tip, interval: 1 Hz, pulse duration: 0.3 s, 20 kHz homogeneous sound, power output: 60 W; Bandelin, Berlin, Germany) while ice-cooling the sample. The cell debris was pelleted at 14000xg, 4°C, 30 min and the supernatant was aliquoted and frozen at -80°C. Prior to the IEF, the protein extract was diluted with thiourea lysis buffer (2M thiourea, 7M urea, 4% CHAPS (w/v), 2% DTT (w/v), 2%

Pharmalyte 3-10 (v/v)) to a final concentration of less than 0.25% SDS. All fluids and devices which came in contact with the bacterial culture prior to the protein extraction had been sterilized to prevent contamination and prewarmed to 30°C to avoid undesired stresses such as cold shock and guarantee defined conditions.

2.4 2D electrophoresis with immobilized pH gradients (IPG-DALT)

Prior to the two-dimensional electrophoresis, the protein concentration was determined with the 2D Quant Kit (Amersham Biosciences, Uppsala, Sweden). Approximately 100 μg of protein extract was loaded onto analytical gels, which were subsequently stained with silver nitrate (section 2.5.1, p. 28 ; [149]) or SYPRO RUBY™ (section 2.5.3; Molecular Probes, Eugene, OR, USA). On preparative gels submitted to MALDI-TOF MS or LC-MS/MS, approximately 500 μg protein extract was loaded. Such gels were stained with Coomassie Blue R-250 (section 2.5.2).

Two-dimensional electrophoresis with immobilized pH gradients was performed as described in [30, 37, 44]. For the first dimension (IPG-IEF) the following immobilized pH gradients were utilized: 2.5-6.5, 3.5-5, 4-7, 4-9, 4.5-5.5, 5-7, 6-12, 9-12 and 10-12. IPG 3.5-7.5 was customized as part of the present study to cover the complete acidic proteome of *Lactococcus lactis* with a single pH gradient. The preparation of this IPG gel is described in section 2.4.1. In the same manner all other utilized IPG gels were cast as previously described in [32]. IPGs 6-12 and 9-12 were cast utilizing N,N-dimethylacrylamide and immobilines up to pK 13 [43].

The rehydration buffer for the IPG-strips contained 6M urea, 2M thiourea, 1% CHAPS (w/v), 0.4% DTT (w/v) and 0.5% Pharmalyte 3-10 (v/v). After IPG strip rehydration, the sample was applied by cup-loading (2 cm from the anode). Isoelectric focusing was performed on the IPGphor in combination with the cup-loading strip holders for alkaline gradients exceeding pH 9 and on the Multiphor II in combination with the DryStrip Kit in all other cases. For evaluation of IEF systems for alkaline gradients (section 3.4.2, p. 91), isoelectric focusing was additionally performed on the IPGphor in combination with universal strip holders (Amersham Biosciences, Uppsala, Sweden). During IEF, IPG-strips were covered with Cover Fluid (Amersham Biosciences, Uppsala, Sweden). The optimized protocol for setting up the reference map was adapted from Wildgruber *et al.*, 2002 [49] and is summarized in Tab. 4.

Tab. 4: Conditions for isoelectric focusing of immobilized pH gradients 6-12 and 9-12

Steps	Current[a)]	Voltage	Duration	Other
sample entry	50 μA	150 V	1 h	18 cm strips
		300 V	1 h	
		600 V	1 h	Temp.: 20°C
gradient	50 μA	600-8000 V	30 min	
IEF until steady state	70 μA	8000 V	25 kVh for IPG 6-12 30 kVh for IPG 9-12	max. Power: 5 W

a) Indicated are values per IPG strip.

After IEF, proteins were reduced and alkylated as described in [30]. The second dimension (SDS-PAGE) was accomplished in 1mm thick vertical gels (12, 13 or 15% T, 2.6% C) using the Ettan DALT II system (Amersham Biosciences, Uppsala, Sweden) [30, 34]. The Precision Protein Standards^{TM} marker (BioRad, Hercules, CA, USA) was used to estimate the molecular weight of the proteins. Gel documentation was performed by scanning with a calibrated flatbed scanner with transparency unit or the Typhoon 9400 (fluorescence imager, Amersham Biosciences, Uppsala, Sweden).

Dependent on the staining method of proteins, ImageMaster 2D (2.6.4) or the DeCyder software (2.7.6) was utilized for the computer-aided gel analysis.

2.4.1 Preparation of immobilized pH gradient (IPG) gels

In the present study, the following IPG slab gels with linear gradients were used: pH 2.5-6.5, 3-12, 3.5-5, 4-7, 4-9, 4-12, 4.5-5-5.5, 6-12 and 9-12. All IPG gels were cast according to Görg *et al.* [32] with the recipes of Righetti [150] and Görg *et al.* [32, 43, 44]. The recipe for the pH gradient from 3.5 to 7.5 (Tab. 5) was several times calculated, cast and tested, until a satisfying separation in the first dimension was achieved. For calculations, the IPGMAKER software was used [151]. Key to a successful separation is a sufficient and evenly distributed buffer concentration over the whole pH gradient. The software only optimizes the gradient for linearity but not for buffer capacity. The optimal buffer capacity and distribution over the gradient have to be determined by calculations based on different choices of immobilines and subsequent IEF tests.

The IPG gels were cast on the hydrophilic side of Gelbond PAGfilms by using a 260 x 200 mm^2 gel cassette. The glass plate with the U-frame was treated with repel-silane to facilitate the detachment of the gel, when disassembling the gel cassette. An acidic and an alkaline pH solution were prepared for each pH gradient according to the published recipes, e.g. IPG 3.5-7.5 in Tab. 5. For homogenous polymerization, the acidic and alkaline solutions were adjusted to pH 7 with sodium hydroxide and hydrochloric acid, respectively. The acidic, dense solution was pipetted into the mixing chamber and mixed with TEMED and ammonium persulfate using a magnetic follower. After ventilation of the connecting tube, the basic, light solution was pipetted into the reservoir of the gradient mixer. Again, TEMED and ammonium persulfate were added and mixed, this time with a spatula, which remained in the reservoir as equivalent for the magnetic follower in the mixing chamber. The gradient was poured at a reproducible speed of the magnetic follower into the precooled mold (refrigerator, 4°C) of the gel cassette. Next, the gel cassette was kept at room temperature for 15 min to allow adequate leveling of the density gradient prior to polymerization for one hour at 50°C. After polymerization, the gel cassette was kept at room temperature for at least 15 min. Then, the IPG gel was removed from the mold and washed six times for 10min with MilliQ H_2O. Finally, it was impregnated with 2% glycerol for 30 min, and dried at room temperature over night in a dust-free cabinet. If not immediately used, the IPG gel was covered with a plastic film and stored at -20°C.

Tab. 5: Recipe for casting linear IPG 3.5-7.5

	pH 3.5		**pH 7.5**	
	mMol/L	µl/15ml	mMol/L	µl/15ml
Immobiline pK 1.0	4.9	368		
Immobiline pK 3.6	4.34	325	2.53	190
Immobiline pK 4.6	6.52	489	5.44	408
Immobiline pK 6.2	4.9	367	4.93	370
Immobiline pK 7.0	2.24	168	6.32	474
Immobiline pK 9.3			6.13	460
Acrylamide/Bis	2 ml		2 ml	
H_20	8.3 ml		11.1 ml	
Glycerol (100%)	3.75 g			
TEMED (100%)	20 µl		20 µl	
APS (40%)	20 µl		20 µl	
Final volume	15 ml		15 ml	

For effective polymerization, acidic and basic solutions were adjusted to pH 7with 4N NaOH and 25% HCl, respectively, before adding TEMED and APS.

2.5 Protein gel staining techniques

2.5.1 Silver staining

Analytical gels were silver stained either according to a modified protocol of Blum *et. al.* or according to a modified protocol of Heukeshoven *et. al.* [149]. The latter was particularly used for staining of alkaline proteins in the IPGs 6-12, 9-12 and 10-12. Detailed manuals can be downloaded at http://www.wzw.tum.de/proteomik/.

2.5.2 Coomassie Brilliant Blue staining

Coomassie Brilliant Blue (CBB) is an anionic triphenylmethane dye that binds noncovalently to the lysyl residues of proteins. After fixation at least for one hour in 40 % ethanol and 10 % acetic acid, the gel was washed 30 min in 25 % ethanol and 8 % acetic acid. Then, it was saturated with dye solution (0.1 % CBB R-250 (w/v) in 45 % ethanol (v/v) and 10 % acetic acid (v/v)) for three hours, and finally destained in several steps with 25 % ethanol and 8 % acetic acid. The detection limit of this stain is better than one microgram of protein per spot. In general, CBB staining was chosen if spots were subjected to further protein analysis methods, such as peptide mass fingerprinting with MALDI-TOF MS or microsequencing with LC-MS/MS.

2.5.3 SYPRO RUBY™ staining

For improved quantification of analytical gels, SYPRO RUBY™ gel stain was used. Gels were stained according to the supplied manual of Molecular Probes. Following fixation of the gels in 40 % ethanol and 10 % acetic acid for at least one hour, each gel was stained over night in 300ml SYPRO RUBY™ (New Formulation; Molecular Probes). Since SYPRO RUBY™ is easily adsorbed by glass, staining was conducted in polypropylene vessels. Next, the gels were destained in 10 % ethanol and 7 % acetic acid for one hour. Finally, the gels were scanned with the Typhoon 9400 Variable Mode Imager at 532 nm for excitation and with 610 BP 30 filter.

2.6 ^{35}S pulse labeling in proteomics

2.6.1 ^{35}S pulse labeling of proteins *in vivo*

Proteins were ^{35}S pulse labeled according to a modified protocol of Kilstrup *et al*., 1997 [7]. First, cells were harvested by centrifugation (10.000xg, 3min) in aliquots of 1 ml and suspended in 1 ml prewarmed SA medium with low Met/Cys content. The temperature of the medium was dependent on the stress treatment (see section 2.2, p. 22). Next, the proteins were labeled *in vivo* by addition of 2 µl ^{35}S-methionine/-cysteine (≈ 30 µCi; Promix) to 1 ml of the bacterial culture. The pulse or labeling duration was dependent on the stress condition and varied from 20 min in case of heat shock up to 30 min following high pressure stress. Then, unlabeled methionine (0.8 mg/ml) and cysteine (0.42 mg/ml) were added to the samples and incubated for 2 min to displace not incorporated radioactive counterparts. After that, 25 µl of chloramphenicol (20mg/ml) was added to inhibit protein synthesis. Finally, cells were washed with prewarmed PBS (30°C, pH 7.2) and proteins were extracted according to the protocol described in section 2.3, p. 24.

Following protein extraction, the counts per minute (cpm) in 1 and 2 µl sample were measured with the 1600 CA Tri-Carb liquid scintillation analyzer (PerkinElmer, Boston, MA, USA) after mixing the sample with 5ml of Ultima Gold scintillation solution (Packard Biosciences, Groningen, Netherlands). Finally, the protein concentration was determined with the 2D Quant Kit (Amersham Biosciences).

2.6.2 2D electrophoresis of ^{35}S labeled proteins

Before 2D electrophoresis, the protein concentration was determined by using the 2D QuantKit (Amersham Biosciences). Approximately 100 µg protein of each sample was applied per IPG strip. For ^{35}S labeled proteins, mainly the specially customized pH gradient from 3.5 to 7.5 was used. 2D electrophoresis was performed as described in section 2.4, p. 25, with the exception that the gels for the second dimension were cast with 0.5% glycerol instead of 5%. Otherwise, the gels cannot be dried for exposition on phosphor imaging screens.

2.6.3 Phosphor imaging of ^{35}S labeled proteins after 2D electrophoresis

Following 2D electrophoresis, ^{35}S labeled proteins were visualized by exposition to phosphor imaging screens. Since the screens are sensitive to fluids and wet gels quench beta particles, all gels were previously dried on Gel-Blotting-Paper (Schleicher&Schuell, Dassel, Germany) for one hour in the Slab Gel Dryer GD2000 under vacuum condition (Amersham Biosciences). Then, gels were exposed three days to Imaging Screens K (BioRad) in exposition cassettes (BioRad). A sheet of Screen Guard Protective Film (BioRad) was placed between the screens and the gels to prevent contamination of the screens. Following exposition, the screens were scanned with the Typhoon 9400 Variable Mode Imager (Amersham, Biosciences) by using the option StoragePhosphorScreens. Finally, the screens were restored by exposition to light for 15 min on the Screen Eraser K (BioRad).

2.6.4 Image analysis after phosphor imaging, silver, CBB or SYPRO RUBY™ staining using ImageMaster 2D

Before the image analysis, the gel images were rotated to the same orientation to minimize the time for protein pattern matching of the gels. Then, the gel images were cut on the edges, because the gels are wider than the IPG strips. By this, detection of non-protein spots outside of the protein pattern was avoided. At the same time, the file size, which needed to be processed, was minimized. After the images were loaded into the ImageMaster 2D Version 4.01, at first, the wizard for spot detection was used and afterwards the values for detection manually adjusted. These values were applied to all gels, which belonged to the same analysis, e.g. reference compared to high pressure stress. Since manual spot addition is not reproducible, it was omitted and if at all manual spot editing was done, only non-protein spots were deleted. Next, the gel with the highest spot number within the reference group was chosen for the creation of a reference gel. The reference gel was used to match same spots of all gels belonging to the reference group. This was first done automatically and then manually. After that, the background of all gels was subtracted by the “mode of non spot” (value: 45) function and the gels were normalized according to the total spot volumes, indicated in percent. Then, an average gel of the reference group was generated and the reference gel replaced by

this average gel. This averaged reference gel was used to match same spots within all gels belonging to one batch of analysis, in which the same parameters such as pH

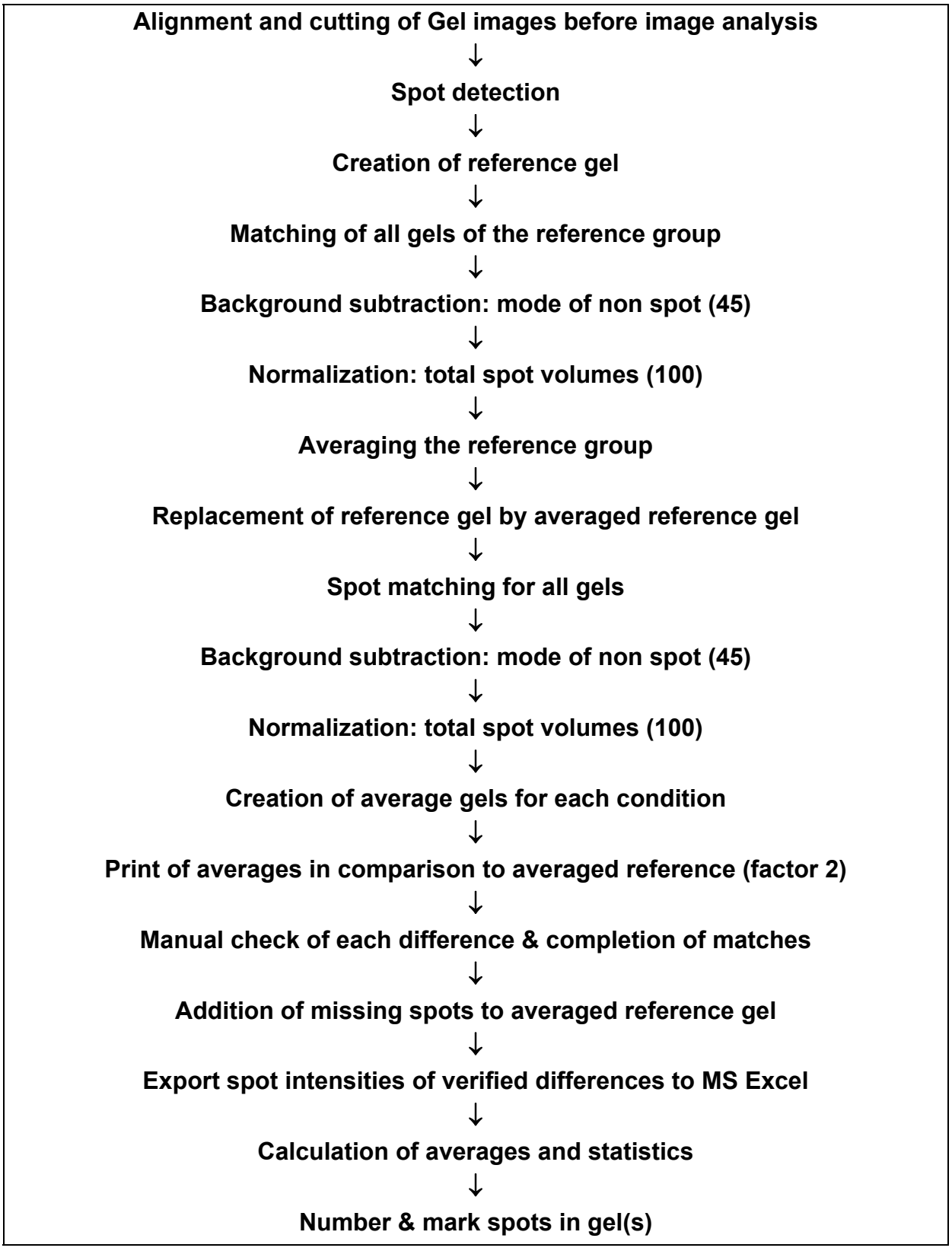

Fig. 4: Workflow of image analysis using ImageMaster 2D

gradient, T value, etc. were applied. Again, the spots were first matched automatically and then manually. Then, background subtraction and normalization was performed as described above and for each group of gels representing the same condition average gels were generated. These average gels were compared to the average reference gel by indicating differences larger than factor two and printed. For quicker software performance, the average gels were removed before proceeding with the analysis.

Each printout displayed the average gel of all gels representing the same condition and the spots with a difference greater than factor two in comparison to the average reference gel. But it also contained spots, which were at this point not included in the averaged reference gel (induced spots) and differences with biological or methodical background. The latter differences characteristically demonstrate large variations of average spot intensities. Thus, each difference was manually checked, matching was completed if necessary, and additional (induced) spots were added to the average reference gel. The latter should not be mixed up with manual spot detection. At this step, the printouts were used to mark all verified differences. The spot intensities of each verified difference was copied into Excel (Microsoft) and averages of all representing one group calculated. Finally, all differences were marked and numbered on a gel, which is representative for one condition.

Since image analysis is a key step in 2D electrophoresis, the complete workflow is summarized in Fig. 4.

2.7 Difference gel electrophoresis (DIGE)

In case of DIGE, the protein samples were labeled with different CyDyes to a low extent before 2D electrophoresis. The minimal labeling ensures that only a single lysine per protein molecule and not more than ~3% of the protein molecules in an extract are labeled. The isoelectric point of proteins is not altered by the covalently linked fluorescent dye, but it causes a size increase of approximately 500 Daltons. Thus, the protein pattern did not significantly differ from silver or Coomassie Blue stained gels. This stands in contrast to saturation labeling, by which all cysteine residues are covalently linked to fluorescent dyes and therefore the protein pattern changes.

After labeling, identical protein amounts of each extract were mixed and loaded on a 2D gel. Approximately the same amount of each fluorescent labeled extract was loaded as was applied for silver staining, e.g. for IPG 3.5.-7.5 approximately 80 μg per dye. The application flow in Fig. 5 gives a short overview of necessary steps in difference gel electrophoresis (DIGE) experiments. Each step is explained in the following subsections.

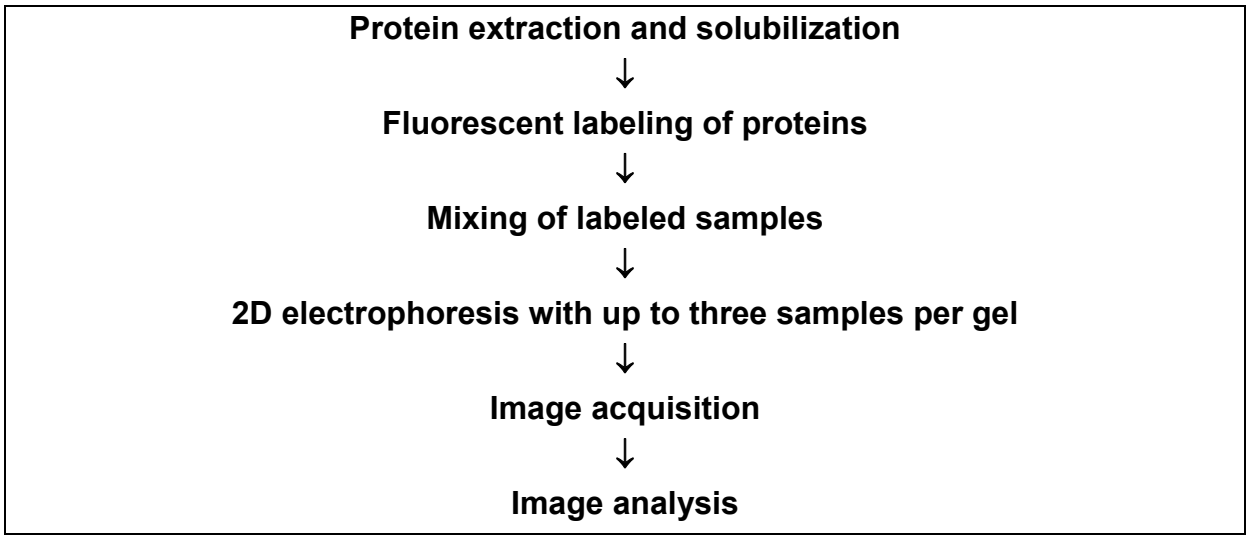

Fig. 5: Application flow for difference gel electrophoresis (DIGE) experiments

2.7.1 Protein extraction and solubilization for DIGE

Protein samples subjected to difference gel electrophoresis (DIGE) were extracted as described in section 2.3, p. 24. Afterwards, the protein content of the extracts was determined by the 2D QuantKit (Amersham Biosciences). The protocol for sample solubilization was slightly modified, because 1% SDS buffer is not compatible with the labeling reaction. The extracts were diluted 1+3 with a modified thiourea lysis buffer (2 M thiourea, 7 M urea, 4% (w/v) CHAPS). Again, the thiourea lysis buffer was modified, because some of its compounds are not compatible with the labeling reaction. The reactive group of the CyDyes is an N-hydroxy succinimidyl (NHS) ester, which covalently attaches to the epsilon amino group of lysyl residues. Primary amines such as carrier ampholytes, as well as reducing agents such as dithiothreitol diminish the labeling efficiency. Therefore, these agents were omitted in the modified thiourea buffer and added after labeling. The final protein concentration

was approximately 3 μg/μl and thus within the margin of 1 and 20 μg/μl indicated by the manufacturer as optimal for the labeling reaction. Referring to the supplemented manual of the CyDyes, the pH optimum for the labeling reaction is pH 8.5 (± 0.1). This pH was guaranteed by Tris within the SDS buffer (section 2.3, p. 24). The pH in the sample was checked with pH indicator strips (pH 4.5–10.0).

2.7.2 Fluorescence labeling of proteins for DIGE

Before first usage of the CyDyes, these were reconstituted in DMF to a stock solution of 1 mM. Only fresh DMF was used (less than three months old after first opening), because it degrades to amine compounds, which interfere with the labeling reaction. According to the supplemented manual of the CyDyes, the reconstituted dyes are stable at –20°C for ~6 months and thus, were only used within this timeframe. Directly before labeling, the CyDye stock solution was diluted with DMF to a final concentration of 0.4 mM (400 pmol/μl). 50 μg of the protein sample was labeled with 400 pmol of the CyDye, while ice-cooling of the sample. If more labeled sample was needed, the amounts were equally adjusted. After exactly 30 minutes, 1 μl of 10 mM lysine was added per 400 pmol of the CyDye to stop the labeling reaction. Again, the mixture was incubated on ice, this time for at least 10 minutes. Finally, 1 μl of 50% DTT (w/v) solution and 1 μl of Pharmalyte (pH 3-10) per 50 μl of sample solution were added.

The dyes and the labeled samples were always ice-cooled and light exposition was avoided to minimize degradation and photo-bleaching. The labeled samples were either directly subjected to 2D electrophoresis or stored at -80°C.

2.7.3 Mixing of the labeled samples for DIGE

According to the experimental outline, the labeled samples were pooled before 2D electrophoresis. In general, at least two extracts representing two different conditions were applied per 2D gel. These extracts were previously labeled with different CyDyes, in general with Cy3 and Cy5. Cy2 was generally used for labeling the internal standard.

The internal standard consisted of a pooled mixture of extracts representing the different conditions. In detail, the pooled mixture, also called internal pooled standard (IPS), consisted of equal protein amounts of each extract, representing the analyzed conditions. For example, in case of heat shock compared to the reference condition, the IPS consisted of two conditions. Since at least three individual samples were extracted per condition, it means that six samples were mixed for preparing the IPS. The IPS was then applied on all gels of this batch analysis.

2.7.4 2D electrophoresis with up to three samples per gel for DIGE

IEF and SDS-PAGE of the mixed samples was performed as described in section 2.4, p. 25, with the exception that low-fluorescence glass cassettes were used in the second dimension. This was done, because the gels are scanned while they are still in the glass cassettes. The IPG strips during IEF and the buffer tank during SDS-PAGE were protected from light to minimize photo-bleaching of the fluorescence dyes.

2.7.5 Image acquisition of DIGE gels

The gels were directly scanned after the second dimension while they were still in the glass cassettes. This ensured that all gels have the same dimensions, which simplifies spot matching of different gels. The exterior of the glass plates was carefully cleaned with deionized H_2O and dried with a lint-free laboratory wipe before the gel cassette was positioned on the Typhoon 9400 Variable Mode Imager (Amersham, Biosciences). Each fluorescent dye was consecutively excited to avoid fluorescence crosstalk and scanned at a resolution of at least 200 μm with the proper filter (Tab. 6).

The sensitivity of the scanner was adjusted by the voltage at the photomultiplier (PMT) according to the maximal linear dynamic range of the CyDyes (five orders of magnitude). After scanning, each gel was loaded into the ImageQuant software and by employment of the volume review it was determined, if the image exceeded the linear dynamic range (Max. Val > 100,000). Then, if necessary, a lower PMT was chosen. In the opposite case, when only a part of the linear dynamic range was covered, the PMT was increased. Furthermore, ImageQuant already provided an

initial image overlay of the scanned channels of one gel (e.g. see Fig. 16, p. 59). Thus, a quick overview of differences between the labeled extracts was obtained.

Tab. 6: Selection guide for proper excitation and filter set for scanning of CyDyes

Dye	Absorption maximum	Fluorescence maximum	Laserlight Wavelength	Emission Filter
Cy2	491 nm	509 nm	488 nm	520 nm, band pass 40
Cy3	553 nm	569 nm	532 nm	580 nm, band pass 40
Cy5	645 nm	664 nm	632.8 nm	670 nm, band pass 40

2.7.6 Image analysis after DIGE with the DeCyder software package

The image analysis in DIGE demands a different software algorithm to enable complete access to the advantages of this technique, such as the inclusion of an internal standard in the software analysis. In addition, the software needs to be capable to overlay protein patterns obtained from the same gel after application of differently Cy labeled protein extracts, instead of matching them. In the present study, the DeCyder software was used for the analysis of DIGE experiments.

Similar to the preparations for analysis of postelectrophoretic stained gels (section 2.6.4, p. 30), the gel images were aligned and cut to the region of interest. For this, a special software was used (ImageQuant Tools, Amersham Biosciences), which processed all scanned channels of a gel at the same time. Following alignment and cutting, one gel was loaded into the DIGE DIA software, by which the optimal settings for spot detection and exclusion were determined. Only one single parameter exists for spot detection (estimated spot number), which was in most cases set to 2500 spots. Higher values merely resulted in the detection of additional non-protein originated spots. The spots without protein origin were excluded by filtering spots with a certain area, peak height, slope or volume. In general, exclusion by peak height and volume delivered the most satisfying result. Then, the determined values were tested on other gels belonging to one batch of analysis, in which the same parameters such as pH gradient, T value, etc. were applied. The determined parameters for spot detection and exclusion were applied to the DIGE Batch Processor (Amersham, Biosciences), in which all gels of a coherent analysis were

processed under the same parameters and automatically matched. In this software, the individual protein patterns of the gels were assigned to groups dependent on the conditions they were representing.

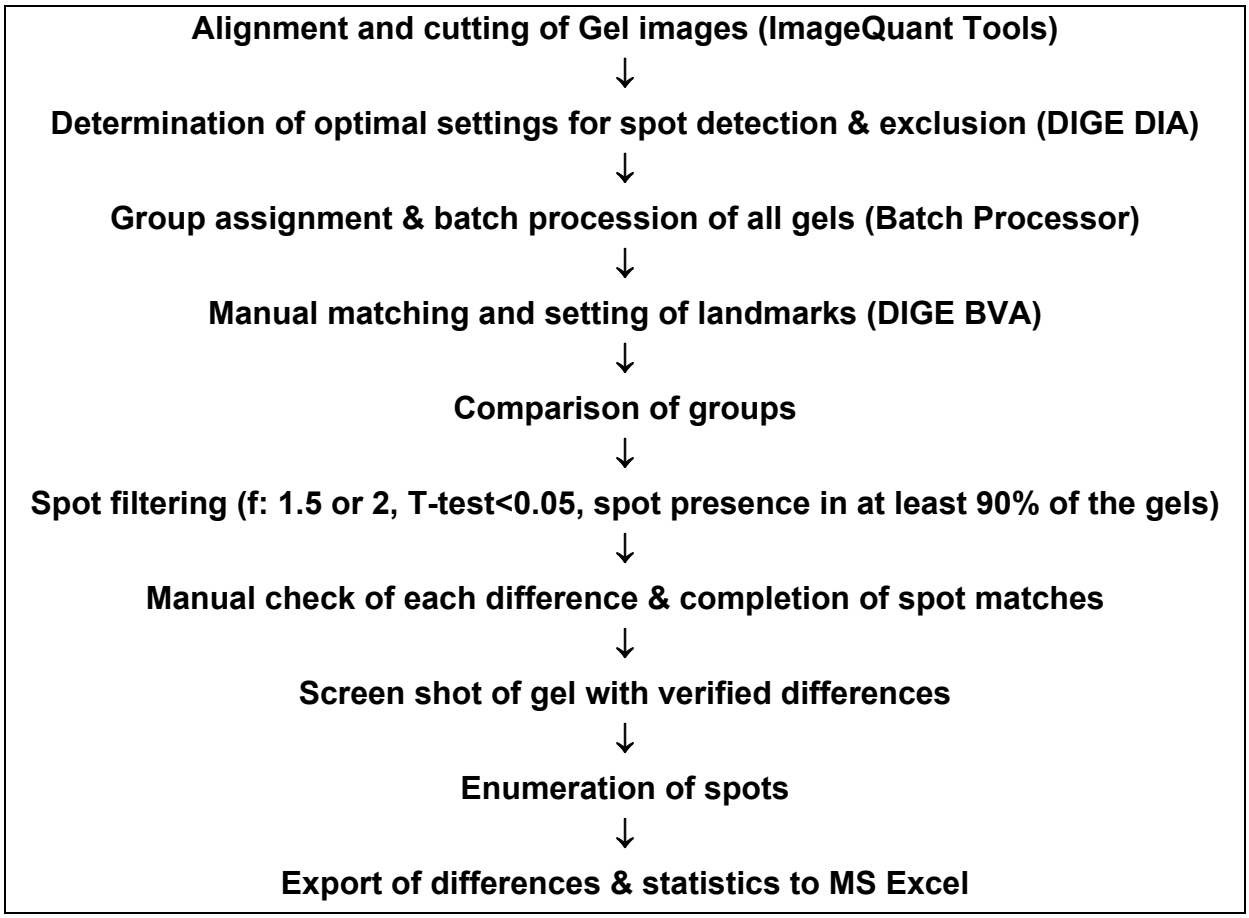

Fig. 6: Workflow of image analysis using the DeCyder software package

After batch processing of the gels, the complete batch was loaded into the BVA software (Amersham, Biosciences) and mismatches within the analysis were manually corrected by setting landmarks and adding spot matches. Then, the groups of gels, which were assigned in the batch processor, were compared and statistical data, such as T-test were calculated. The obtained data were filtered according to the parameters of interest. In general, the parameters were set to a difference of factor 1.5 or 2 between the groups, a T-test below 0.05, and a presence of the spot in at least 90% of the analyzed protein patterns. For example, in the simplest case, in which only two conditions were compared, three gels were made. Each gel was loaded with two labeled extracts representing the different conditions and one labeled

extract representing the internal standard. Therefore, three protein patterns were obtained per gel, which means in total nine for three gels. After filtering for differences by using the mentioned parameters, only spots, which were detected eight times within the nine protein patterns, were displayed. Despite this critical selection, each difference was further manually checked and in some cases spot matches again completed. The confirmed spots were marked and a screen shot of the gel generated. Analytical spot data, such as T-test and ratios of the differences, were exported to Excel (Microsoft) and numbered. Finally, the spots in the gel of the screen shot were numbered referring to the MS Excel sheet.

2.8 Protein identification

Spots of interest were identified by matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) in case of *Lactococcus lactis* and by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) in case of *Lactobacillus sanfranciscensis*. The different methods were necessary, because the genome of *Lactococcus lactis* is completely sequenced [8], whereas almost no sequence data of *Lactobacillus sanfranciscensis* were available at time of the present study. By spot digestion and MALDI-TOF MS a peptide mass fingerprint was obtained, which is supposed to be unique for each protein [63, 64]. This fingerprint was compared with the *in silico* digest of the translated genome of *Lactococcus lactis*. In LC-MS/MS, the digested spots were further fragmented by Collision-induced Dissociation (CID). Thus, the peptides break up at, for the apparatus, typical sites [67, 68]. In case of LC-MS/MS with a Q-TOF, mainly b- and y-ions are obtained. The amino acid sequence was deduced by determination of the differences between the y-ion peeks in the spectrum (e.g. Fig. 13, p. 53). Depending on size and solubility, the sequence of each peptide in the spot digest can be determined by LC-MS/MS. Finally, the spot was identified by homology to sequences in the NCBI non redundant (NR) protein database.

2.8.1 Peptide mass fingerprinting MALDI-TOF MS

Spots were excised from Coomassie stained gels and destained for 30 minutes using 100 µl acetonitrile (50%) and 5mM $(NH_4)HCO_3$ (50%); Next, spots were dehydrated with acetonitrile and dried at 40°C for 30 minutes using a Speedvac.

For the tryptic digestion, 20 µg trypsin (Promega, Madison, WI, USA) was dissolved in 100 µl 1mM HCl and directly before use, 150 µl 5mM $(NH_4)HCO_3$ were added to the trypsin solution (final concentration:12.5 ng/µl). 10 µl of this solution was pipetted on each dried protein spot and incubated for 30 minutes at 0°C. The supernatant was discarded to minimize auto-digestion of trypsin. Then 20 µl 5mM $(NH_4)HCO_3$ was added and the sample was incubated for 8h at 37°C.

On the sample slides 0.25 µl α-cyano-4-hydroxycinnamic acid in acetonitrile (1 mg/ml) were pipetted and air dried (seed-layer). Then 0.5 µl digested sample was added to the slide and mixed with 0.5 µl matrix solution (15 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile + 0.5% trifluoroacetic acid) and air dried for 10 minutes.

Mass spectrometry was performed by using the Ettan z² MALDI-ToF (Amersham Biosciences, Uppsala, Sweden) with UV nitrogen laser (337 nm) and harmonic reflectron; Mode: Positive-ion-reflectron-mode at 20 kV with delayed extraction mode und low mass rejection; Calibration: Peptide-Samples (Angiotensine II, ACTH 1-39) and internal standard (trypsine auto-digestion fragments). For each spectrum, 200 single shots were accumulated.

Proteins were identified by searching the monoisotopic masses against the database of *Lactococcus lactis* IL1403 curated by NCBI (accession: NC_002662) with the implemented software ETTAN MALDI-TOF Pro Evaluation Module Ver. 2.0 (Amersham Biosciences). One missed cleavage per peptide was allowed, and a mass tolerance of 10 ppm was used in all searches. Partial modifications of proteins by carbamidomethylation of cysteines and oxidization of methionines were taken into account. Proteins were considered as identified, when at least five peptides were matched, the expectation value was smaller than 0.01 and the result could be reproduced with a corresponding spot picked of a parallel gel. Agreement of theoretical and approximate experimental isoelectric point and molecular weight were generally included in the identification process of proteins too.

2.8.2 Peptide fragmentation LC-MS/MS

In-gel digestion with trypsin was performed according to published methods [69-71] modified for use with a robotic digestion system [152] (Investigator ProGest, Genomic Solutions, Huntington, UK). Excised gel pieces were washed with 50mM ammonium hydrogen carbonate buffer and dehydrated with acetonitrile. Then, gel pieces were dried at 60°C, prior to addition of modified trypsin (Promega, Madison, WI; 10 µL at 6.5 ng/µL in 25mM ammonium hydrogen carbonate). Digestion proceeded for 8 hours at 37°C and products were recovered by sequential extractions with 25mM ammonium hydrogen carbonate, 5% formic acid, and acetonitrile. The pooled extracts were lyophilized and redissolved in 0.1% formic acid prior to mass spectrometry.

Tandem electrospray mass spectra were recorded using a Q-TOF hybrid quadrupole / orthogonal acceleration time of flight spectrometer (Micromass, Manchester, UK) interfaced to a Micromass CapLC capillary chromatograph. Samples were dissolved in 7 µl of 0.1% aqueous formic acid, and 1.4 µl aliquots were injected onto a 300 µm x 15 mm desalting column, packed with Pepmap C18 (LC Packings, Amsterdam, NL), and washed for 3 min with 0.1% aqueous formic acid (with the stream select valve diverting the column effluent to waste). The flow rate was then reduced to 1 µl per min, the stream select valve was switched to the data acquisition position, and an acetonitrile 0.1% formic acid gradient (5% to 70% acetonitrile over 20 minutes) was initiated to elute peptides into the mass spectrometer.

The capillary voltage was set to 3,500 V, and data dependent MS/MS acquisitions were performed on precursors with charge states of 2, 3 or 4 over a survey mass range 540-1000. Known trypsin autolysis products and keratin derived precursor ions were automatically excluded. The collision gas was argon, and the collision voltage was varied between 18 and 45 V depending on the charge state and mass of the precursor. Product ion spectra were charge-state de-encrypted and de-isotoped with a maximum entropy algorithm (MaxEnt 3, Micromass). Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in SWISS-PROT/TrEMBL using ProteinLynx Global Server (Version 1, Micromass). One missed cleavage per peptide was allowed, and an initial mass tolerance of 50 ppm was used in all searches. Cysteines were assumed to be carbamidomethylated, but

other potential modifications were not considered in the first pass search. When this approach failed, amino acid sequences were deduced manually from the charge state de-encrypted spectra, and searched against the NCBI's non-redundant database using BLAST [153].

2.9 Resources and algorithms for the *in silico* analysis of *L. lactis*

The base for all calculations were the 2266 annotated protein sequences included in the database of *Lactococcus lactis* IL1403 curated by NCBI (accession: NC_002662). Mr and pI were calculated in batch for all proteins by using the pI/MW Prediction Tool (http://proteome.ibi.unicamp.br/tools/pimw/index.htm) with default settings for pK values. The codon adaptation index (CAI) was generated by application of the software CodonW [154] as previously described [24]. The same software was applied for the calculation of the grand average of hydropathicity (GRAVY) of each protein.

2.10 Resources, algorithms and software used for the dynamic online database

The online database was realized with the relational database management system MySQL (http://www.mysql.com/) on an Apache web server with Linux platform. The database mainly consists of three tables: the protein information, the gel data and the spot coordinates. The protein table contains the 2266 annotated protein sequences included in the database of *Lactococcus lactis* IL1403 curated by the NCBI (accession: NC_002662) [155], as well as the data calculated for the *in silico* analysis, namely the predicted Mr and pI, the CAI and the GRAVY value of each protein. Furthermore, the functional classification according to the MOLOKO website (http://spock.jouy.inra.fr/RL000801.html) [8] and the predicted cellular localization calculated with the PSORT algorithm [156] were added to the protein table. Data of the alkaline reference maps (see section 3.4.3, p. 93) and the corresponding spot coordinates on the gels were integrated in the remaining two tables. Two identifiers linked to the Entrez (NCBI; http://www.ncbi.nlm.nih.gov/entrez) [155] and Swiss-Prot (http://www.expasy.org/sprot/) [157] databases are also part of the stored data for easily accessible cross references.

Access to the online MySQL database was realized by scripts written in PreHypertextProcessor language (PHP). The scripts are interpreted on the web server and serve as interface between user and database. Even tools for the administration of the database were written in PHP scripts.

For the realization of dynamically displayed circles in spot size on the reference gels, the GD library was used (http://www.boutell.com/gd/). Javascripts were used for several functions, such as the realization of pop-up windows, alerts or the mouse-over information display in the reference maps. Therefore, full functionality is only provided if Javascript is enabled.

3 Results

Before the differential analyses of *Lactobacillus sanfranciscensis* and *Lactococccus lactis* were started, especially customized protocols for sample preparation and 2D electrophoresis were established for these two organisms. This was in particular necessary for *Lactobacillus sanfranciscensis*, because at the beginning of the present thesis, to our knowledge, no established sample preparation for 2D electrophoresis has been published for this organism. All stress analyses were performed in the early logarithmic growth phase of the bacteria to avoid undesirably influences such as adaptation to accumulated catabolites, acidification of the medium or induction of the stringent stress response due to starvation. Therefore, typical growth curves of each organism were determined to grow reproducible cultures for comparative analyses.

The protein extraction described in section 2.3, p. 24, was established as a result of the comparison of several different extraction methods. Different buffers for protein solubilization were tested as well as different methods for cell disruption. The diameter of the probe tip of the ultrasonic homogenizer, for instance, ensured optimal cell disruption in a 1.5 ml eppendorf tube with 200 µl solubilization buffer, while foaming or spraying of the solution was minimized. SDS instead of urea or a combination of urea/thiourea as primary solvent circumvents carbamylation of the proteins due to local heating at the probe tip and urea degradation while sonication. In addition, SDS provides better solubilization of the proteins, which resulted in higher protein concentrations in extracts and more spots on 2D gels (Fig. 7). Reproducibility of the extraction method was high, which was indicated by almost similar protein concentrations in individually prepared extracts (10-20% deviation) and 2D patterns (Fig. 8). Stored at -80°C, SDS extracts were highly stable and could still be used for 2D electrophoresis after at least two years. Just before IEF, SDS extracts were diluted 1+3 with a compatible buffer for IEF. This buffer contained saturating amounts of thiourea and urea, and relatively high amounts of CHAPS to keep the proteins after dilution in solution. No precipitation was observed after dilution. Identification of proteins within SDS extracts separated after dilution by 2D electrophoresis, demonstrated that several membrane associated proteins and components of transporters were dissolved by this method (e.g. see Tab. 16, p. 86). This indicates that more than the usually extracted cytoplasmatic proteins were solubilized.

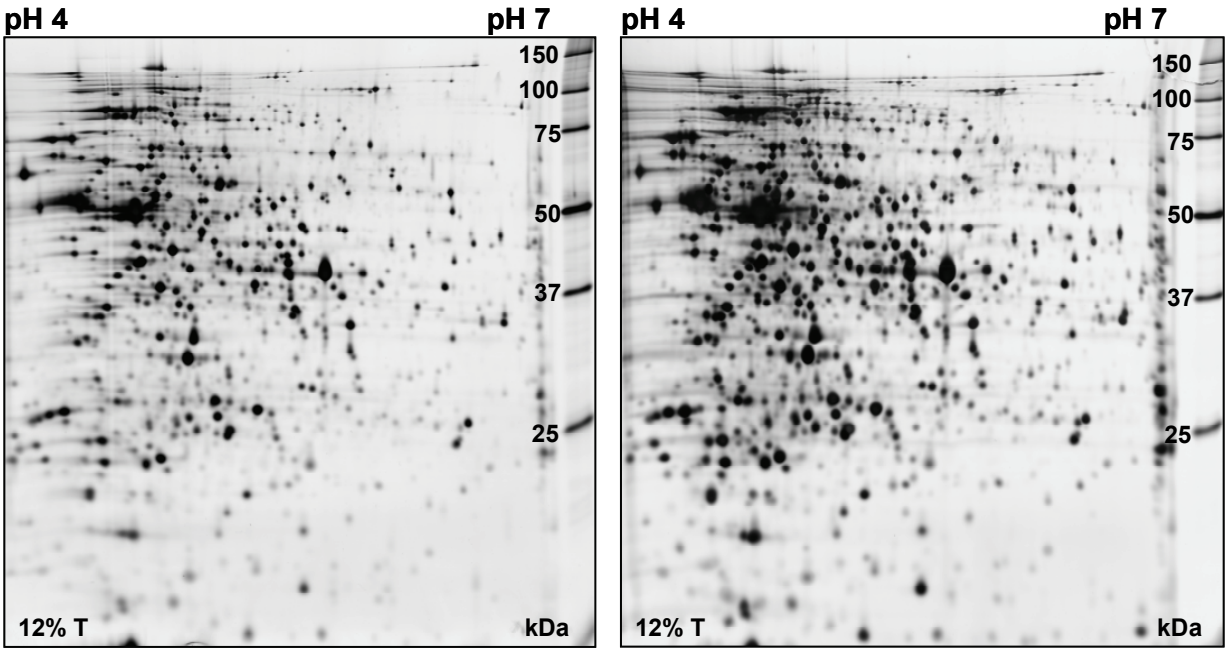

Fig. 7: Optimization of protein extraction by comparison of different buffers for solubilization of proteins. Starting with equal aliquots of lactococcal culture at OD 0.5, proteins were either extracted in 200 µl urea lysis buffer or 200 µl 1% SDS buffer (100 mM Tris, pH 9.5), and equal volumes were applied on 2D gels (IPG-Dalt), silver stain. Extraction with SDS buffer results in a higher quantitative and qualitative amount of spots on 2D gels.

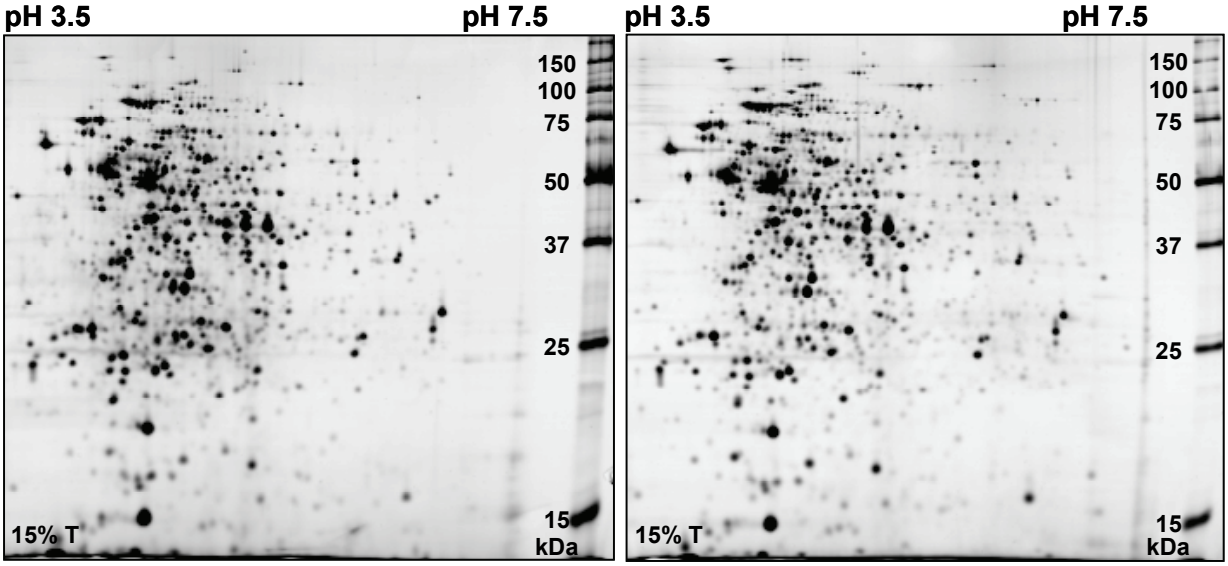

Fig. 8: Reproducibility of optimized and standardized protein extraction, and 2-DE running conditions in a customized IPG 3.5-7.5, silver stain. Two individual samples of *Lactococcus lactis* were grown, extracted and separated in parallel (IPG-Dalt).

Protocols for IEF were mainly adapted from previous protocols. An overview of IEF protocols is given in the 2D manual, which can be downloaded at http://www.wzw.tum.de/proteomik/. IPG 3.5-7.5 was designed to cover all acidic and neutral proteins of *L. lactis* with one pH gradient, in order to minimize gels needed in analyses with radioactive pulse labeling and DIGE. Several gradients were calculated and tested, before a satisfying separation was achieved. The final recipe of IPG 3.5-7.5 is indicated in Tab. 5, p. 27. The protocol for IEF of IPG 3.5-7.5 was adapted from IPG 4-7. Due to the obligatory dilution of SDS extracts before IEF, especially the separation of preparative amounts proved to be difficult. Here, slow sample entries at 150V, 300V and 600V for 2h at each step and regular changes of filter paper at the electrodes facilitated the application of up to 160 µl diluted extract per IPG strip with negligible precipitation at the sample cups. A more thorough optimization of IEF conditions was necessary for the separation of alkaline proteins and is described in section 3.4.2, p. 91. The optimized protocol is indicated in Tab. 4, p. 26.

Finally, the stress treatments were optimized to obtain protein patterns nearly unbiased of methodical or environmental variances except the conditions of interest. Thus, cultures subjected to the same analysis were always grown on the same batch of medium. One large culture was grown and split just before the stress treatment to obtain reference and stressed cultures with same background. Cold shock was avoided by prewarming of media, buffers and vessels. Acidification was monitored and prevented by buffering the medium or medium exchange prior to the stress treatment. Reference and stressed cultures were grown to similar optical densities ($\Delta OD<0.1$) to exclude cell density dependent differences in the protein patterns. Special preventative measures to avoid other stresses depended on the analyzed treatment and are described in sections 2.2, p. 22.

3.1 Detection of pressure dependent proteins in *Lactobacillus sanfranciscensis*

First, high pressure treatment, sample preparation and 2D electrophoresis were optimized and standardized for reproducibility and high resolution as briefly described in section 3. Then, the stress response of *Lactobacillus sanfranciscensis* treated with pressures ranging from 25 up to 200 MPa was analyzed by utilizing different protein detection techniques and computer-aided image analysis. DIGE was not employed for this analysis, because the technique was not available at that time. For the detection of pressure dependent proteins, we compared the protein patterns of atmospherically grown *Lactobacillus sanfranciscensis* with pressure treated ones. The bacterial culture was split just before the high pressure treatment, in order to gain a reference culture grown in the same manner. Additionally, another protein extract was produced of cells with the same optical density as the pressurized bacteria had after the pressure treatment. In this way, differences in the protein pattern due to different cell densities or variances in the cell culture can be excluded. However, no differences between the two controls were observed. The extension of the experiments to a comparison of the protein patterns of *Lactobacillus sanfranciscensis* upon pressure treatment from 0.1 to 200 MPa in 25 MPa steps allowed a kinetic analysis of the protein levels under high pressure. Possible altered expression due to heating or cooling of the bacteria while turning the pressure on or off, was excluded from the analysis by randomizing the gradient of the pressure ramp in several experiments and elimination of spots, which showed altered intensities, but not in a kinetic manner to pressure. However, the temperature increased only 2 to 3 °C due to compression (200 MPa) in comparison to 13°C used for heat shock analysis, and thus was negligible. At least three individual protein extracts per pressure condition were produced.

During optimization of pressure treatments, the optical density, the microscopic appearance as well as the pH of bacterial cultures before and after treatments were monitored and compared. The optical density slightly increased during pressure treatments up to 125 MPa (stated as ΔOD_{578} in Fig. 9), while the microscopy and the pH measurements showed no significant changes. Filament formation was not observed unlike investigations with *E. coli* under pressure [158] and thus, is not responsible for the increase of the optical density during pressure treatments.

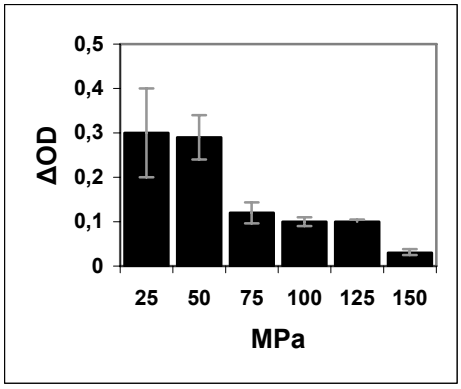

Fig. 9: Changes in the optical density (ΔOD_{578}; mean values with standard deviations) of *Lactobacillus sanfranciscensis* cultures at high hydrostatic pressures. Values resemble the difference between the measurements that were performed directly before and after pressure treatment for 1h.

Image analysis of protein extracts focused in the pH range from 4 to 7 (Fig. 10a) revealed altered spot intensities of five proteins (spots H to L). Four of them were increased after pressure treatment (spots H to K), but their maximum intensity occurred at different pressure levels. An enlargement of this pH range by using zoom-in gels revealed another three induced (spots C, E and F) and two repressed proteins (spots D and G) in the pH gradient from 4.5 to 5.5 (Fig. 10b). Extending the search down to pH 3.5 and up to 12 (Fig. 10c and d) showed two additional spots, which were induced (spots B and M) and another repressed spot (spot A). Some of the proteins were just slightly beyond our specification of induction or repression and for this reason we evaluated their expression by comparing SYPRO RUBY™ stained gels too (Fig. 11, p. 50). This fluorescence dye delivers a linear range for quantification at least over three orders of magnitude [159]. Only spots, which exhibited a differential expression in silver and SYPRO RUBY™ stained gels were regarded as stress relevant. Additionally, the intensity of each spot was determined in at least two different pH gradients. For example, intensities of spots A and B were confirmed by using an IPG 2.5-6.5 as well as IPG 3.5-5 as demonstrated in Fig. 12, p. 50. In total, at least six gels per pressure condition were processed. Finally, 13 spots met our specification of pressure dependent proteins (Tab. 7, p. 51).

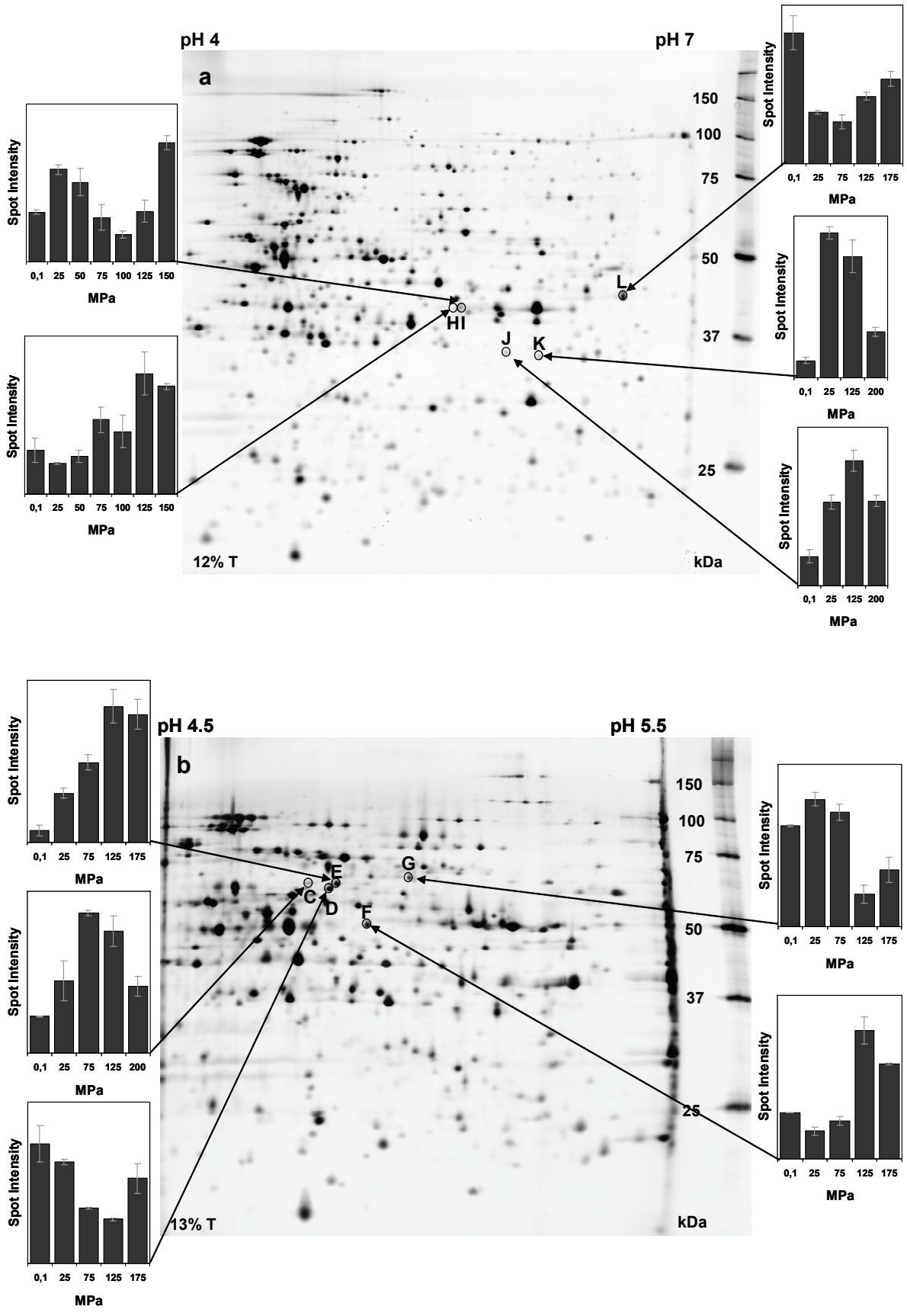

Fig. 10 a, b: Legend see next page

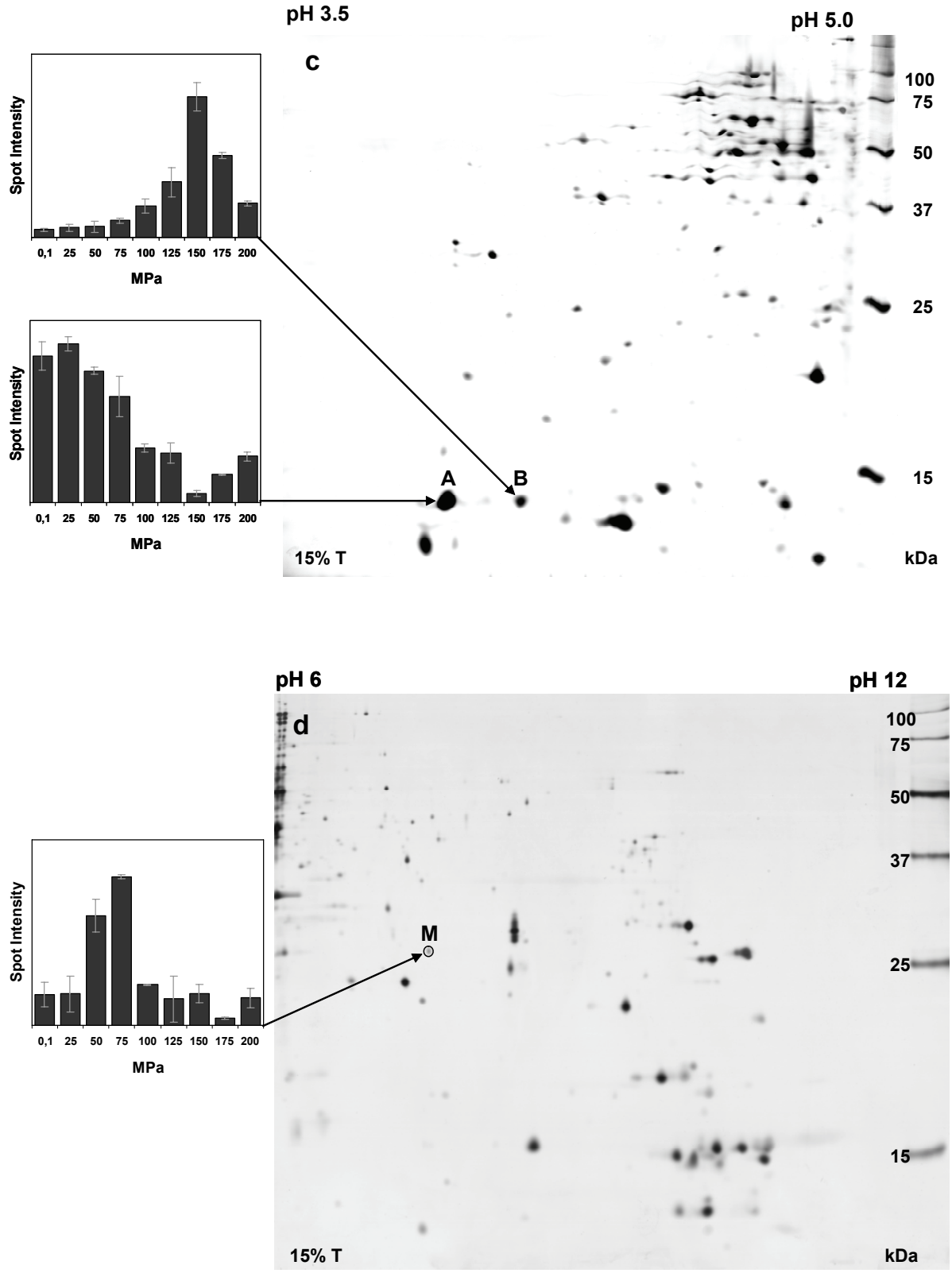

Fig. 10 a-d: IPG-DALT of *Lactobacillus sanfranciscensis* proteins, silver stain. The diagrams beside the gels demonstrate changes in the intensity of the same spots in different gels depending on the applied pressure (mean values with standard deviations; 0.1 MPa represents the untreated control); a: IPG 4-7, b: IPG 4.5-5.5, c: IPG 3.5-5.0, d: IPG 6-12.

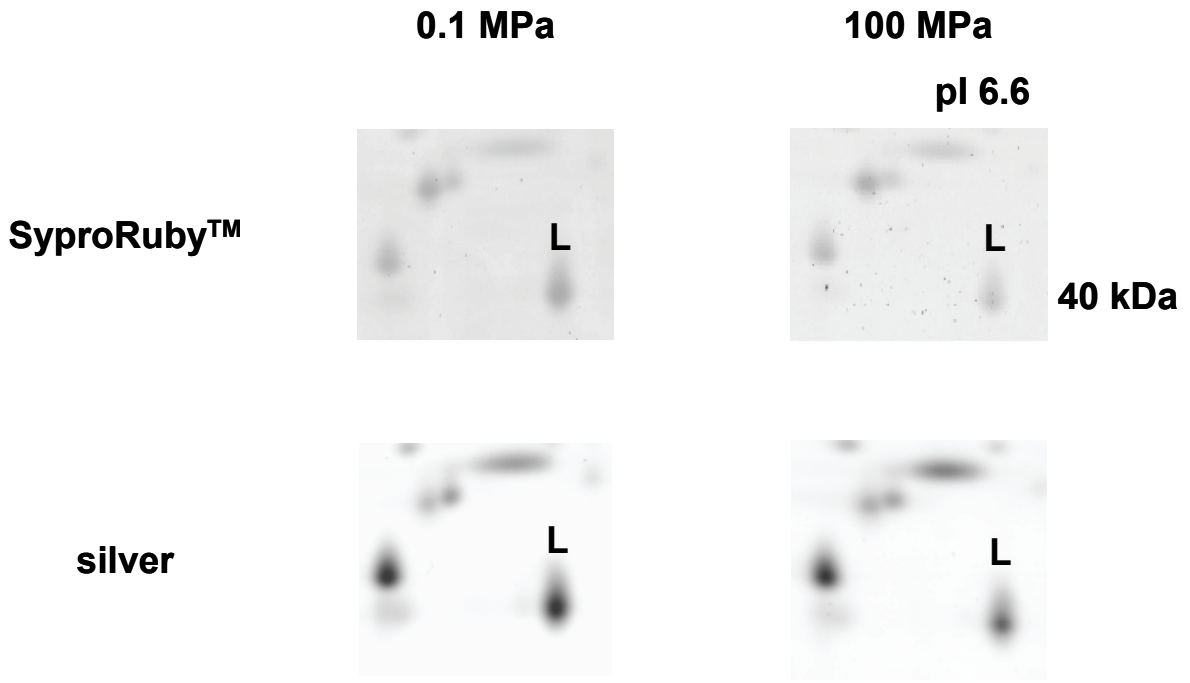

Fig. 11: Comparison of spot L on SYPRO RUBY™ (top) and silver (bottom) stained gels. Display details show IPG-DALTs (IPG 4-7) of *Lactobacillus sanfranciscensis* proteins, extracted from atmospherically grown (left) and 1h, 100 MPa (right) treated bacteria.

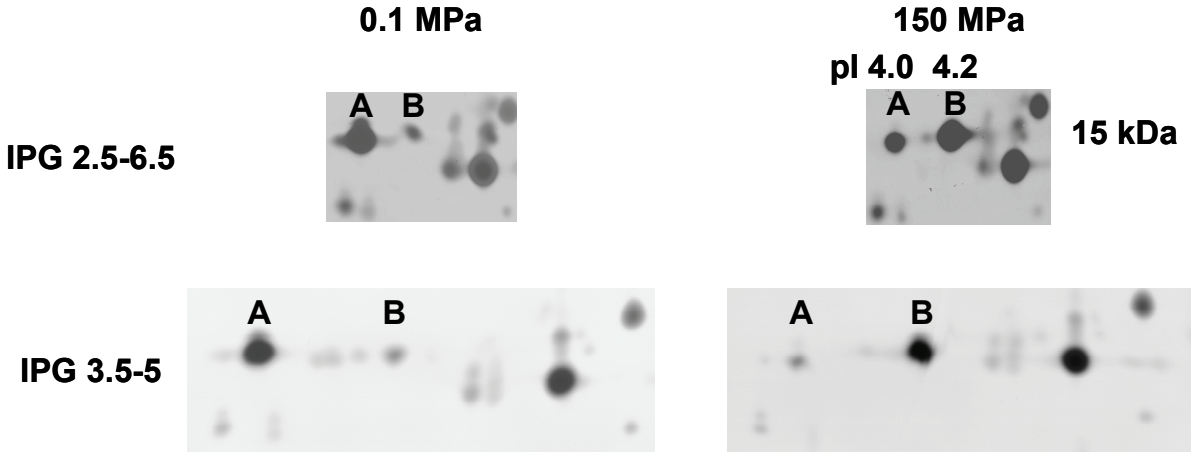

Fig. 12: Comparison of spot A and B after isoelectric focusing on IPG 2.5-6.5 (top) and 3.5-5 (bottom). Display details show IPG-DALTs of *Lactobacillus sanfranciscensis* proteins (silver stain), extracted from atmospherically grown (left) and 1h, 150 MPa (right) treated bacteria.

Tab. 7: Pressure dependent spots with intensity levels greater than factor 2.0 or less than 0.5 at pressure treatments.

Spot	pI[a)]	Mr[a)] (kDa)	Max[b)]
A	4.0	15	150r
B	4.2	15	150i
C	4.7	65	75-125i
D	4.8	60	75-125r
E	4.8	65	125-175i
F	4.8	50	125i
G	5.0	65	125r
H	5.6	40	125-150i
I	5.7	40	150i
J	5.9	35	125i
K	6.1	35	25i
L	6.6	40	25-75r
M	7.5	25	75i

a) pI and Mr are estimated values of the image analysis and rounded to 0.1 pH units and 5kDa, respectively.
b) Max indicates the pressure in MPa which caused the maximum induction (i) or repression (r).

The pressure level, which induced the most intense change in the intensity, is individual for each spot. Spot B for example was maximally induced at 150 MPa, while 75 MPa were needed for the maximal induction of spot M. Furthermore, some of the pressure dependent spots reached a plateau in their intensity over a range of up to 50 MPa (e.g. spot E), while others showed a distinct peak in their (intensity/pressure diagram) expression profile (e.g. spot B).

A closer view at the expression profiles of spots A and B revealed that they followed opposite trends over the pressure range from 0.1 to 200 MPa (i.e. at 150 MPa for 1h, the more acidic spot is maximally repressed, while the other one is maximally induced). Supposing a conversion from one into the other, these spots were further analyzed by MALDI-TOF MS.

3.1.1 Characterization of pressure dependent proteins by MALDI-TOF MS

Peptide mass fingerprints of spots A and B were generated by tryptic digestion followed by MALDI-TOF MS. Peptides of the cleavage appear as discrete peaks in the obtained spectra. Thus, they are characteristic for each protein. The MS spectra of spots A and B consisted of five major peaks, which had the same m/z values in both spectra (Tab. 8). Indeed, this indicated that both spots represent the same protein.

Tab. 8: Monoisotopic masses of spots A and B generated by MALDI-TOF MS after tryptic digestion.

Spot	**Masses of major peaks in spectra**				
A	2211.11	2239.20	2303.26	2750.32	3011.50
B	2211.10	2239.12	2303.11	2750.35	3011.47

3.1.2 Characterization of pressure dependent spots by LC-MS/MS

Since similar MALDI-TOF MS spectra are a good indication but no proof for being the same protein, we decided to sequence spots A and B. We chose tryptic digestion followed by LC-MS/MS as approach to this problem, because by this method, several sequences are obtained for one protein, which increases the probability of identification in homology search. The derived sequences of spots A and B supported the results obtained by peptide mass fingerprinting. For peptides of equivalent size, the same sequence information was deduced. The sequences (Tab. 9, p. 54) were further used for homology search. The best hits of BLAST search against NCBIs non-redundant (NR) sequence database revealed similarities to cold shock proteins of *Lactococcus lactis*.

Furthermore, spots C to F were identified by this method (Tab. 9, p. 54). Proteins C and E both showed homology to ribokinase (RbsK) of *Lactobacillus sakei*. A typical MS/MS spectrum of one peptide of spot E is shown in Fig. 13. The sequence deduced from this spectrum by interpretation of y-ions was VVDTTAAGDTFLGSLASKLELD. The occurrence of y-ions is characteristic for the Q-TOF, which was used for LC-MS/MS. The nomenclature for sequence ions in mass spectrometry is presented in Fig. 3, p. 10. Several spectra were produced for each

fragment to obtain trustworthy sequences. Protein D demonstrated high similarities to GMP synthase of *Lactococcus lactis* subsp. *lactis*. Three sequences derived from spot F matched to elongation factor Tu of three diverse organisms.

The sequences were submitted to the Swiss-Prot database and were assigned to the entries P83529-P83541. Recently, one cold shock protein could be identified in the genome of *Lactobacillus sanfranciscensis* and it was sequenced [4]. P83529 (GYGFLTTDD) and P83530 (VTLDVED) match to 100% internal fragments of this cold shock protein with the exception that leucine in P83529 is isoleucine in the sequenced gene. Isoleucine and leucine have the same mass and thus, cannot be differentiated in LC-MS/MS.

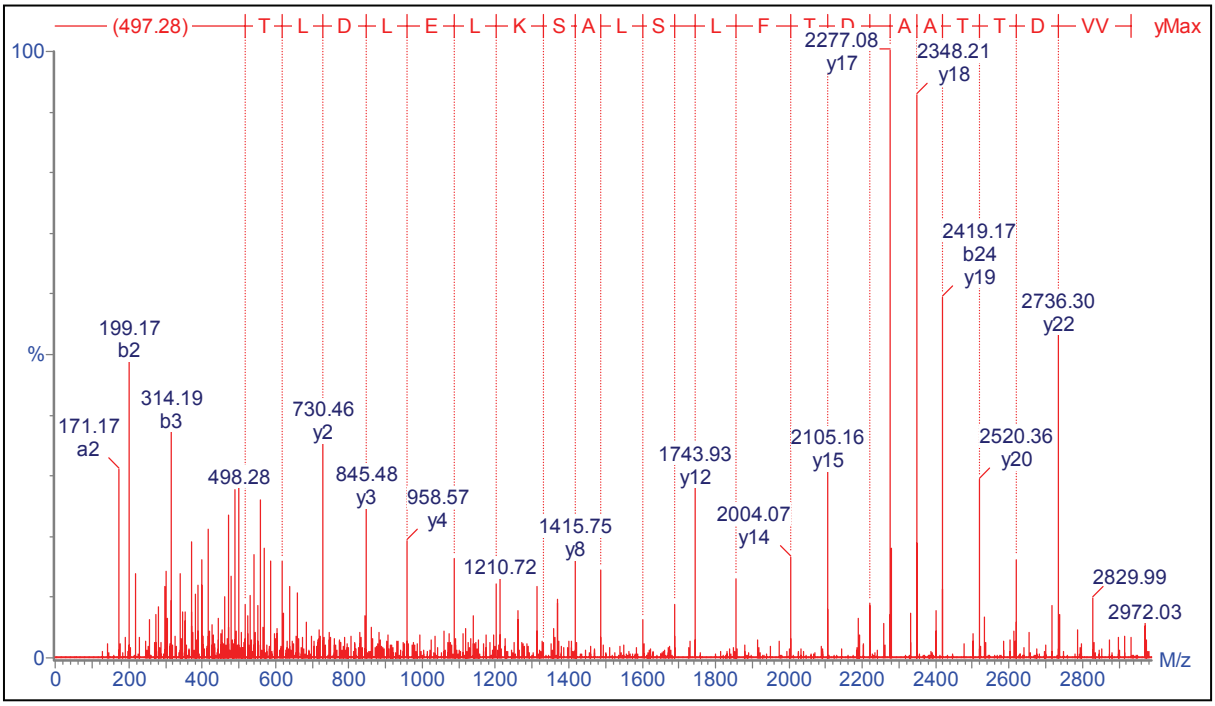

Fig. 13: Spectrum of one fragmented peptide of spot E produced by Collision-induced Dissociation and using a Q-TOF. The amino acid sequence was deduced by interpretation of the y-ions and reads as follows: VVDTTAAGDTFLGSLASKLELD.

Tab. 9: Peptide sequences of pressure dependent spots of *Lactobacillus sanfranciscensis* determined by peptide fragmentation by LC-MS/MS

Spot	Deduced sequences	Protein identified	E*
A and B	GYGFLTTDD, VTLDVED, GYGFLTTDD, SLGSGGSL, GVPTVNAV, DTVNAA, RGDE	cold shock protein CspE *Lactococcus lactis* subsp. *lactis* probable cold shock protein transcriptional regulator CspA6 *Sinorhizobium meliloti*	0.018 0.82
C and E	VVDTTAAGDTFLGSLASKLELDE, SGADTTFLTK, AQLQNPVGYELQHK, GSFFATPDDRH, PYLLNLPPEK, LRLDLSNLR	ribokinase RbsK *Lactobacillus sakei*	5.7e-15
D	ALGDQLLSVFVDHT, LVDEVA	GMP synthase *Lactococcus lactis* subsp. *lactis*	0.019
F	VADEVELVGLVED, TLDEGQAGDNLVDQ, DLLSEYGYD	elongation factor Tu *Streptococcus salivarius* elongation factor Tu *Chlorobium vibrioforme* translation elongation factor EF-Tu (tuf) - *Spirochaeta aurantia*	0.28 0.39 0.6

*E refers to the expectation value of the BLAST/FASTS search against NCBI's non-redundant database.

In case of spot F, the expectation values of the BLAST search were poor, but three individual matches to the same protein are regarded as sufficient proof for protein identification.

3.2 Analysis of the heat shock response of *L. lactis* with advanced proteomic tools

At the time when heat shock response of *L. lactis* was investigated, protein identification was primarily performed by immunoblotting. Therefore, the identified proteins were confined to prominent heat shock proteins for which antibodies were available [5-7]. Now, the genome of *L. lactis* is sequenced [8] and enables protein identification by MALDI-TOF MS. Furthermore, advanced proteomic techniques such as DIGE or improved image analyzing tools are available. Therefore, the heat shock response of *L. lactis* was again analyzed.

At first, the heat shock response of *Lactococcus lactis* IL1403 was analyzed with DIGE after exposure of the bacteria at 43°C for 20 or 45 min. After protein extraction, each IPG strip was loaded with a mixture of even parts of Cy3 labeled reference extract (grown at 30°C), Cy5 labeled heat shock extract (either shocked for 20 or 45 min), and Cy2 labeled combination of both extracts. As a result, each Cy label produced one characteristic protein pattern, after scanning 2D gels. The Cy2 labeled combination was prepared of even parts of both conditions and in batch for all three gels of one experiment. In the image analysis of the protein patterns with the DeCyder software, the Cy2 labeled combination of extracts was used as internal pooled standard, as described in [56].

3.2.1 45 min heat shock analyzed with DIGE

The spot detection by the software resulted in the detection of 892 spots for the gel (IPG 3.5-7.5), which was set as master gel for this analysis. After matching of the nine protein patterns obtained from the three gels in this experiment, spots were filtered according to their averaged ratio from heat shock to reference condition and their significance (T-test). At first, 30 spots were selected by setting filter parameters for averaged ratio to greater than 2 or smaller than -2, and for p value to smaller than 0.05. According to their averaged ratio, 11 spots demonstrated induced and 19 repressed spot intensities at heat shock. The p value ranged from 0.048 down to 3.4 x 10^{-7} and the averaged ratios from as high as 8.99 down to -10.05 (Tab. 10, p. 60). For reasons discussed later in this report, spots with averaged ratios greater than 1.5 or smaller than -1.5 are presented too. With these settings, 41 additional spots were

filtered, 5 of them with increased and 36 with decreased spot intensities at heat shock. The p value ranged within this group from 0.023 down to 1.7 x 10^{-6}. Filtered spots with averaged ratios greater than 2 or smaller than -2 are marked black in Fig. 14, and spots with averaged ratios greater than 1.5 or smaller than -1.5 are marked green. The image was derived from the DeCyder software analysis, which means that the polygons around the marked spots resemble the analyzed spot area and the dots within the polygons mark the highest intensity of the spots. The grey-scale image cannot correctly display four to five orders of magnitude in linearity, which are provided by the CyDyes. Therefore, a multicolored image overlay of protein patterns derived from one reference and one heat shock condition is shown in Fig. 16, p. 59. For a better overview, only spots with averaged ratios greater than 2 or smaller than -2 are marked in this image. The extract representing the reference condition was labeled with Cy3 (green) and the other, representing the heat shock condition, was labeled with Cy5 (red). The color selection in the image overlay results in yellow for spots, which do not change their spot intensity upon heat treatment, and green or red for those spots, which demonstrate decreased or increased spot intensities at heat shock, respectively. The comparison of the image overlay with the results of the DeCyder software analysis shows that differences in spots intensities with averaged ratios greater than 2 or smaller than -2 in the software analysis, are evident in the image overlay too. On the other hand, green and red spots in the image overlay (Fig. 16, p. 59) are not always confirmed as differences by the software analysis, although they indicate changes in spot intensity upon heat shock. In most cases, the averaged ratios of these spots were after normalization of the gels within the range of 1.5 and -1.5, but poor significance due to high variability in spot intensity was another reason for the difference between image overlay and software analysis, and demonstrates the necessity of the latter. A representative example of software analysis is shown in Fig. 15, p. 58 for spot 18.

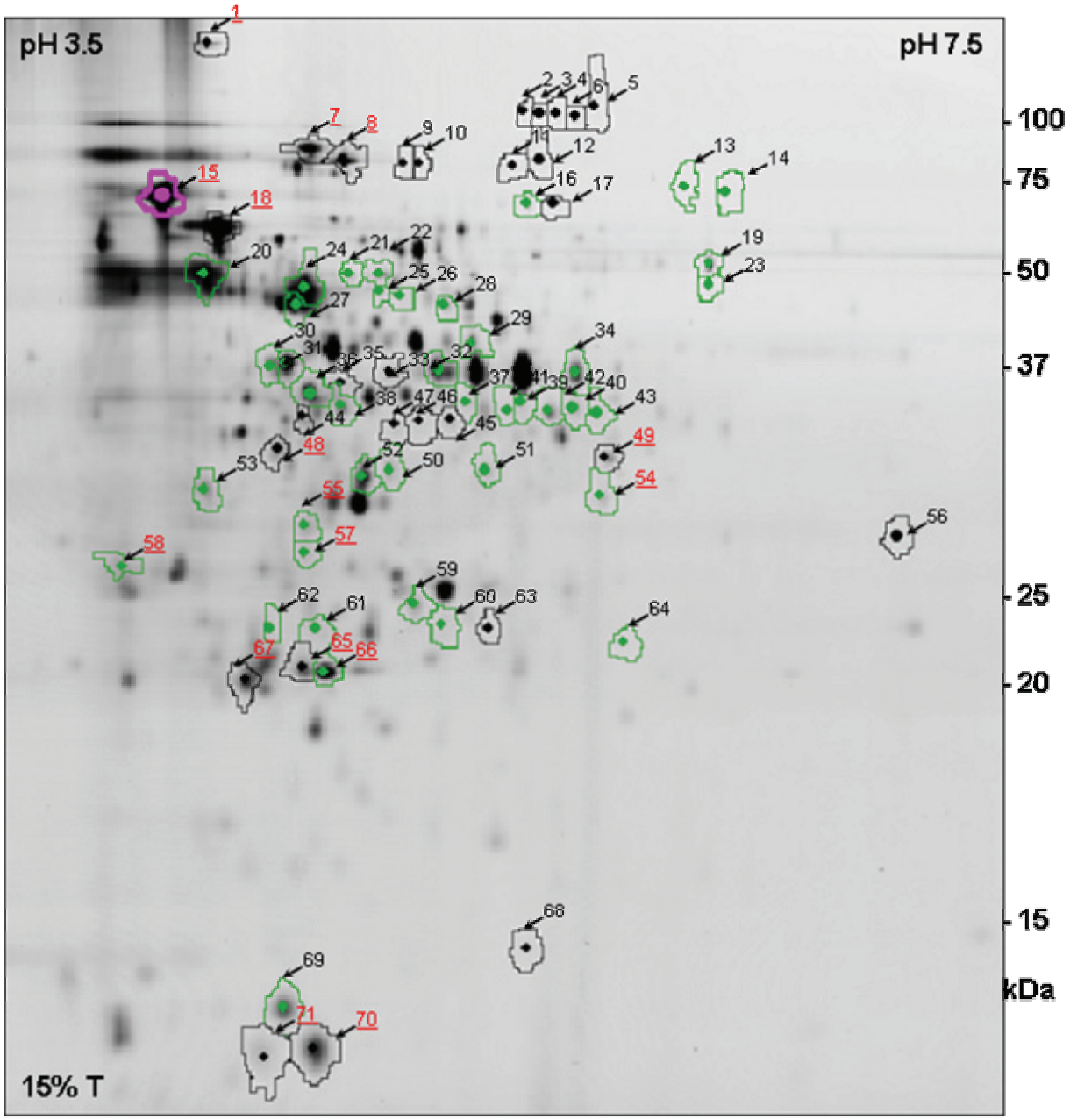

Fig. 14: Protein pattern representing 45 min heat shock at 43°C of *L. lactis* IL1403. Following extraction, proteins were Cy5 labeled for difference gel electrophoresis (DIGE) and separated by 2D electrophoresis in the pH range from 3.5-7.5 (IPG-DALT). Polygons around spots mark differences greater than factor 2 (black) or 1.5 (green) determined by image analysis with the DeCyder. Numbered spots refer to Tab. 10, p. 60, and indicate spots with increased (red) or decreased intensity after heat shock.

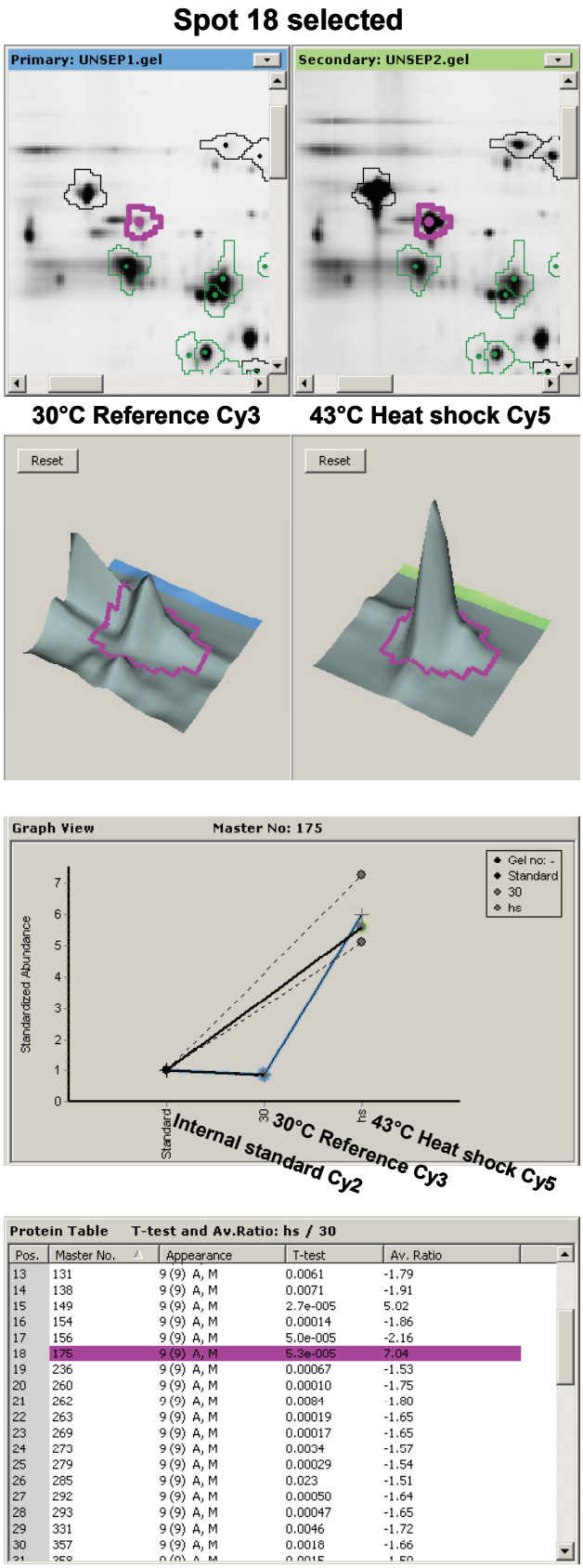

Image view

3D view

Graph view

Indicated are the ratios of corresponding spots of three gel patterns per condition in comparison to the internal standard. Deviation in case of the reference condition is minimal and thus, the three values are overlying in the graph. Blue and green are the values of the selected spots in the image and 3D view.

Table view

Spot 18 corresponds to spot 175 in the mastergel and was matched in nine protein patterns. The averaged ratio in the comparison of heat shock to reference condition is 7.04 (p: 0.000053).

Fig. 15: 2D gel analysis of heat shock in comparison with reference using the DeCyder software. Displayed are the software components used for verification of differences in spot patterns. The example shows spot 18 from Fig. 14, Fig. 16 and Tab. 10, p. 60.

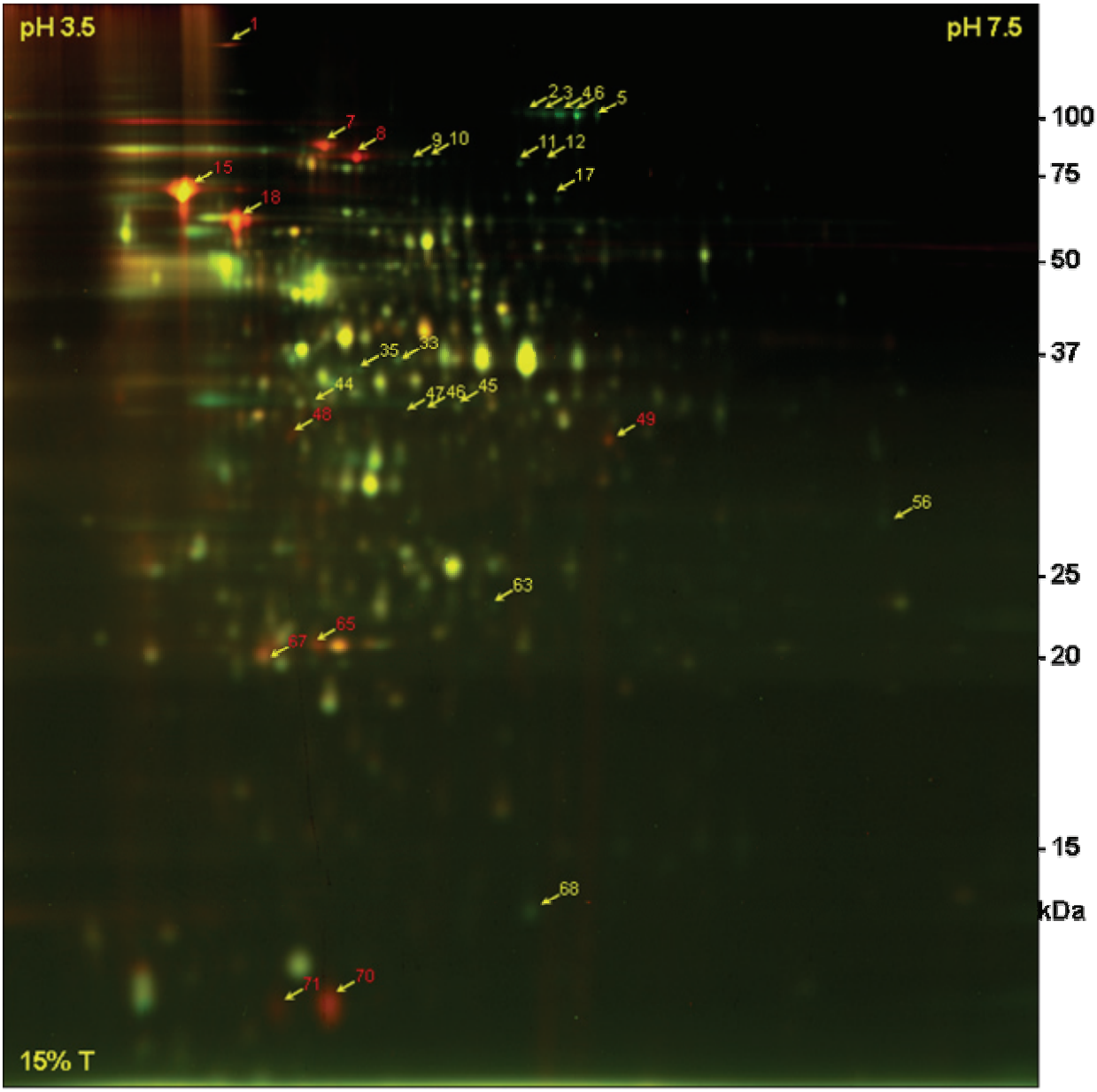

Fig. 16: Image overlay of two protein patterns representing reference and 45 min heat shock at 43°C of *L. lactis* IL1403. Following extraction, proteins were Cy3 (green: reference) and Cy5 (red: heat shock) labeled for difference gel electrophoresis (DIGE) and separated by 2D electrophoresis in the pH range from 3.5-7.5 (IPG-DALT). Yellow indicates similarities, whereas green and red indicate decreased or increased spot intensities, respectively. Numbers mark spot differences greater than factor 2 determined by image analysis with the DeCyder (see Tab. 10).

Next, the 71 filtered spots showing differences in the software analysis were assigned to corresponding spots on Coomassie stained gels, for further analysis by peptide mass fingerprinting (PMF). This approach was chosen, because direct identification by PMF of spots automatically picked from DIGE gels resulted in poor coverage or not interpretable spectra. The utilization of a relative large picker head

(2mm in diameter), and picking at the lower part of the spots to consider the slight mass shift added by the Cy dyes, did not improve the quality of the spectra. Thus, the poor spectra probably occurred due to the low protein concentration on DIGE gels compared to Coomassie stained gels. Nevertheless, the few identified spots from DIGE gels confirmed the assignment of spots to Coomassie stained gels. In total, 47 proteins were identified in the analysis of 45 min heat shock from spots of Coomassie gels. Some of them occurred more than once on the same gel, for example up to five times in case of alcohol-acetaldehyde dehydrogenase. Among those proteins, which are increased in their intensities upon heat shock, are the known heat shock proteins DnaK, GroEL and GroES and proteins belonging to the Clp proteolytic complex, but also proteins undetected in previous studies such as DpsA and SodA. In the group of proteins, which are decreased in their spot intensities, are among others several proteins implicated in the energy metabolism, the translation and notably the purine metabolism. All identified spots are listed in Tab. 10.

Tab. 10: Differentially expressed proteins of *L. lactis* IL1403 after 45 min heat shock at 43°C determined by DIGE in the pH range from 3.5-7.5 and DeCyder analysis. Identification was performed at least two times per spot by peptide mass fingerprint (e<0.01).

No[a)]	Master No.	Av. Ratio	T-test	PID[b)]	Protein
1	29	2.1	7.2E-03	12725073	non-heme iron-binding ferritin
2	80	-5.86	1.6E-04	12725215	alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)
3	81	-9.12	2.9E-06	12725215	alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)
4	82	-9.61	3.4E-07	12725215	alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)
5	83	-4.79	1.8E-03	12725215	alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)
6	85	-10.05	5.7E-07	12725215	alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)
7	98	3.57	4.4E-04	12723443	ATP-dependent protease ATP-binding subunit
8	117	3.66	6.1E-03	12724524	ClpB protein
9	118	-2.13	3.7E-03		
10	119	-2.42	1.6E-02		
11	120	-2.05	3.7E-02	12723148	HYPOTETICAL PROTEIN
12	121	-2.12	4.8E-02		
13	131	-1.79	6.1E-03		
14	138	-1.91	7.1E-03		
15	149	5.02	2.7E-05	12723891	DnaK protein
16	154	-1.86	1.4E-04		
17	156	-2.16	5.0E-05	12723372	CTP synthetase
18	175	7.04	5.3E-05	12723267	60 KD chaperonin
19	236	-1.53	6.7E-04	12723077	IMP dehydrogenase (EC 1.1.1.205)
20	260	-1.75	1.0E-04	12723548	enolase (EC 4.2.1.11)
21	262	-1.8	8.4E-03		
22	263	-1.65	1.9E-04	12723016	Glu-tRNA amidotransferase subunit B
23	269	-1.65	1.7E-04	12723077	IMP dehydrogenase (EC 1.1.1.205)
24	273	-1.57	3.4E-03	12724893	elongation factor Tu
25	279	-1.54	2.9E-04		
26	285	-1.51	2.3E-02		

No[a)]	Master No.	Av. Ratio	T-test	PID[b)]	Protein
27	292	-1.64	5.0E-04		
28	293	-1.65	4.7E-04	12724651	adenylosuccinate lyase (EC 4.3.2.2)
29	331	-1.72	4.6E-03		
30	357	-1.66	1.8E-03		
31	358	-1.5	1.5E-03	12725212	elongation factor Ts
32	362	-1.53	1.1E-02	12725315	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
33	373	-2.06	8.6E-03	12724153	malate oxidoreductase (EC 1.1.1.38)
34	375	-1.61	2.0E-03	12725315	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
35	381	-2.19	4.4E-04	12724226	Trp-sensitive phospho-2-dehydro-deoxyheptonate aldolase (EC 4.1.2.15)
36	385	-1.52	3.4E-04	12724312	L-lactate dehydrogenase (EC 1.1.1.27)
37	404	-1.73	1.7E-06		
38	408	-1.73	1.0E-03		
39	409	-1.82	1.0E-04		
40	410	-1.71	2.7E-04		
41	411	-1.98	1.2E-02		
42	414	-1.6	9.8E-03	12724321	UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)
43	415	-1.8	6.6E-03		
44	423	-2.02	1.1E-04	12724157	citrate lyase beta chain (EC 4.1.3.6)
45	426	-2.04	9.9E-03	12724888	ribose-phosphate pyrophosphokinase (EC 2.7.6.1)
46	428	-2.27	4.9E-04	12723900	conserved hypothetical protein
47	434	-2.08	1.5E-04	12725023	UDP-glucose 4-epimerase (EC 5.1.3.2)
48	455	2.48	1.3E-02		
49	461	3.27	1.7E-06	12723425	cysteine synthase (EC 4.2.99.8)
50	469	-1.78	2.0E-04	12724959	HYPOTHETICAL PROTEIN
51	470	-1.53	7.6E-03	12723642	dihydroxynaphthonic acid synthase (EC 4.1.3.36)
52	475	-1.69	4.3E-04	12725213	30S ribosomal protein S2
53	492	-1.66	1.8E-03	12723048	glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24)
54	502	1.64	1.1E-03	12724529	Oxidoreductase
55	530	1.93	1.6E-03		
56	547	-2.5	5.1E-03	12723453	NADH-dependent enoyl-ACP reductase
57	557	1.75	2.3E-02		
58	560	1.91	7.3E-03		
59	600	-1.52	3.0E-04	12724720	cytidine monophosphate kinase (EC 2.7.4.14)
60	614	-1.66	4.0E-03	12723547	HYPOTHETICAL PROTEIN
61	616	-1.67	2.2E-02		
62	619	-1.82	5.6E-04		
63	622	-2.26	1.1E-02	12724428	basic membrane protein A
64	633	-1.96	2.1E-04	12723032	Oxidoreductase
65	649	3.1	6.0E-03	12723282	superoxide dismutase
66	652	1.91	1.8E-04	12723282	superoxide dismutase
67	661	2.01	2.7E-03	12723580	ATP-dependent Clp protease proteolytic subunit
68	805	-2.15	2.4E-04	12725171	zinc transport transcriptional regulator
69	825	-1.61	1.3E-03	12722964	phosphocarrier protein HPr
70	840	8.99	7.3E-05	12723266	10 KD chaperonin
71	842	3.73	9.6E-05	12723266	10 KD chaperonin

a) Spot numbers refer to marked spots in Fig. 14, p. 57, and Fig. 16, p. 59
b) PID, identifier according to GenBank (NCBI)

3.2.2 20 min heat shock analyzed with DIGE

The master gel for this analysis was produced similar to that of the analysis after 45 min heat shock, but contained 1436 spots. The reason for about 50% more spots on this master gel is found in the exclusion settings of the software. This time, the settings were not as strict as in the 45 min analysis, because otherwise some spots with low intensity would have been excluded from the analysis. The reason for a different master gel in this analysis is that the experiment was performed in parallel to radioactive pulse labeling for highest comparability (same timeframe). Thus, two reference extracts for the different timeframes were produced. The master gel of this analysis (Fig. 17) looks slightly different from that in Fig. 14, p. 57, because a higher contrast was chosen to display spots with low intensity, which are not visible in Fig. 14, p. 57.

Software analysis revealed 10 spots with an averaged ratio of at least 2 and a T-test smaller than 0.05 (Tab. 11, p. 64). Spots were subjected to PMF in the described manner and again, DnaK, GroEL and GroES were the most prominent proteins, but the Clp proteins, which were detected after 45 min heat shock, were lacking. Lowering the filter settings in the software analysis down to an averaged ratio of 1.5, revealed seven more proteins and spot identification showed that one was ClpE. Only one spot was detected with an averaged ratio below -2. This spot for example was identified as YxdB and did not change in the analysis of 45 min heat shock. Including spots with an average ratio below -1.5, added only one more spot to the analysis, which was not identified. In total, 13 of 19 spots, which fitted to the filter settings, were identified.

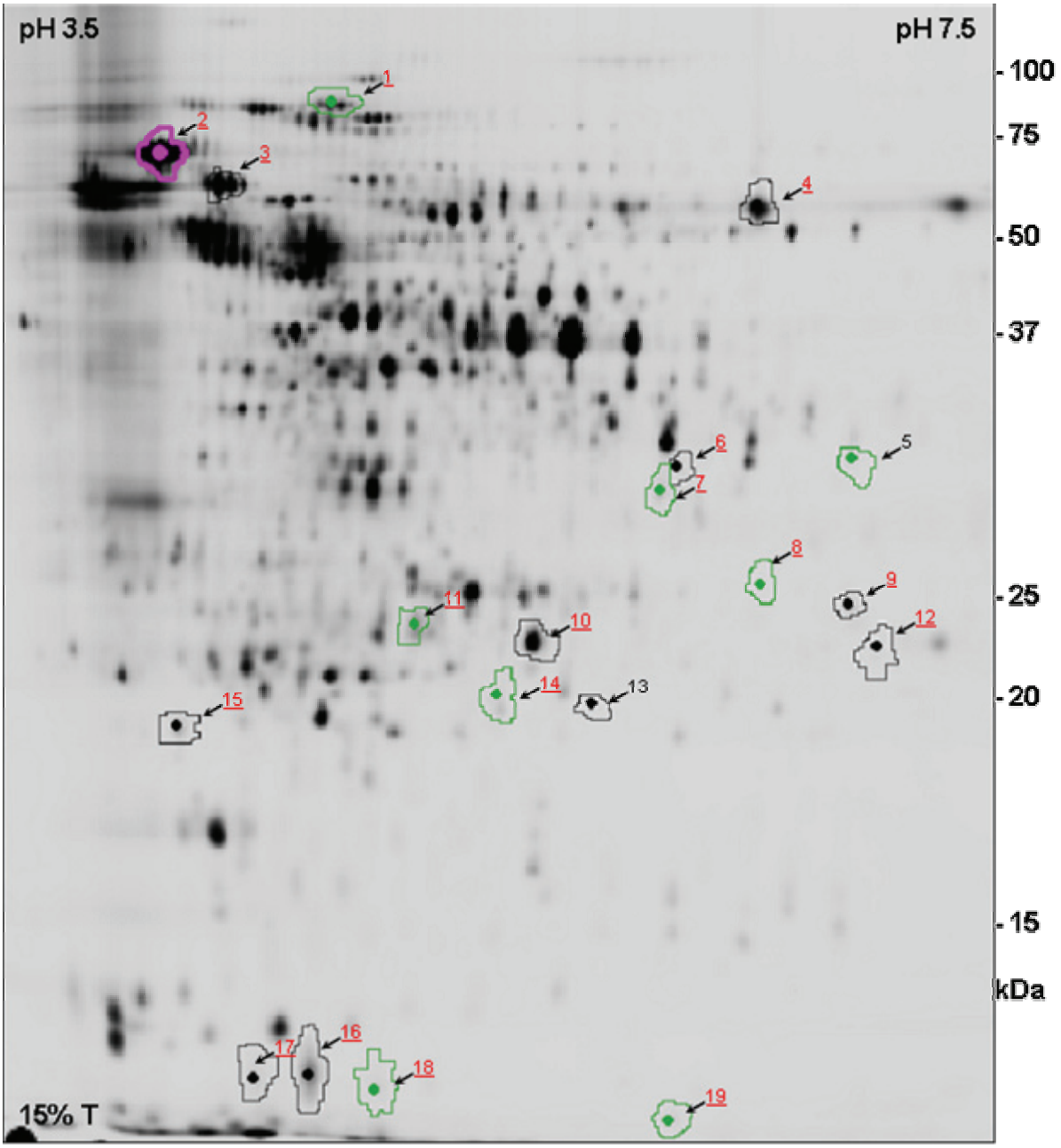

Fig. 17: Protein pattern representing 20 min heat shock at 43°C of *L. lactis* IL1403. Following extraction, proteins were Cy5 labeled for difference gel electrophoresis (DIGE) and separated by 2D electrophoresis in the pH range from 3.5-7.5 (IPG-DALT). Polygons around spots mark differences greater than factor 2 (black) or 1.5 (green) determined by image analysis with the DeCyder. Numbered spots refer to Tab. 11 and indicate spots with increased (red) or decreased intensity after heat shock.

Tab. 11: Differentially expressed proteins of *L. lactis* IL1403 after 20 min heat shock at 43°C determined by DIGE in the pH range from 3.5-7.5 and DeCyder analysis. Identification was performed at least two times per spot by peptide mass fingerprint (e<0.01).

No.[a)]	Master No.	Av. Ratio	T-test	PID[b)]	Protein
1	85	1.78	2.1E-03	12723443	ATP-dependent protease ATP-binding subunit
2	152	2.71	1.3E-04	12723891	DnaK protein
3	197	3.37	1.0E-06	12723267	60 KD chaperonin
4	205	2.29	1.3E-02	-	
5	530	-1.55	2.9E-02	-	
6	537	2.03	7.2E-03	12725053	HYPOTHETICAL PROTEIN
7	563	1.56	7.8E-03	12724529	oxidoreductase
8	660	1.64	2.5E-02	-	
9	685	2.05	8.5E-03	-	
10	723	2.23	1.5E-03	12724428	basic membrane protein A
11	724	1.93	1.7E-02	12722960	acyl carrier protein phosphodiesterase
12	745	3.74	3.6E-02	-	
13	805	-2.07	1.8E-03	12725316	HYPOTHETICAL PROTEIN
14	807	1.68	4.3E-02	12724522	phosphoribosylglycinamide formyltransferase (EC 2.1.2.2)
15	842	2.06	3.9E-02	12725108	UNKNOWN PROTEIN
16	1070	4.06	2.4E-05	12723266	10 KD chaperonin
17	1073	4.1	1.8E-03	12723266	10 KD chaperonin
18	1077	1.77	7.7E-05		
19	1106	1.8	2.3E-02	12723869	conserved hypothetical protein

a) Spot numbers refer to marked spots in Fig. 17
b) PID, identifier according to GenBank (NCBI)

3.2.3 20 min heat shock analyzed with ^{35}S pulse labeling

The heat shock response of *Lactococcus lactis* was also analyzed at the protein synthesis level by radioactive pulse labeling and compared to the analysis of total protein level by DIGE. For this, the cells were labeled for 20 min with ^{35}S-methionine/cysteine at reference and heat shock conditions. After 2D electrophoresis of the obtained protein extracts, the protein patterns were visualized by phosphor imaging, and analyzed with the ImageMaster 2D software. The crystalline character of phosphor imaging screens prevented a proper automatic spot detection and thus, initially more than 2000 spots were detected, but in average about 600 spots per gel were matched after manual examination. The normalized spot volumes were exported to Excel (Microsoft) and averaged ratios as well as T-tests were calculated. In total, 103 spots fitted to the selection of spots with averaged ratios outside the range from -2 to 2 (Fig. 18). A large portion of the spots (80% of 103) were decreased in their intensity by the heat treatment, similar to the observation after 45 min heat shock with DIGE at the lowered filter settings (77% of 71). The T-test for 17

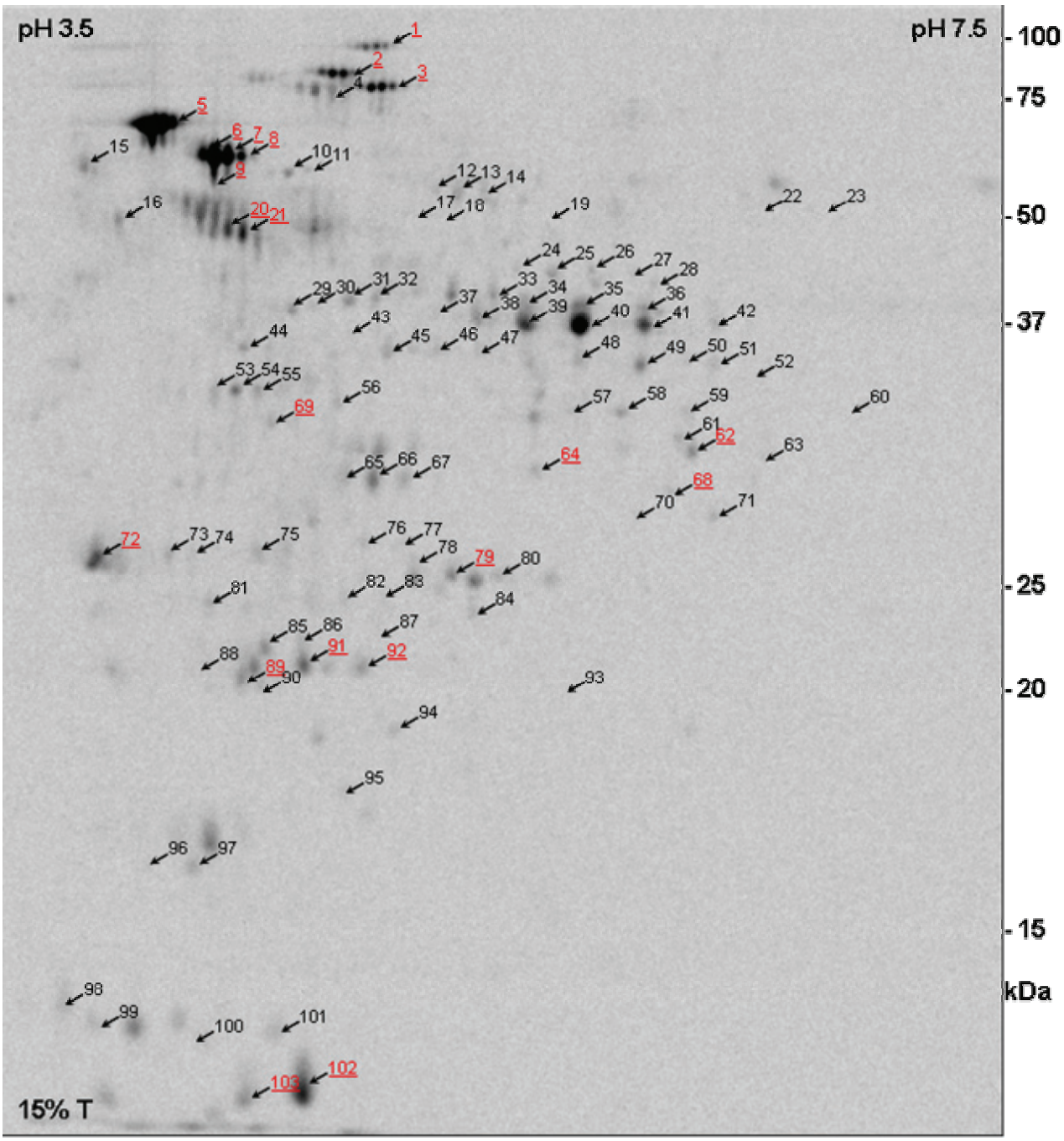

Fig. 18: Protein pattern representing 20 min heat shock at 43°C of ^{35}S pulse labeled *L. lactis* IL1403. Following extraction, proteins were separated by 2D electrophoresis in the pH range from 3.5-7.5 (IPG-DALT) and visualized by phosphor imaging. Numbers mark spot differences greater than factor 2 determined by image analysis with the ImageMaster 2D (see Tab. 12). Numbers of spots with increased intensity are marked red.

of the 103 spots missed the criteria of $p<0.05$ (up to 0.28 for spot 4) and for 11 spots, no T-test was calculable. In these cases, the poor significance occurred, because the spots were not detected in all analyzed gels and/or the variation near the detection

limit is high. For example, spot 43 is present in all gels of the reference condition, but missing in one gel of the heat shock condition and the spot volumes in the latter case are close to the detection limit. If only one or no corresponding spot was detected in one set of gels, this spot was indicated as 100 (induced) in Tab. 12.

The assignment of spots to the protein pattern of Coomassie stained gels for further identification by PMF was more difficult compared to DIGE gels, because the protein patterns of pulse labeled samples resemble snapshots of the protein expression. Thus, spots not merely differ in intensity, but are completely absent or unique in protein patterns. In order to simplify spot assignment, one sample was co-labeled with ^{35}S-Met/Cys and Cy2. The image overlay of the obtained protein patterns enabled spot assignment comparable to that of DIGE gels. By this methodical approach, 67 spots were identified (65%), which represents a comparable percentage to the identification rate of the DIGE analysis. Similar to the analysis of 20 and 45 min heat shock with the DIGE technique, the characteristic group of heat shock proteins and also the Clp proteins are evidently increased in their intensity after pulse labeling during heat stress. Interestingly, some more proteins like the regulators LlrA and PurN, which differed not in the DIGE analysis, were detected.

Tab. 12: Differentially expressed proteins of *L. lactis* IL1403 after 20 min heat shock at 43°C determined by ^{35}S pulse labeling, 2D electrophoresis in IPG 3.5-7.5 and ImageMaster 2D analysis. Identification was performed at least two times per spot by peptide mass fingerprint (e<0.01).

No.[a)]	Master No.	Av. Ratio[b)]	T-test	PID[c)]	Protein
1	2125	100.0		12724524	ClpB protein
2	2118	100.0		12723443	ATP-dependent protease ATP-binding subunit
3	2119	100.0		12724524	ClpB protein
4	312	-2.1	2.8E-01	12722901	dihydrolipoamide acetyltransferase component of PDH complex (EC 2.3.1.12)
5	364	3.4	6.7E-04	12723891	DnaK protein
6	403	13.3	6.7E-05	12723267	60 KD chaperonin
7	404	14.3	3.0E-03	12723267	60 KD chaperonin
8	2120	100.0			
9	2121	100.0		12723768	dipeptidase
10	434	-2.6	5.4E-03	12724481	GMP synthase (EC 6.3.5.2)
11	437	-4.4	9.8E-03	12722900	lipoamide dehydrogenase component of PDH complex (EC 1.8.1.4)
12	461	-6.5	2.2E-03	12724313	pyruvate kinase (EC 2.7.1.40)
13	456	-4.3	2.9E-03	12724313	pyruvate kinase (EC 2.7.1.40)
14	457	-5.4	1.9E-04	12724313	pyruvate kinase (EC 2.7.1.40)
15	417	-6.5	1.1E-01	12723436	trigger factor
16	501	-6.0	5.8E-03	12724901	cell division protein FtsZ
17	488	-10.6	9.7E-02	12723016	Glu-tRNA amidotransferase subunit B
18	489	-18.0	9.3E-02		

No.[a)]	Master No.	Av. Ratio[b)]	T-test	PID[c)]	Protein
19	482	-42.8	1.9E-01	12723014	Glu-tRNA amidotransferase subunit A
20	521	5.9	2.2E-02	12724911	aminopeptidase C
21	526	100.0			
22	470	-22.5	5.8E-04	12723077	IMP dehydrogenase (EC 1.1.1.205)
23	473	-39.6	8.0E-03	12723077	IMP dehydrogenase (EC 1.1.1.205)
24	560	-5.2	1.0E-02		
25	567	-5.4	1.7E-02	12723496	serine hydroxymethyltransferase (EC 2.1.2.1)
26	565	-8.0	3.0E-02	12723496	serine hydroxymethyltransferase (EC 2.1.2.1)
27	571	-9.9	7.1E-05		
28	582	-5.1	8.3E-04		
29	642	-4.0	9.1E-04	12725212	elongation factor Ts
30	643	-4.5	7.2E-04	12725212	elongation factor Ts
31	614	-3.8	2.3E-03	12723104	phosphoglycerate kinase (EC 2.7.2.3)
32	615	-3.9	6.3E-04	12723104	phosphoglycerate kinase (EC 2.7.2.3)
33	587	-2.6	1.5E-02	12724509	phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21)
34	604	-4.5	1.9E-03		
35	616	-2.8	4.5E-03	12725315	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
36	622	-3.4	4.0E-03		
37	633	-15.9	2.2E-02	12724153	malate oxidoreductase (EC 1.1.1.38)
38	630	-6.1	2.2E-03	12725315	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
39	605	-3.6	4.2E-03	12725315	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
40	639	-2.2	4.9E-03	12725315	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
41	644	-2.8	3.9E-03	12725315	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
42	645	-5.0	5.7E-04	12725214	conserved hypothetical protein
43	665	-20.0	6.4E-02	12724226	Trp-sensitive phospho-2-dehydro-deoxyheptonate aldolase (EC 4.1.2.15)
44	700	-3.7	2.0E-02		
45	689	-3.5	3.6E-04	12724723	mannose-specific PTS system component IIAB (EC 2.7.1.69)
46	686	-4.2	4.6E-03		
47	685	-7.1	2.0E-02		
48	692	-3.9	2.5E-03		
49	702	-2.9	4.1E-04		
50	704	-7.5	9.7E-02	12722912	fatty acid/phospholipid synthesis protein
51	701	-5.0	2.9E-04		
52	729	-20.1	8.5E-02	12723220	GTP-binding protein Era
53	764	-4.4	6.3E-02		
54	770	-2.8	1.9E-03		
55	765	-3.5	7.5E-05		
56	776	-6.7	1.7E-02		
57	777	-6.1	2.6E-04		
58	778	-4.0	5.3E-04	12724314	6-phosphofructokinase (EC 2.7.1.11)
59	775	-6.8	3.1E-04		
60	780	-18.2	9.1E-02	12723216	oligopeptide ABC transporter ATP binding protein
61	820	-4.2	1.4E-04	12723425	cysteine synthase (EC 4.2.99.8)
62	2122	100.0		12725053	HYPOTHETICAL PROTEIN
63	862	-15.0	5.2E-04		
64		100.0		12725330	regulator of purine biosynthetic genes
65	898	-8.1	1.7E-03	12724945	fructose-bisphosphate aldolase (EC 4.1.2.13)
66	906	-3.5	8.4E-03	12724945	fructose-bisphosphate aldolase (EC 4.1.2.13)
67	905	-4.3	1.1E-03	12724945	fructose-bisphosphate aldolase (EC 4.1.2.13)
68		100.0		12724529	oxidoreductase
69	2124	100.0			

No.[a)]	Master No.	Av. Ratio[b)]	T-test	PID[c)]	Protein
70	954	-7.5	1.1E-02	12724564	dihydrodipicolinate reductase (EC 1.3.1.26)
71	944	-6.1	2.1E-04	12724918	pyrroline-5-carboxylate reductase (EC 1.5.1.2)
72	1058	37.4	1.8E-03	12723890	stress responce protein GrpE
73	1037	-7.3	2.5E-02	12724096	triosephosphate isomerase (EC 5.3.1.1)
74	1025	-22.3	1.9E-02		
75	1033	-3.1	1.2E-02	12723011	transcriptional regulator
76	1015	-5.5	5.5E-03	12724936	HYPOTHETICAL PROTEIN
77	1012	-14.8	1.3E-01		
78	1054	-4.9	1.5E-01	12725086	arginine deiminase (EC 3.5.3.6)
79	1071	5.1		12724600	two-component system regulator
80	1067	-3.7	1.6E-03	12725101	HYPOTHETICAL PROTEIN
81	1110	-4.4	4.3E-02		
82	1104	-8.5	7.8E-03	12723447	polypeptide deformylase
83	1100	-16.2	3.0E-03	12725056	UMP-kinase (EC 2.7.4.-)
84	1103	-10.7	7.8E-04	12723547	HYPOTHETICAL PROTEIN
85	1184	-2.6	1.4E-03		
86	1177	-6.4	5.6E-02		
87	1144	-20.6	1.0E-01	12723866	purine nucleoside phosphorylase (EC 2.4.2.1)
88	1260	-5.6	1.8E-03		
89	1256	4.0	1.9E-02	12723580	ATP-dependent Clp protease proteolytic subunit
90	1266	-4.0	2.5E-02		
91	1217	4.1	5.3E-03	12723282	superoxide dismutase
92	2123	100.0			
93	1247	-17.3	1.9E-02	12725316	HYPOTHETICAL PROTEIN
94	1326	-4.6	1.1E-04		
95	1451	-5.2	2.1E-03		
96	1599	-6.8	9.9E-02		
97	1593	-3.3	2.5E-03		
98	1861	-27.1	1.7E-01		
99	1839	-24.0	5.5E-05	12724655	thioredoxin
100	1950	-15.7	6.3E-02		
101	1947	-7.9	1.3E-03	12722964	phosphocarrier protein HPr
102	2031	17.3	3.5E-04	12723266	10 KD chaperonin
103	2048	35.0		12723266	10 KD chaperonin

a) Spot numbers refer to marked spots in Fig. 18, p. 65
b) An average ratio of 100 marks spots, which were only detected under heat shock conditions
c) PID, identifier according to GenBank (NCBI)

The image analysis by ImageMaster 2D facilitates one more detailed fine tuning in spot detection by which the spots 6, 7 and 8 were detected individually. These spots were detected as one spot in the DeCyder analysis of DIGE gels. In this special case it might not cause a problem, because spots 6 and 7 were both identified as GroEL and spot 8 is probably GroEL too. In case that those spots would represent different proteins, for example narrower IPGs would improve the spot detection [42]. However, spot 35 was detected individually with the ImageMaster 2D, although it probably belongs to spot 40 (GAPDH). All identified spots of the DIGE as well as the pulse labeling analysis are listed in Tab. 13 and their averaged ratios are compared, as far as they exceed at least 1.5 or -1.5.

Tab. 13: Comparison of differentially expressed proteins of *L. lactis* IL1403 after 20 or 45 min heat shock at 43°C and in the pH range from 3.5-7.5 determined by DIGE and ^{35}S pulse labeling. DIGE gels were analyzed with DeCyder and phosphor imaging gels with ImageMaster 2D.

Protein (EC number)	Mr[a)]	pI[a)]	45 min[b)] DIGE	20 min[b)] DIGE	20 min[b)] ^{35}S
AMINO-ACID BIOSYNTHESIS					
Trp-sensitive phospho-2-dehydro-deoxyheptonate aldolase (EC 4.1.2.15)	38842.7	5.08	-2.2		-20.0
cysteine synthase (EC 4.2.99.8)	32358.02	5.76	3.3		-4.2
dihydrodipicolinate reductase (EC 1.3.1.26)	28490.82	5.8			-7.5
serine hydroxymethyltransferase (EC 2.1.2.1)	44789.99	5.45			-6.7
pyrroline-5-carboxylate reductase (EC 1.5.1.2)	27972.09	5.8			-6.1
BIOSYNTHESIS OF COFACTORS, PROSTHETIC GROUPS, AND CARRIERS					
dihydroxynaphthonic acid synthase (EC 4.1.3.36)	30880.28	5.5	-1.5		
thioredoxin	11672.41	4.32			-24.0
CELL ENVELOPE					
basic membrane protein A	36652.03	8.68	-2.3	2.2	
UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	34873.02	5.5	-1.6		
ENERGY METABOLISM					
alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)	98220.83	5.71	-7.9		
arginine deiminase (EC 3.5.3.6)	46043.16	5.15			-4.9
citrate lyase beta chain (EC 4.1.3.6)	33307.51	4.9	-2.0		
enolase (EC 4.2.1.11)	46911.77	4.68	-1.8		
fructose-bisphosphate aldolase (EC 4.1.2.13)	31989.56	5.04			-5.3
UDP-glucose 4-epimerase (EC 5.1.3.2)	36229.98	5.18	-2.1		
glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	35819.46	5.57	-1.6		-3.5
L-lactate dehydrogenase (EC 1.1.1.27)	35050.69	4.97	-1.5		
malate oxidoreductase (EC 1.1.1.38)	40475.63	5.21	-2.1		-15.9
dihydrolipoamide acetyltransferase component of PDH complex (EC 2.3.1.12)	56316.88	4.95			-2.1
lipoamide dehydrogenase component of PDH complex (EC 1.8.1.4)	49866.82	4.9			-4.4
6-phosphofructokinase (EC 2.7.1.11)	35805.61	5.68			-4.0
phosphoglycerate kinase (EC 2.7.2.3)	42070.09	5.06			-3.8
pyruvate kinase (EC 2.7.1.40)	54254.84	5.27			-5.4
triosephosphate isomerase (EC 5.3.1.1)	26905.42	4.63			-7.3
oxidoreductase YbiE	23260.49	5.8	-2.0		
oxidoreductase YphC	31519.72	5.78	1.6	1.6	100.0
FATTY ACID AND PHOSPHOLIPID METABOLISM					
acyl carrier protein phosphodiesterase	24662.79	5.28		1.9	
NADH-dependent enoyl-ACP reductase	26382.42	6.44	-2.5		
fatty acid/phospholipid synthesis protein	34786.01	5.72			-7.5
PURINES, PYRIMIDINES, NUCLEOSIDES AND NUCLEOTIDES					
cytidine monophosphate kinase (EC 2.7.4.14)	24548.3	5.36	-1.5		
purine nucleoside phosphorylase (EC 2.4.2.1)	25427.15	5.15			-20.6
GMP synthase (EC 6.3.5.2)	56780.71	4.86			-2.6
IMP dehydrogenase (EC 1.1.1.205)	52826.54	5.95	-1.6		-31.1
ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	35278.25	5.34	-2.0		
adenylosuccinate lyase (EC 4.3.2.2)	49687.41	5.37	-1.7		
phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21)	39159.49	5.22			-2.6
phosphoribosylglycinamide formyltransferase (EC 2.1.2.2)	20482.26	5.32		1.7	
CTP synthetase	59552.1	5.57	-2.2		

Protein (EC number)	Mr[a)]	pI[a)]	45 min[b)] DIGE	20 min[b)] DIGE	20 min[b)] ^{35}S
UMP-kinase (EC 2.7.4.-)	25677.42	5.25			-16.2
glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24)	32130.68	4.66	-1.7		
REGULATORY FUNCTIONS					
transcriptional regulator CodY	29128.45	4.79			-3.1
GTP-binding protein Era	34737.04	5.88			-20.1
two-component system regulator LlrA	26684.36	5.32			5.1
regulator of purine biosynthetic genes	30360.87	5.49			100.0
zinc transport transcriptional regulator	16366.72	5.56	-2.2		
STRESS RELATED PROTEINS					
ClpB protein	97335.08	5.09	3.7		100.0
ATP-dependent protease ATP-binding subunit ClpE	83144.86	5.02	3.6	1.8	100.0
ATP-dependent Clp protease proteolytic subunit	22082.23	4.98	2.0		4.0
DnaK protein	64987.16	4.62	5.0	2.7	3.4
non-heme iron-binding ferritin	16652.83	4.69	2.1		
cell division protein FtsZ	43988.22	4.54			-6.0
60 KD chaperonin	57201.42	4.75	7.0	3.4	13.8
10 KD chaperonin	10220.82	5.03	6.4	4.1	26.2
stress responce protein GrpE	20580.95	4.43			37.4
superoxide dismutase	23253.88	5.03	2.5		4.1
trigger factor	46930.55	4.43			-6.5
TRANSLATION					
polypeptide deformylase	23769.28	5.03			-8.5
Glu-tRNA amidotransferase subunit A	52071.24	5.51			-42.8
Glu-tRNA amidotransferase subunit B	54730.99	5.32	-1.7		-10.6
aminopeptidase C	49914.1	4.74			5.9
dipeptidase PepV	51943.44	4.75			100.0
30S ribosomal protein S2	28538.73	5.08	-1.7		
elongation factor Ts	36669.66	4.92	-1.5		-4.2
elongation factor Tu	43211.91	4.89	-1.6		
TRANSPORT AND BINDING PROTEINS					
oligopeptide ABC transporter ATP binding protein OptF	35014.99	6.04			-18.2
mannose-specific PTS system component IIAB (EC 2.7.1.69)	35064.49	5.14			-3.5
phosphocarrier protein HPr	9179.45	5.05	-1.6		-7.9
UNKNOWN					
HYPOTETICAL PROTEIN YciC	78771.34	5.63	-2.1		
HYPOTHETICAL PROTEIN YgdA	21282.95	5.36	-1.7		-10.7
conserved hypothetical protein YjfJ	11941.49	5.84		1.8	
conserved hypothetical protein YjiF	35697.64	5.21	-2.3		
HYPOTHETICAL PROTEIN YtgG	26545.46	5.02			-5.5
HYPOTHETICAL PROTEIN YtjH	27295.82	5.85	-1.8		
HYPOTHETICAL PROTEIN YuiC	32312.76	5.78		2.0	100.0
HYPOTHETICAL PROTEIN YvdE	26511.12	5.41			-3.7
UNKNOWN PROTEIN YveC	18490.78	4.56		2.1	
conserved hypothetical protein YwcC	37287.83	5.74			-5.0
HYPOTHETICAL PROTEIN YxdB	19508.19	5.43		-2.1	-17.3

a) Calculated values

b) Averaged ratios were assigned from Tab. 10, p. 60, Tab. 11, p. 64, and Tab. 12, p. 66. If the protein occurred in more than one spot on the gel, ratios were again averaged. An average ratio of 100 marks spots, which were only detected under heat shock conditions. Differences greater than factor 2 are marked in red for induction and green for repression.

3.3 Analysis of high pressure effects on *L. lactis* at protein level

For the analysis of the late high pressure stress response and recovery of *Lactococcus lactis* IL1403 with 2D electrophoresis, two methodical approaches were chosen. First, alterations at the total protein level were determined with the DIGE technique and second, changes of proteins expressed during the first 30 min after pressure treatment were analyzed by ^{35}S pulse labeling. Thus, in particular the observed alterations in protein patterns after pulse labeling were expected to represent the late phase of stress response and initial recovery of the bacteria. In both analytical approaches, protein patterns of cells treated with 60 or 90 MPa for 30 min were compared to patterns of atmospherically grown cells (reference). The two pressure steps were selected, because it was reported that piezosensitive bacteria stop growing at 50 to 60 MPa [129] and protein synthesis is completely inhibited *in vivo* between 83 to 110 MPa [21]. Indeed, measurements of the optical density before and after the pressure treatment indicated negligible growth of *L. lactis* IL1403 in SA medium at 60 MPa ($\Delta OD < 0.05$ in 30 min). Similar results were recently published for the growth of *L. lactis* MG1363 in GM17 medium (2.3% growth at 50 MPa for 20h compared to growth under atmospheric conditions [128]). Since radioactive pulse labeling during high pressure treatment was not manageable in the way it was performed by Welch *et al.*, 1993 [21] and in order to avoid contamination of the device for pressure application, protein synthesis was not monitored at high pressure.

3.3.1 Analysis of high pressure response by DIGE

Protein extracts of pressure stressed bacteria subjected to DIGE analysis were Cy5 labeled and compared to Cy3 labeled reference extracts. Furthermore, an internal standard was used for improved spot matching and diminishing of influences by gel to gel variances on the analysis. The protein patterns were analyzed with the DeCyder and spots filtered according to their averaged ratio and statistical significance (T-Test). Referring to the analysis of heat shock response in *L. lactis* with DIGE (see section 4.2, p. 109), only spots with at least 1.5-fold difference (averaged ratio) and $p<0.05$ were regarded as altered due to stress. Smaller differences are probably influenced by biological and methodical aspects and were excluded from

the analysis. Similar to the heat shock analysis, image overlays merely provide preliminary results (Fig. 19 and Fig. 21, p. 74). Only the software analysis with the DeCyder and manual inspection of each proposed difference results in the significant differences marked in Fig. 20 and Fig. 22, p. 75, and listed in Tab. 14, p. 77.

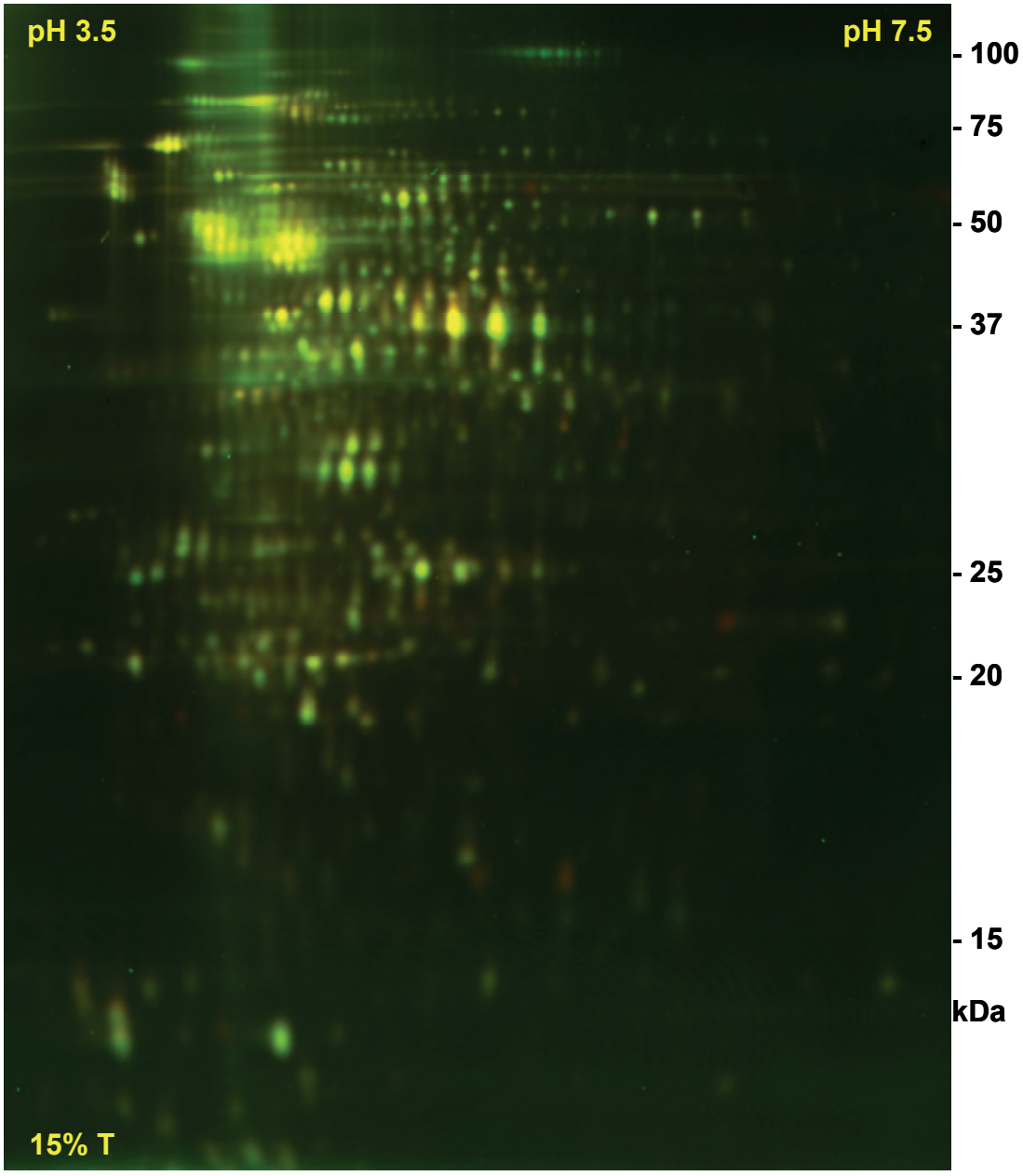

Fig. 19: Image overlay of two protein patterns representing reference and high pressure at 60 MPa for 30 min of *L. lactis* IL1403, DIGE in IPG 3.5-7.5 and **15% T**.

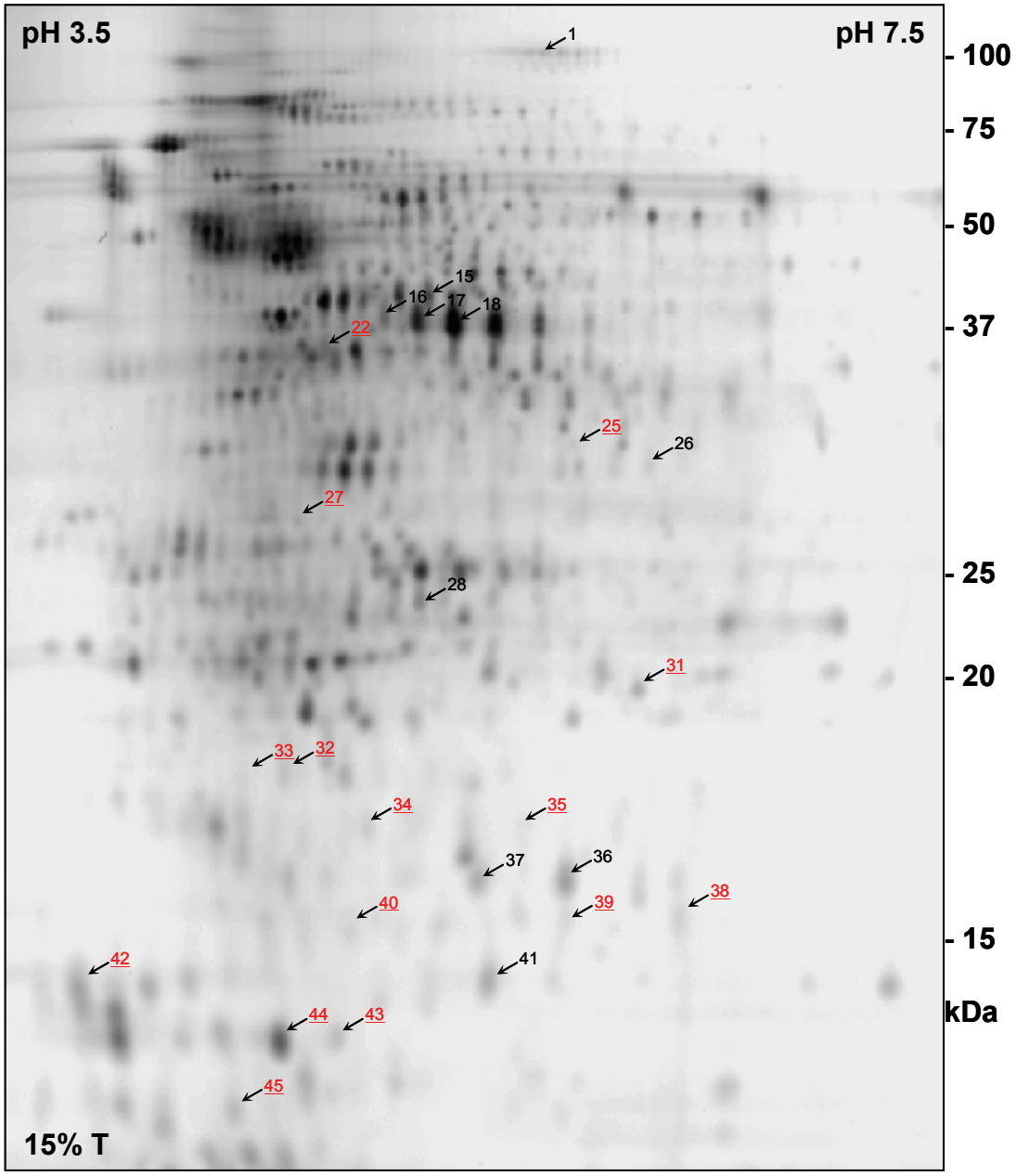

Fig. 20: Results of DeCyder analysis of high pressure stress response of *L. lactis* IL1403, DIGE in IPG 3.5-7.5 and 15% T. Numbered spots refer to Tab. 14, p. 77, and indicate spots with increased (red) or decreased intensity greater than factor 1.5 after pressure treatments at 60 or 90 MPa for 30 min.

At first, 2D gels with 15% acrylamide content in the second dimension were compared. In this case, the differential spot analysis of cells treated with 60 MPa revealed 10 spots with increased and 9 spots with decreased spot intensity (Tab. 14, p. 77). Most of the observed differences occurred in the low molecular weight area on the gel (Fig. 20). In addition, the samples were separated on 2D gels with low acrylamide content in the second dimension (11%), in order to improve the resolution for the detection of proteins with high molecular masses. In that case, only one spot

was increased and 14 decreased on gels representing 60 MPa stressed cells. At least nine spots on the gels with 11% AA content are located in the high molecular weight area (Fig. 22, p. 75). This indicates indeed improved spot analysis in that area, and thus complements the analysis of gels with 15% AA content. Therefore, each of the following analyses was performed after separation of the samples in gels with 11 and 15% AA content.

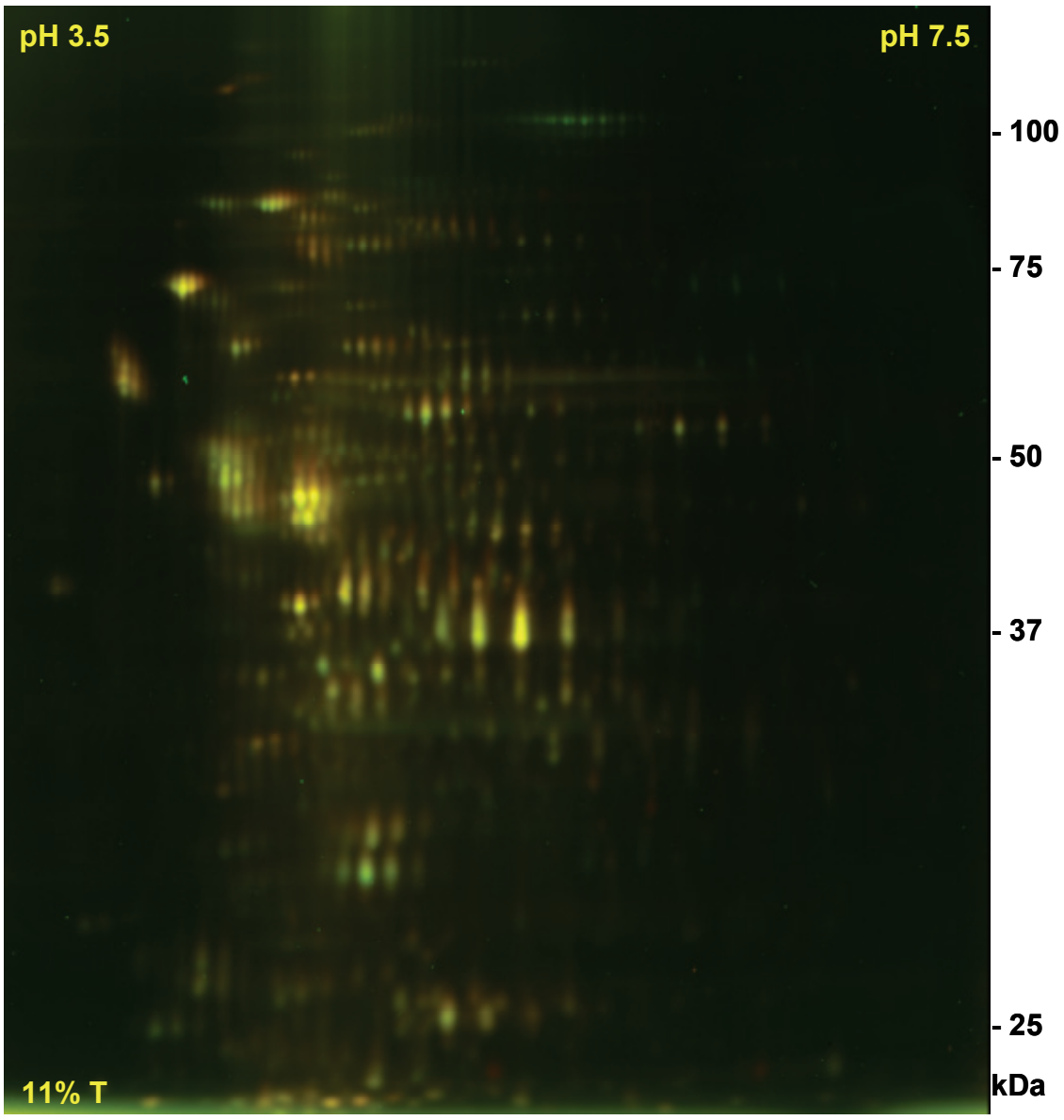

Fig. 21: Image overlay of two protein patterns representing reference and high pressure at 60 MPa for 30 min of *L. lactis* IL1403, DIGE in IPG 3.5-7.5 and **11% T.**

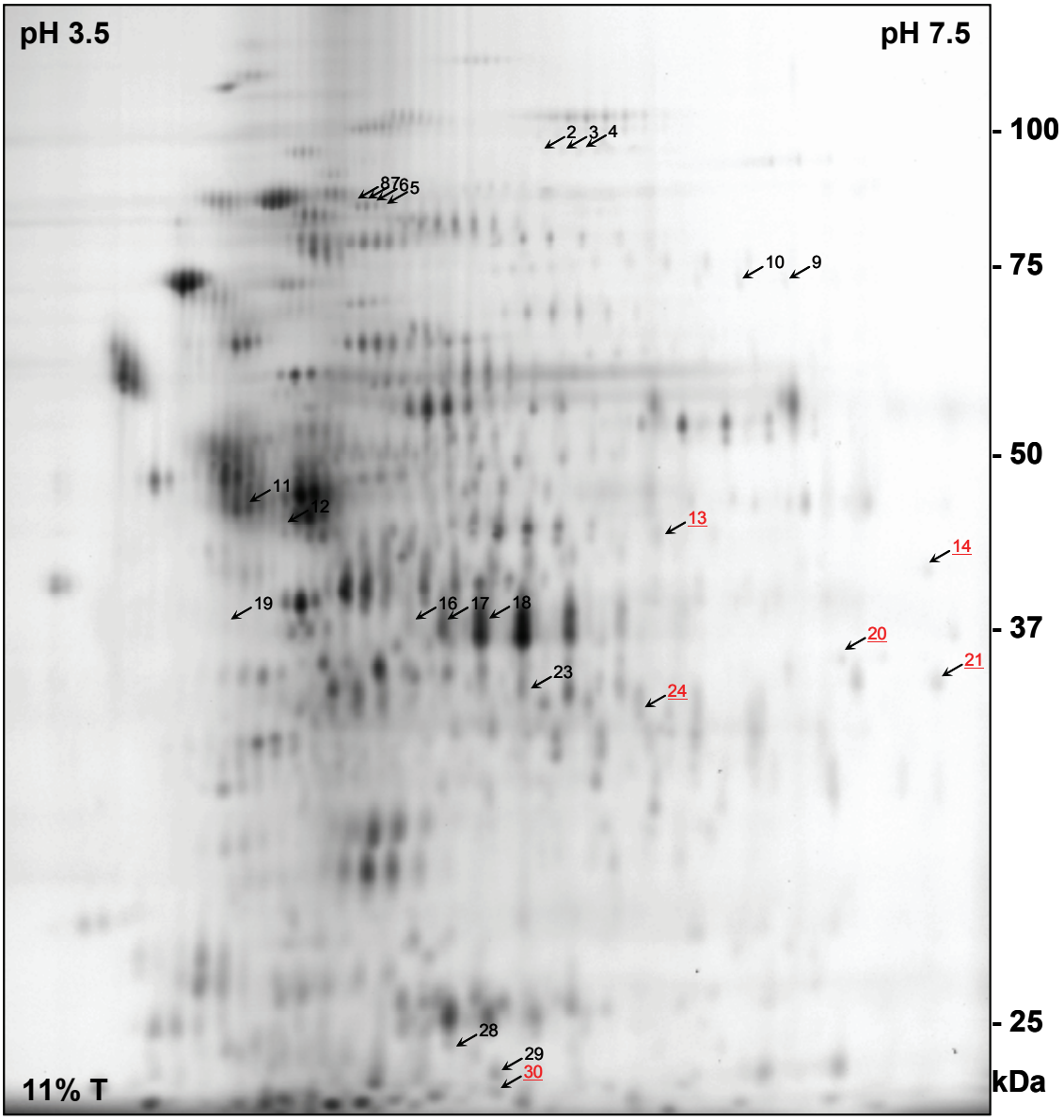

Fig. 22: Results of DeCyder analysis of high pressure stress response of *L. lactis* IL1403, DIGE in IPG 3.5-7.5 and 11% T. Numbered spots refer to Tab. 14, p. 77, and indicate spots with increased (red) or decreased intensity greater than factor 1.5 after pressure treatments at 60 or 90 MPa for 30 min.

The spots with differential intensity in the image analysis were assigned to corresponding spots on Coomassie stained gels for further analysis by peptide mass fingerprint as described in section 3.2.1, p. 55, because direct identification of DIGE spots often resulted in poor coverage or not interpretable spectra. 15 spots of the differential analysis of 60 MPa stressed cells were identified by PMF. Most of the identified proteins are implicated in the energy metabolism, four are uncharacterized (hypothetical) proteins and one is a known stress protein of *L. lactis* (ClpB). Spots 16, 17 and 18 are probably corresponding spots on gels with 15% and 11% AA content,

and thus bear same numbers in Fig. 20, p. 73 and Fig. 22. Identification of these three spots supported the spot assignments.

The comparison of bacteria stressed with 90 MPa and the reference group revealed 19 spots with increased and 17 spots with decreased intensity after pressure treatment. The distribution of the determined spots in the gels with different AA content was similar to that of the 60 MPa analysis. The application of a common internal standard in both DIGE analyses facilitated confident spots assignment of gels with same AA content. According to this, 9 spots were increased and 13 decreased after 60 and 90 MPa pressure stress (Tab. 14, p. 77). Besides spots 32 and 33, the corresponding averaged ratios in the two analyses did not diverge largely from each other. Since more than 60% of the pressure dependent spots determined by DIGE analysis were altered at 60 as well as at 90 MPa, identification of spots with altered intensity after 90 MPa stress added only four further proteins to the list in Tab. 14, p. 77. Two of them are uncharacterized (hypothetical) proteins, one is an oxidoreductase and the remaining is implicated in the synthesis of aromatic amino acids.

In total, the DIGE analysis showed that 45 spots were differentially expressed at high pressure and 22 of them were identified. Most of the spots, which were not identified, mark low abundant proteins and were barely visible on Coomassie stained gels. The largest group of identified proteins is implicated in energy metabolism. Remarkably, the second largest group consists of six uncharacterized (hypothetical) proteins.

Tab. 14: Differentially expressed proteins of *L. lactis* IL1403 after pressure treatments at 60 or 90 MPa determined by DIGE in the pH range from 3.5-7.5 and DeCyder analysis. Identification was performed at least two times per spot by peptide mass fingerprint (e<0.01).

	60 MPa[b]				90 MPa[b]					
	11% T		15% T		11% T		15% T			
No.[a]	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	PID[c]	Protein
1			3.1E-02	-1.6			2.1E-02	-1.6	12725215	alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)
2	7.3E-03	-1.6			4.5E-03	-1.7			-	
3	2.1E-03	-1.8			3.9E-03	-1.6			-	
4	7.9E-03	-2.2			1.2E-03	-2.1			-	
5	5.2E-03	-1.7			2.2E-01	-1.1			-	
6	1.6E-05	-1.6			3.9E-02	-1.2			-	
7	2.9E-03	-1.8			3.8E-02	-1.3			12724524	ClpB protein
8	6.5E-04	-1.9			3.8E-04	-1.3			-	
9	2.6E-02	-1.6			6.4E-04	-1.9			-	
10	1.5E-02	-1.5			2.5E-04	-1.8			-	
11	9.4E-02	-1.4			2.0E-02	-1.6			-	
12	4.1E-02	-1.5			7.4E-03	-1.9			-	
13	1.1E-02	1.7			5.0E-03	2.1			12723499	D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)
14	5.1E-01	1.1			3.6E-02	1.8			-	
15			1.1E-02	-1.5			1.2E-01	-1.3	12724509	phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21)
16	2.1E-04	-2.0	4.6E-03	-1.9	8.7E-04	-1.8	1.3E-02	-1.7	12724153	malate oxidoreductase (EC 1.1.1.38)
17	2.1E-04	-2.1	7.7E-04	-2.0	4.3E-04	-1.8	1.1E-02	-1.6	12725315	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
18	7.1E-04	-2.1	4.6E-03	-1.6	3.1E-03	-1.6	1.6E-01	-1.2	12725315	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
19	1.0E-01	-1.4			8.6E-04	-1.7			-	
20	2.1E-01	1.2			2.9E-03	2.2			12725003	oxidoreductase
21	1.2E-01	-1.2			7.6E-03	1.6			-	
22			5.3E-02	1.4			3.7E-03	1.8	12724226	Trp-sensitive phospho-2-dehydro-deoxyheptonate aldolase (EC 4.1.2.15)
23	2.5E-03	-1.7			1.2E-02	-1.5			-	
24	2.0E-01	1.2			2.0E-02	1.6			12723220	GTP-binding protein Era
25			3.8E-02	2.0			7.8E-01	1.1	12725053	HYPOTHETICAL PROTEIN
26			3.3E-02	-1.8			4.8E-02	-1.8	-	
27			6.8E-01	1.2			6.1E-03	1.8	12724665	HYPOTHETICAL PROTEIN

No. a)	60 MPa b) 11% T T-test	60 MPa b) 11% T Av. Ratio	60 MPa b) 15% T T-test	60 MPa b) 15% T Av. Ratio	90 MPa b) 11% T T-test	90 MPa b) 11% T Av. Ratio	90 MPa b) 15% T T-test	90 MPa b) 15% T Av. Ratio	PID c)	Protein
28	8.8E-03	-1.6	2.2E-02	-1.4	2.0E-02	-1.4	1.7E-02	-1.6	12723547	HYPOTHETICAL PROTEIN
29	2.7E-02	-1.6			9.5E-01	-1.0			12724428	basic membrane protein A
30	7.9E-01	-1.1			1.7E-02	1.8			-	
31			1.2E-02	1.5			5.7E-03	2.1	12725316	HYPOTHETICAL PROTEIN
32			2.4E-02	1.9			3.6E-03	4.8	-	
33			2.2E-01	1.5			2.0E-02	3.6	-	
34			1.3E-01	1.6			3.8E-02	1.7	12724862	conserved hypothetical protein
35			6.9E-02	1.6			1.0E-02	1.7	-	
36			1.7E-02	-2.1			3.0E-02	-1.8	12724785	ATP synthase epsilon subunit (EC 3.6.1.34)
37			4.3E-03	-2.2			3.9E-02	-1.8	12723203	phosphoglycerate mutase (EC 5.4.2.1)
38			1.2E-03	2.1			7.6E-02	1.6	-	
39			1.1E-02	2.4			8.3E-02	1.4	-	
40			6.8E-03	1.5			8.9E-03	1.9	12723005	HYPOTHETICAL PROTEIN
41			1.1E-02	-1.8			5.8E-02	-1.3	12725171	zinc transport transcriptional regulator
42			3.2E-03	1.3			4.2E-02	1.5	12724655	thioredoxin
43			4.5E-02	1.6			2.1E-02	1.7	-	
44			3.2E-02	1.3			1.6E-02	1.6	12722964	phosphocarrier protein HPr
45			2.6E-02	1.6			8.7E-02	1.8	-	

a) Spot numbers refer to marked spots in Fig. 20, p. 73, and Fig. 22, p.75
b) Differences greater than factor 1.5 are marked in red for induction and green for repression.
c) PID, identifier according to GenBank (NCBI)

3.3.2 Analysis of protein expression after pressure stress by ^{35}S pulse labeling

Similar to the DIGE analyses, protein patterns obtained after pulse labeling and representing the reference, 60 or 90 MPa conditions, were compared in 2D gels with 11 and 15% AA content. Image analysis was performed with the ImageMaster 2D as described in section 2.6.4, p. 30, and spots with twofold increase or decrease in spot intensity after pressure treatment were regarded as influenced by high pressure. Smaller differences were biased by methodical and biological variances and only indicated in Tab. 15, p. 82, if the spots were significantly altered in a parallel analysis using another AA content or pressure.

The analysis of differential expression after 60 MPa revealed 18 spots with increased and 26 spots with decreased spot intensity. After treatment at 90 MPa, 52 spots were increased and 46 decreased. Comparison of the analyses showed that many of the differentially expressed spots were even more influenced by 90 MPa than by 60 MPa treatments (e.g. spots 2-4, 25-29). Other spots indicated similar (e.g. spots 10, 11) and three even opposite alterations (spots 34, 35 and 64).

It has been tried to assign corresponding spots on gels with different AA content in the way it was done for DIGE gels (Tab. 15, p. 82). In 50 cases of the 98 determined differences, spots were found in similar positions on gels with 11% and 15% AA content. In 46 of these 50 cases similar differential expression was determined, while in the remaining 4 cases the criteria of twofold difference was only met on gels with either 11% or 15% AA content (spots 17, 34, 41 and 74). In comparison to DIGE analyses, averaged spot ratios varied more between the gels with different AA content. For example, spot 72 indicated an increase of 8.7-fold in gels with 11% AA content compared to 2.5-fold increase in gels with 15% AA content (60 MPa values). Several reasons might cause the differences between the sets of gels, e.g. spot areas are overlapping or their size largely differs in the two sets of gels. Gel to gel variances are probably another source for the observed differences. The influence of gel to gel variances is diminished by application of an internal standard in DIGE gels [56] and thus, explains the higher coherence of DIGE analyses. However, in all cases in which spots were found on gels with 11% and 15% AA content, they were either both increased or both decreased. Furthermore, all differences greater than twofold were confirmed at least in one set of gels per spot by

statistical significance ($p<0.05$). For example, spot 6 is 1.4-fold decreased in gels with 11% T representing 60 MPa, but 2.2-fold in gels with 15% T and in the latter case the T-Test is 0.017. Therefore, all differences indicated in Tab. 15, p. 82, were regarded as stress related.

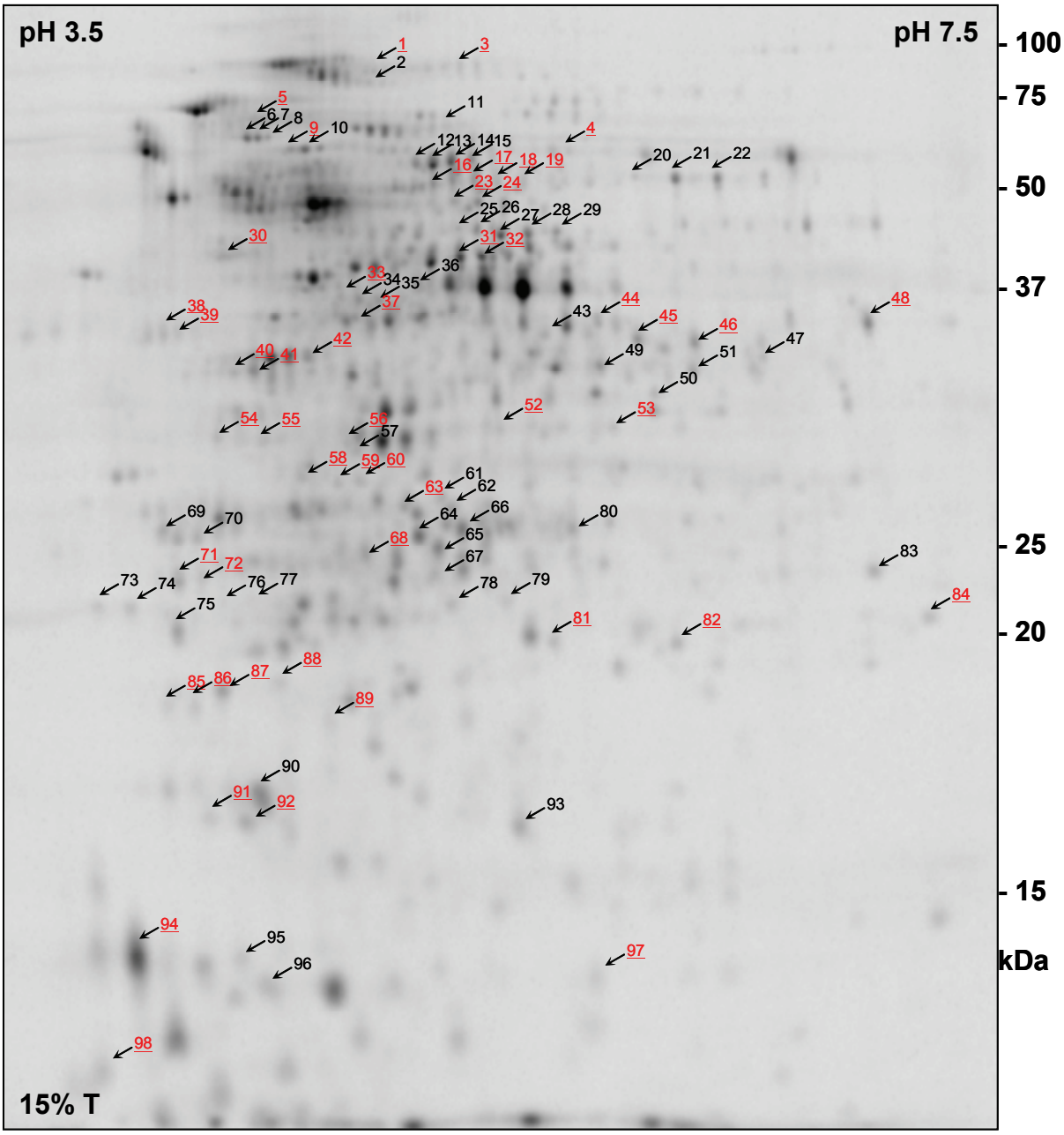

Fig. 23: Protein pattern representing ^{35}S pulse labeled proteins of *L. lactis* IL1403 within the first 30 min after high pressure treatment at 60 MPa. Following extraction, proteins were separated by 2D electrophoresis in the pH range from 3.5-7.5 (IPG-DALT) and visualized by phosphor imaging. Numbers mark spots with differences greater than factor 2 after treatments at 60 or 90 MPa determined by image analysis with the ImageMaster 2D (see Tab. 15, p. 82). Increased spots were marked red.

Assignment of ^{35}S labeled spots to corresponding spots on Coomassie stained gels for subsequent identification by PMF was improved by co-labeling of ^{35}S pulse labeled samples with Cy2 and assignment by way of Cy2 co-detected spots. In total, 57 different proteins were identified in 66 distinct spots. Among the identified proteins with increased intensities after 60 MPa stress were proteins with regulatory functions (e.g. Era) and several uncharacterized proteins. On the other hand, proteins implicated in e.g. purine and energy metabolism, but also the heat shock protein GroEL, were decreased. Since most of the spots with altered intensity after 60 MPa stress were similarly or even more influenced after 90 MPa, the major groups of proteins decreased in the latter case were the same. Increased proteins after 90 MPa treatment belonged to several functional classes. At least four of them were assigned to regulatory functions (Era, PurR, YnaB and ZitR) and three were stress related proteins (ClpB, RecA and YnaB). Others are implicated in cell envelope synthesis, transcription and translation. A relatively large part is formed by currently uncharacterized proteins (Tab. 15). All identified pressure related proteins were categorized into functional groups and the results of the gel analyses summarized in Tab. 16, p. 86.

Tab. 15: Differentially expressed proteins of *L. lactis* IL1403 within the first 30 min after pressure treatments at 60 or 90 MPa determined by ^{35}S pulse labeling, 2D electrophoresis in IPG 3.5-7.5 and ImageMaster 2D analysis. Identification was performed at least two times per spot by peptide mass fingerprint (e<0.01).

	60 MPa[b]				90 MPa[b]					
	11% T		15% T		11% T		15% T			
No.[a]	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	PID[c]	Protein
1	8.1E-01	-1.1			5.0E-03	2.7			12724524	ClpB protein
2	1.0E-02	-2.0	8.2E-02	-1.9	3.6E-03	-4.8	9.4E-03	-3.6	12724634	transketolase (EC 2.2.1.1)
3	2.1E-02	1.5			9.8E-04	2.4			12723918	ribonucleoside-diphosphate reductase alpha chain (EC 1.17.4.1)
4	1.4E-01	1.6			3.8E-03	3.2			12723372	CTP synthetase
5		100.0				8.7			12722914	ABC transporter ATP binding protein
6	1.1E-01	-1.4	1.7E-02	-2.2	5.7E-04	-2.3	7.4E-03	-3.8	12723267	60 KD chaperonin
7	9.3E-03	-1.5	1.4E-02	-2.1	1.4E-03	-2.6	2.6E-03	-3.9	12723267	60 KD chaperonin
8	6.6E-01	-1.1	4.5E-02	-2.5	3.9E-02	-4.0	2.9E-02	-6.1		
9	4.0E-01	1.6			8.9E-04	6.5				
10	2.1E-03	-2.2	2.5E-01	-2.2	8.5E-04	-2.1	1.2E-01	-3.3	12724481	GMP synthase (EC 6.3.5.2)
11	1.8E-02	-2.0	3.2E-02	-2.3	8.4E-03	-2.4	2.6E-02	-2.3		
12		-100.0	6.2E-02	-2.3		-100.0	6.0E-03	-6.0	12724313	pyruvate kinase (EC 2.7.1.40)
13	1.3E-02	-2.1	1.7E-02	-2.6	2.2E-03	-3.8	2.9E-03	-7.6	12724313	pyruvate kinase (EC 2.7.1.40)
14	4.0E-02	-2.0	3.7E-02	-1.9	2.5E-03	-5.1	3.9E-03	-4.5	12724313	pyruvate kinase (EC 2.7.1.40)
15	2.7E-02	-1.8		-3.3	1.4E-03	-3.9	1.6E-03	-7.3		
16		100.0				100.0			12725089	UDP-N-acetylmuramate-alanine ligase (EC 6.3.2.8)
17	2.8E-01	1.7	2.1E-01	1.9	1.3E-01	1.6	9.9E-03	3.5	12724202	glucose inhibited division protein GidC
18	7.1E-01	1.3	4.6E-01	1.3	4.2E-04	9.1	3.0E-03	4.8	12723014	Glu-tRNA amidotransferase subunit A
19	5.4E-01	1.5		1.5	6.4E-02	11.6	1.1E-02	6.7	12723014	Glu-tRNA amidotransferase subunit A
20			2.0E-02	-3.0			4.8E-03	-3.4	12723077	IMP dehydrogenase (EC 1.1.1.205)
21	3.0E-02	-1.7	7.3E-01	-1.2	1.3E-02	-2.2	3.5E-02	-2.0	12723077	IMP dehydrogenase (EC 1.1.1.205)
22	2.5E-03	-1.6	9.1E-01	-1.1	1.0E-04	-2.2	5.5E-02	-3.1		
23	8.0E-01	-1.1			2.7E-03	2.8				
24	1.7E-02	1.5	2.2E-01	1.9	2.1E-06	4.1	2.9E-03	2.6	12723210	D-Ala-D-Ala adding enzyme (EC 6.3.2.15)
25	1.4E-02	-2.7	1.6E-01	-3.8	1.0E-02	-3.5		-14.0	12724651	adenylosuccinate lyase (EC 4.3.2.2)

No.[a]	60 MPa[b] 11% T T-test	60 MPa[b] 11% T Av. Ratio	60 MPa[b] 15% T T-test	60 MPa[b] 15% T Av. Ratio	90 MPa[b] 11% T T-test	90 MPa[b] 11% T Av. Ratio	90 MPa[b] 15% T T-test	90 MPa[b] 15% T Av. Ratio	PID[c]	Protein
26	1.3E-02	-2.3	7.9E-02	-2.6	3.0E-03	-4.4	3.4E-03	-5.0	12724993	adenylosuccinate synthase (EC 6.3.4.4)
27	6.6E-05	-2.9	3.6E-02	-2.5	1.7E-05	-5.4	1.3E-02	-5.1	12723496	serine hydroxymethyltransferase (EC 2.1.2.1)
28	3.2E-02	-2.7	8.8E-02	-2.8	7.0E-03	-6.2	1.8E-02	-3.7	12723496	serine hydroxymethyltransferase (EC 2.1.2.1)
29	3.2E-02	-2.5	2.0E-01	-2.2	9.1E-03	-8.0	1.5E-01	-5.1	12723078	GTP-binding protein
30			1.3E-01	1.5			2.0E-03	2.6	12723209	D-alanine-D-alanine ligase (EC 6.3.2.4)
31	3.0E-01	1.2			1.3E-03	2.7				
32	7.0E-03	1.9	4.5E-01	1.5	7.6E-05	3.4	2.2E-02	2.1	12723223	RecA protein
33					1.7E-02	3.8		100.0		
34	6.6E-02	-2.6	3.0E-01	-1.6	5.5E-03	2.3	1.6E-01	1.9	12724226	Trp-sensitive phospho-2-dehydro-deoxyheptonate aldolase (EC 4.1.2.15)
35	1.6E-01	-2.2	9.9E-01	-1.0	1.2E-02	2.8	1.3E-01	2.6	12724226	Trp-sensitive phospho-2-dehydro-deoxyheptonate aldolase (EC 4.1.2.15)
36	6.7E-02	-5.2	5.1E-02	-2.2	4.8E-02	-2.6	1.8E-02	-2.3	12724153	malate oxidoreductase (EC 1.1.1.38)
37						100.0		100.0		
38			1.4E-01	1.7			2.9E-03	3.3	12723917	ribonucleoside-diphosphate reductase beta chain (EC 1.17.4.1)
39			2.0E-01	1.6			1.1E-02	3.8		
40			3.5E-02	1.6			2.3E-02	3.5	12723876	thioredoxin reductase (EC 1.6.4.5)
41	1.1E-01	1.4	2.9E-01	-1.2	1.2E-03	2.8	8.3E-02	1.7	12723876	thioredoxin reductase (EC 1.6.4.5)
42			3.8E-01	1.1			6.1E-03	2.1		
43	7.8E-01	1.1	8.1E-01	-1.1		-100.0	1.7E-02	-4.2	12724608	aspartate carbamoyltransferase (EC 2.1.3.2)
44			4.3E-01	1.4			6.7E-03	2.6	12722912	fatty acid/phospholipid synthesis protein
45	4.0E-03	4.2	6.7E-01	1.2	2.0E-04	13.6	2.7E-02	3.1	12723220	GTP-binding protein Era
46	6.0E-02	2.7	1.5E-01	2.6	1.5E-05	9.3	5.1E-03	3.7		
47			2.3E-01	-1.6			1.9E-02	-23.8		
48	2.0E-02	1.8			8.2E-04	5.3			12725003	oxidoreductase
49	9.4E-01	-1.0	4.5E-01	-1.2	1.3E-02	-2.6	6.2E-02	-2.0		
50			1.2E-02	-2.1			4.0E-03	-2.4	12724215	oxidoreductase
51	1.4E-02	-1.4	3.8E-01	-1.2	1.5E-04	-12.1	1.9E-05	-12.4	12723216	oligopeptide ABC transporter ATP binding protein
52	2.1E-01	-1.2			3.3E-03	2.2			12725330	regulator of purine biosynthetic genes
53	7.4E-01	1.2	4.3E-01	1.5	1.6E-02	4.9	1.4E-02	3.7	12725053	HYPOTHETICAL PROTEIN
54			1.6E-02	2.2			4.3E-03	4.9	12724246	transcriptional regulator
55	2.9E-02	2.5	2.5E-01	1.2	3.8E-02	7.9	4.8E-03	2.9		

No.[a]	60 MPa[b] 11% T T-test	60 MPa[b] 11% T Av. Ratio	60 MPa[b] 15% T T-test	60 MPa[b] 15% T Av. Ratio	90 MPa[b] 11% T T-test	90 MPa[b] 11% T Av. Ratio	90 MPa[b] 15% T T-test	90 MPa[b] 15% T Av. Ratio	PID[c]	Protein
56				100.0				100.0		
57			4.6E-01	-1.3			7.0E-02	-2.7	12724030	conserved hypothetical protein
58		-1.2	6.5E-01	1.1		10.2	1.5E-02	2.6		
59			1.9E-01	1.7			1.5E-02	2.6		
60		100.0	5.9E-02	5.0		100.0	1.3E-02	9.7	12723433	conserved hypothetical protein
61	1.3E-01	-1.7	1.2E-02	-3.6	7.2E-03	-9.5	6.4E-03	-6.8		
62			1.8E-01	-1.3			4.2E-03	-2.0	12723143	acetyltransferase
63	2.9E-02	3.1			1.7E-02	2.7		100.0		
64	1.1E-01	1.9	6.3E-03	1.2	3.6E-03	-3.3	1.9E-04	-2.6	12724782	glutamine ABC transporter ATP-binding protein
65			1.5E-01	-1.5			7.0E-03	-2.8	12724720	cytidine monophosphate kinase (EC 2.7.4.14)
66	9.6E-01	-1.0	2.5E-02	-1.3	2.2E-03	-3.8	7.9E-04	-3.4	12723203	phosphoglycerate mutase (EC 5.4.2.1)
67	2.9E-02	-2.6	7.8E-03	-3.2	3.8E-02	-2.9	5.5E-03	-3.4	12724611	pyrimidine operon regulator
68			1.1E-01	1.9			1.5E-03	3.1	12723447	polypeptide deformylase
69			7.5E-01	-1.1			8.3E-03	-2.7		
70			3.0E-02	-1.8			1.4E-02	-2.2	12723142	HYPOTHETICAL PROTEIN
71	4.2E-02	3.2	1.9E-01	2.0	4.2E-04	3.1	3.8E-02	2.5	12725158	transcription antitermination protein
72	8.9E-02	8.7	7.1E-02	2.5	5.7E-02	8.1	3.4E-02	4.0		
73	5.7E-01	-1.1	8.5E-02	-1.6	2.6E-03	-5.3	6.3E-03	-2.7		
74	6.3E-02	1.6	7.4E-01	1.1	2.1E-02	-3.0	1.1E-03	-1.9	12724703	UNKNOWN PROTEIN
75	2.3E-01	-1.5	4.7E-01	-1.2	9.0E-03	-2.2	4.4E-03	-2.5	12723518	UNKNOWN PROTEIN
76			1.1E-02	-3.5			1.8E-02	-2.6		
77			2.8E-03	-5.4			1.4E-03	-6.4		
78	3.8E-02	-1.8	2.8E-03	-3.5	2.6E-03	-5.3	1.5E-03	-4.4	12724109	xanthine phosphoribosyltransferase (EC 2.4.2.-)
79			4.5E-02	-2.2			2.2E-02	-3.0		
80			3.3E-01	-1.2			6.6E-03	-3.7	12723693	3-oxoacyl-acyl carrier protein reductase (EC 1.1.1.100)
81			3.2E-01	1.2			9.6E-05	5.5	12725316	HYPOTHETICAL PROTEIN
82			2.5E-02	3.5			8.4E-03	10.1	12725316	HYPOTHETICAL PROTEIN
83	1.1E-01	-1.5	4.0E-02	-2.6	3.5E-03	-3.0	1.4E-02	-4.2	12724952	uracil phosphoribosyltransferase (EC 2.4.2.9)
84	1.4E-02	3.3	4.6E-01	1.5	2.4E-03	8.4	1.8E-03	4.1	12724433	conserved hypothetical protein
85			1.4E-02	1.8			3.5E-03	6.3		
86			3.0E-02	4.6			4.3E-03	11.9	12725108	UNKNOWN PROTEIN

No.[a]	60 MPa[b] 11% T T-test	60 MPa[b] 11% T Av. Ratio	60 MPa[b] 15% T T-test	60 MPa[b] 15% T Av. Ratio	90 MPa[b] 11% T T-test	90 MPa[b] 11% T Av. Ratio	90 MPa[b] 15% T T-test	90 MPa[b] 15% T Av. Ratio	PID[c]	Protein
87			6.3E-02	3.0			8.0E-03	5.2		
88			6.6E-02	2.4			2.0E-02	4.3		
89			1.7E-01	1.8			8.5E-03	6.4	12723919	ribonucleotide reductase
90			5.1E-01	-1.2			2.3E-03	-2.4	12725073	non-heme iron-binding ferritin
91			3.9E-01	1.3			2.3E-02	2.0		
92			2.6E-01	1.5			1.4E-02	2.5		
93			1.6E-01	-1.2			1.1E-03	-4.0	12723452	hydroxymyristoyl-acyl carrier protein dehydratase
94			9.9E-02	1.9			2.3E-02	2.2	12724655	thioredoxin
95			9.2E-01	1.0			7.9E-03	-4.8		
96			5.0E-03	-3.1			3.9E-03	-3.0	12723113	ABC transporter ATP binding protein
97			1.2E-03	4.2			2.7E-03	8.6	12725171	zinc transport transcriptional regulator
98			1.4E-01	2.9			4.7E-02	3.1		

a) Spot numbers refer to marked spots in Fig. 23, p. 80
b) An average ratio of 100 marks spots, which were only detected after pressure treatment. Differences greater than factor 2 are marked in red for induction and green for repression.
c) PID, identifier according to GenBank (NCBI)

Tab. 16: Comparison of differentially expressed proteins of *L. lactis* IL1403 in the pH range from 3.5-7.5 and in course of high pressure treatments at 60 or 90 MPa determined by DIGE and ^{35}S pulse labeling. DIGE gels were analyzed with DeCyder and phosphor imaging gels with ImageMaster 2D.

Protein	Mr[a)]	pI[a)]	DIGE[b)] 60 MPa 11%T	DIGE[b)] 60 MPa 15%T	DIGE[b)] 90 MPa 11%T	DIGE[b)] 90 MPa 15%T	^{35}S pulse labeling[c)] 60 MPa 11%T	^{35}S pulse labeling[c)] 60 MPa 15%T	^{35}S pulse labeling[c)] 90 MPa 11%T	^{35}S pulse labeling[c)] 90 MPa 15%T
AMINO-ACID BIOSYNTHESIS										
Trp-sensitive phospho-2-dehydro-deoxyheptonate aldolase (EC 4.1.2.15)	38842.7	5.08		1.4		1.8	-2.4	-1.3	2.6	2.2
serine hydroxymethyltransferase (EC 2.1.2.1)	44789.99	5.45					-2.8	-2.7	-5.8	-4.4
D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	43637.68	5.97	1.7		2.1					
BIOSYNTHESIS OF COFACTORS, PROSTHETIC GROUPS, AND CARRIERS										
thioredoxin	11672.41	4.32		1.3		1.5		1.9		2.2
thioredoxin reductase (EC 1.6.4.5)	33894.43	4.76					1.4		2.8	2.6
CELL ENVELOPE										
basic membrane protein A	36652.03	8.68	-1.6		-1.0					
D-alanine-D-alanine ligase (EC 6.3.2.4)	38693.07	4.66						1.5		2.6
UDP-N-acetylmuramate-alanine ligase (EC 6.3.2.8)	51740.1	5.14					100.0		100.0	
D-Ala-D-Ala adding enzyme (EC 6.3.2.15)	48823.3	5.42					1.5	1.9	4.1	2.6
ENERGY METABOLISM										
alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)	98220.83	5.71		-1.6		-1.6				
ATP synthase epsilon subunit (EC 3.6.1.34)	15669.77	5.73		-2.1		-1.8				
glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	35819.46	5.57	-2.1	-1.8	-1.7	-1.4				
malate oxidoreductase (EC 1.1.1.38)	40475.63	5.21	-2.0	-1.9	-1.8	-1.7	-5.2	-2.2	-2.6	-2.3
phosphoglycerate mutase (EC 5.4.2.1)	26329.75	5.3		-2.2		-1.8	-1.0	-1.3	-3.8	-3.4
pyruvate kinase (EC 2.7.1.40)	54254.84	5.27					-2.0	-2.3	-4.5	-6.1
transketolase (EC 2.2.1.1)	71725.86	5.06					-2.0	-1.9	-4.8	-3.6
oxidoreductase YmgK	32156.52	5.88						-2.1		-2.4
oxidoreductase YudI	36596.24	6.53	1.2		2.2		1.8		5.3	
FATTY ACID AND PHOSPHOLIPID METABOLISM										
3-oxoacyl-acyl carrier protein reductase (EC 1.1.1.100)	25598.5	5.72						-1.2		-3.7
hydroxymyristoyl-acyl carrier protein dehydratase	16796.42	5.51						-1.2		-4.0

Protein	Mr[a)]	pI[a)]	DIGE[b)] 60 MPa 11%T	DIGE[b)] 60 MPa 15%T	DIGE[b)] 90 MPa 11%T	DIGE[b)] 90 MPa 15%T	^{35}S pulse labeling[c)] 60 MPa 11%T	^{35}S pulse labeling[c)] 60 MPa 15%T	^{35}S pulse labeling[c)] 90 MPa 11%T	^{35}S pulse labeling[c)] 90 MPa 15%T
fatty acid/phospholipid synthesis protein	34786.01	5.72						1.4		2.6
PURINES, PYRIMIDINES, NUCLEOSIDES AND NUCLEOTIDES										
cytidine monophosphate kinase (EC 2.7.4.14)	24548.3	5.36						-1.5		-2.8
GMP synthase (EC 6.3.5.2)	56780.71	4.86					-2.2	-2.2	-2.1	-3.3
IMP dehydrogenase (EC 1.1.1.205)	52826.54	5.95					-1.7	-2.1	-2.2	-2.7
ribonucleoside-diphosphate reductase alpha chain (EC 1.17.4.1)	81495.45	5.22					1.5		2.4	
ribonucleoside-diphosphate reductase beta chain (EC 1.17.4.1)	37615.07	4.45						1.7		3.3
ribonucleotide reductase	15660.84	4.8						1.8		6.4
adenylosuccinate synthase (EC 6.3.4.4)	47325.72	5.41					-2.3	-2.6	-4.4	-5.0
adenylosuccinate lyase (EC 4.3.2.2)	49687.41	5.37					-2.7	-3.8	-3.5	-14.0
phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21)	39159.49	5.22		-1.5		-1.3				
aspartate carbamoyltransferase (EC 2.1.3.2)	34558.26	5.52					1.1	-1.1	-100.0	-4.2
CTP synthetase	59552.1	5.57					1.6		3.2	
uracil phosphoribosyltransferase (EC 2.4.2.9)	23230.28	6.54					-1.5	-2.6	-3.0	-4.2
xanthine phosphoribosyltransferase (EC 2.4.2.-)	21806.28	5.3					-1.8	-3.5	-5.3	-4.4
REGULATORY FUNCTIONS										
GTP-binding protein Era	34737.04	5.88	1.2		1.6		4.2	1.2	13.6	3.1
regulator of purine biosynthetic genes	30360.87	5.49					-1.2		2.2	
pyrimidine operon regulator	19830.79	5.26					-2.6	-3.2	-2.9	-3.4
transcriptional regulator HdiR	28894.04	4.6						2.2		4.9
GTP-binding protein	42247.53	6.43					-2.5	-2.2	-8.0	-5.1
zinc transport transcriptional regulator	16366.72	5.56		-1.8		-1.3		4.2		8.6
STRESS RELATED PROTEINS										
ClpB protein	97335.08	5.09	-1.8		-1.3		-1.1		2.7	
non-heme iron-binding ferritin	16652.83	4.69						-1.2		-2.4
glucose inhibited division protein GidC	49991.91	5.43					1.7	1.9	1.6	3.5
60 KD chaperonin	57201.42	4.75					-1.5	-2.2	-2.4	-3.9
RecA protein	41477.3	5.39					1.9	1.5	3.4	2.1
TRANSCRIPTION										
transcription antitermination protein	21110.02	4.55					3.2	2.0	3.1	2.5
TRANSLATION										
polypeptide deformylase	23769.28	5.03						1.9		3.1

Protein	Mr[a)]	pI[a)]	DIGE[b)] 60 MPa 11%T	DIGE[b)] 60 MPa 15%T	DIGE[b)] 90 MPa 11%T	DIGE[b)] 90 MPa 15%T	^{35}S pulse labeling[c)] 60 MPa 11%T	^{35}S pulse labeling[c)] 60 MPa 15%T	^{35}S pulse labeling[c)] 90 MPa 11%T	^{35}S pulse labeling[c)] 90 MPa 15%T
Glu-tRNA amidotransferase subunit A	52071.24	5.51					1.4	1.4	10.4	5.8
TRANSPORT AND BINDING PROTEINS										
glutamine ABC transporter ATP-binding protein	27071.34	5.19					1.9	1.2	-3.3	-2.6
oligopeptide ABC transporter ATP binding protein OptF	35014.99	6.04					-1.4	-1.2	-12.1	-12.4
phosphocarrier protein HPr	9179.45	5.05		1.3		1.6				
ABC transporter ATP binding protein YahG	61381.42	4.71					100.0		8.7	
ABC transporter ATP binding protein YcfB	27287.6	7.67						-3.1		-3.0
UNKNOWN										
HYPOTHETICAL PROTEIN YbfE	16615.02	5.25		1.5		1.9				
HYPOTHETICAL PROTEIN YchG	23316.58	4.55						-1.8		-2.2
acetyltransferase YchH	26776.81	5.34						-1.3		-2.0
conserved hypothetical protein YfdE	29070.21	5.14					100.0	5.0	100.0	9.7
UNKNOWN PROTEIN YgaJ	20659.97	4.49					-1.5	-1.2	-2.2	-2.5
HYPOTHETICAL PROTEIN YgdA	21282.95	5.36	-1.6	-1.4	-1.4	-1.6				
conserved hypothetical protein YkiF	30048.58	4.99						-1.3		-2.7
conserved hypothetical protein YogG	22190.23	6.43					3.3	1.5	8.4	4.1
HYPOTHETICAL PROTEIN YraB	14979.72	4.71		1.2		1.8				
UNKNOWN PROTEIN YreC	21670.31	9.14					1.6	1.1	-3.0	-1.9
conserved hypothetical protein YtaA	15700.1	5.2		1.6		1.7				
HYPOTHETICAL PROTEIN YuiC	32312.76	5.78		2.0		1.1	1.2	1.5	4.9	3.7
UNKNOWN PROTEIN YveC	18490.78	4.56						4.6		11.9
HYPOTHETICAL PROTEIN YxdB	19508.19	5.43		1.5		2.1		2.4		7.8

a) Calculated values; Averaged ratios were assigned from b) Tab. 14, p. 77, and c) Tab. 15, p. 82. If the protein occurred in more than one spot on the gel, ratios were again averaged. An average ratio of 100 marks spots, which were only detected after high pressure treatment. Differences greater than factor 1.5 (DIGE) and factor 2 (^{35}S pulse labeling) are marked in red for induction and green for repression.

3.4 Establishing alkaline reference maps for *L. lactis*

Previous proteomic studies on *L. lactis* were mainly focused on proteins in the pH range from 4 to 7 (reviewed in [2, 23]). For this pH range, two reference maps were published [24, 89], but the alkaline proteins remained uninvestigated. Since *L. lactis* is fully sequenced, the theoretical alkaline proteome of this bacterium was analyzed. Following this *in silico* analysis, IEF protocols were optimized and several IEF systems were compared to achieve a highly reproducible protein pattern in the alkaline range. The separated proteins were subsequently identified by MALDI-TOF MS to establish a reference map in this range.

3.4.1 Analyzing the alkaline *in silico* proteome of *L. lactis*

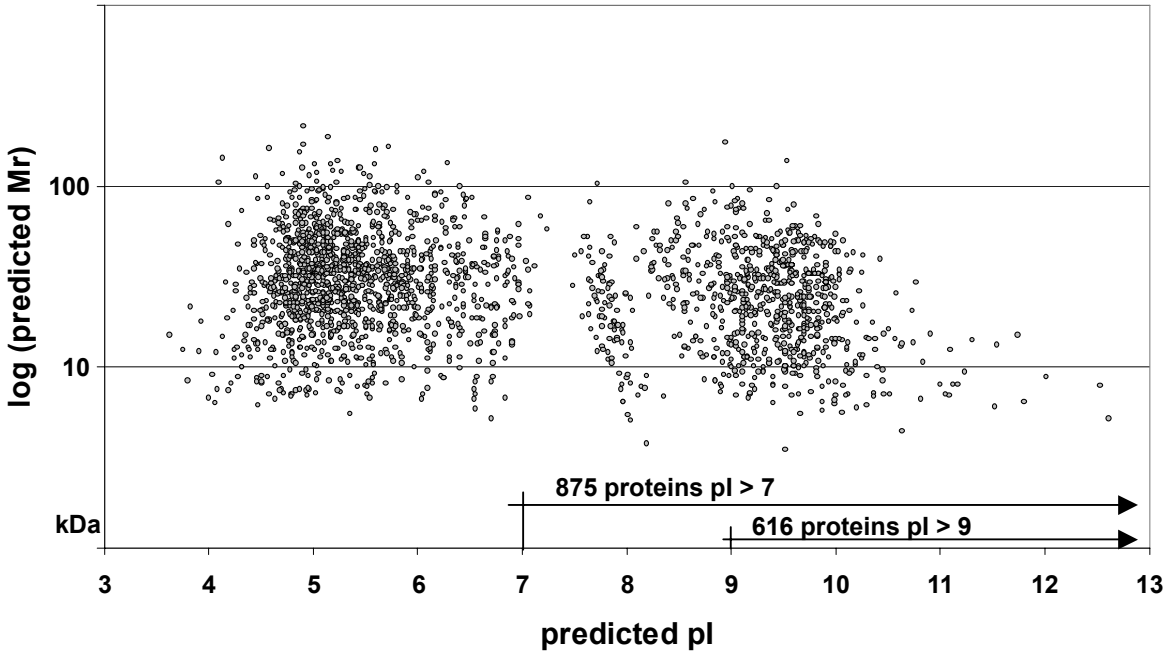

Fig. 24: *In silico* proteome of *Lactococcus lactis* IL1403. The calculated molecular weights of all 2266 lactococcal proteins annotated in the database curated by NCBI (accession: NC_002662) are plotted against the predicted isoelectric points.

The delineation of the theoretical proteome of *Lactococcus lactis* in Fig. 24 shows an uneven distribution of proteins, which is similar to those of other microorganisms [45, 46, 77]. First analysis about the predicted proteome of

Lactococcus lactis was already published in [24]. Since that analysis mainly focused on acidic proteins, here, we concentrated on alkaline proteins. In contrast to the former analysis, which is based on 2310 proteins published after primary analysis of the genome of *L. lactis* [8], the present analysis is based on the database entry curated by NCBI for *Lactococcus lactis*, which includes 2266 annotated proteins. Referring to this, 875 proteins have predicted pIs above pH 7 and 616 above pH 9, respectively. The molecular weight of these proteins ranges from 4 to 176 kDa, with almost 90% of the proteins between 10 and 100 kDa. 660 of the proteins with predicted pIs above 7 are transposases, prophage-encoded proteins, unknown or hypothetical proteins, which are possibly not expressed in general or under the chosen growth conditions.

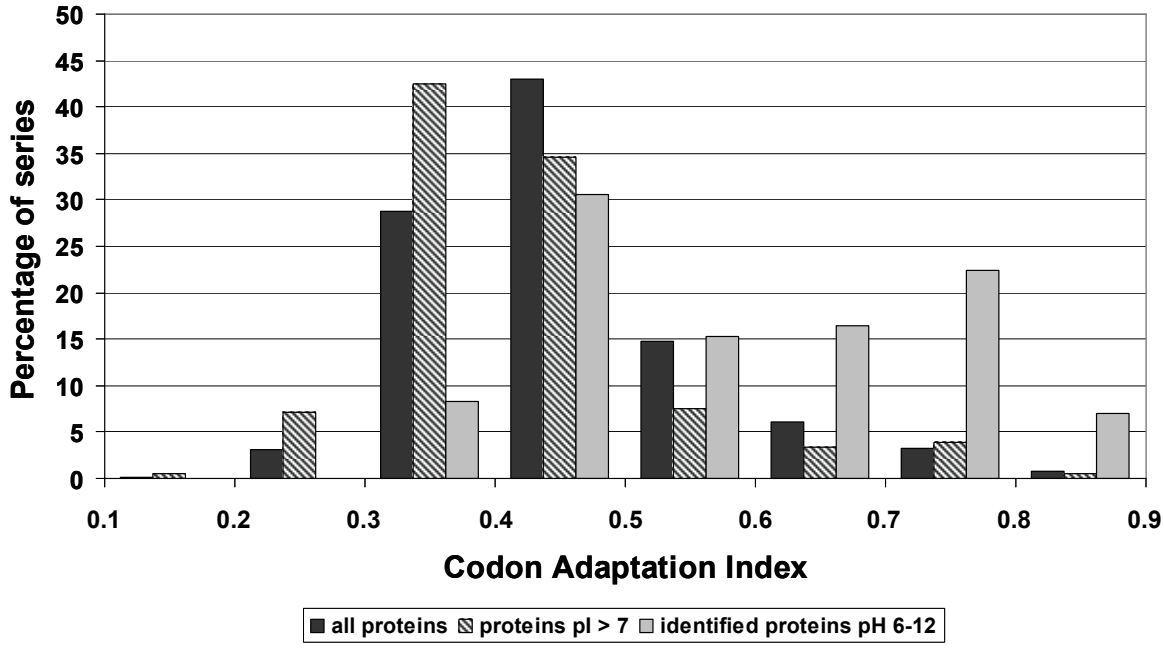

Fig. 25: Frequency distribution of the codon adaptation index (CAI) within the *in silico* proteome of *Lactococcus lactis* IL1403. The number of proteins in percent per series was plotted against the CAI. The series consist of all 2266 lactococcal proteins annotated in the database curated by NCBI (accession: NC_002662), all 875 lactococcal proteins with isoelectric point above seven or all 85 identified proteins of the present analysis (Tab. 17, p. 97).

The codon adaptation index (CAI) is an instrument to predict the expression level of proteins from the genome level via synonymous codon usage bias [160]. In case of lactococcal proteins, the CAI ranges from 0.168 to 0.897. The frequency distribution of the CAI for the 875 proteins of our interest is depicted in Fig. 25.

According to this, approximately 8% of the proteins have a CAI above 0.6 and probably are highly abundant proteins. Nearly 70% among those are ribosomal proteins.

For evaluation of hydrophobic proteins, the grand average of hydropathicity (GRAVY) was calculated for each lactococcal protein (Fig. 26). In general, hydrophobic proteins have a GRAVY value above zero [161]. Among the selected proteins with predicted pIs above 7, 345 (39%) possess a positive GRAVY. This time, 100 (29%) proteins with unknown function are clearly dominating this group.

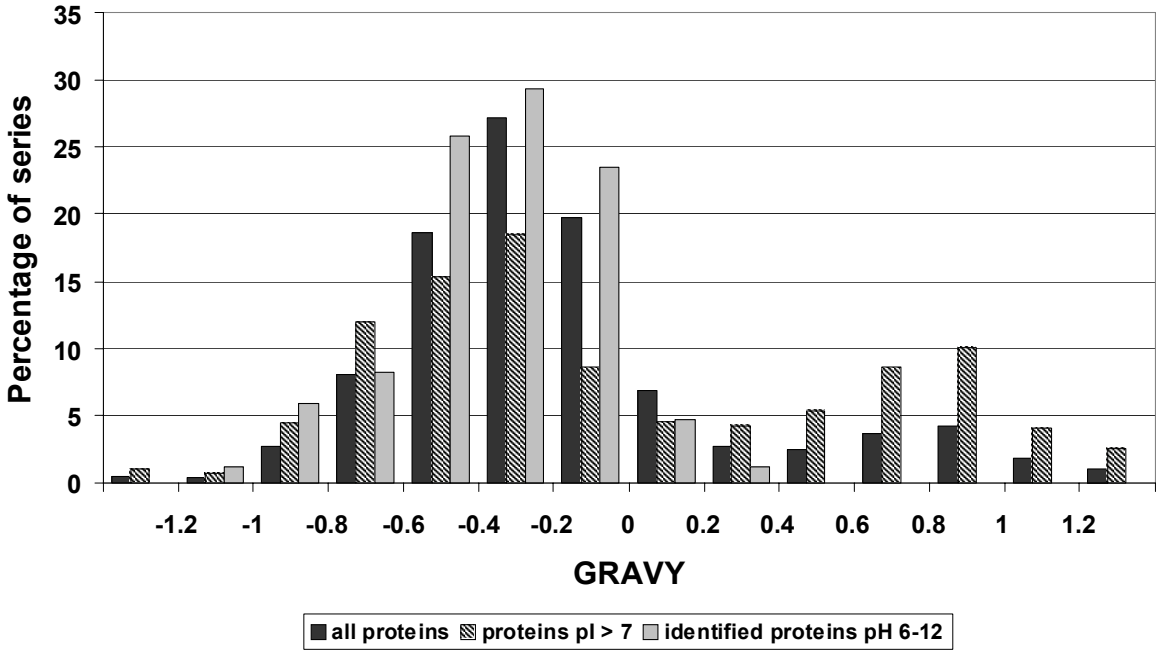

Fig. 26: Frequency distribution of the grand average of hydropathicity (GRAVY) within the *in silico* proteome of *Lactococcus lactis* IL1403. The number of proteins in percent per series was plotted against GRAVY values. The series consist of all 2266 lactococcal proteins annotated in the database curated by NCBI (accession: NC_002662), all 875 lactococcal proteins with isoelectric point above seven or all 85 identified proteins of the present analysis (Tab. 17, p. 97).

3.4.2 Optimization of IEF conditions for the alkaline pH range

The quality of the protein pattern after using different electrophoresis systems for isoelectric focusing in immobilized pH gradients from 6 to 12 was compared. For this, the same lactococcal extract was separated on the Multiphor II in combination with the DryStrip Kit and on the IPGphor in combination with the cup-loading strip

holder. In the second dimension, all IPG strips were applied on SDS gels, cast and separated at the same time in the same apparatus. Every condition was repeated three times. The results in Fig. 27 show representative gels of each condition. The protein pattern of samples focused on Multiphor II after in-gel rehydration is dominated by horizontal streaks (Fig. 27A). Application of the sample at the anodic side improves the quality of the pattern in general, but still streaking is observable, especially towards the cathodic end (Fig. 27B). Best results were obtained after application of the sample via cup-loading at the anodic side and increasing the final voltage up to 8000 V (Fig. 27C; IEF protocol adapted from Wildgruber *et al.*, 2002 [49]). For this, the IPGphor was applied.

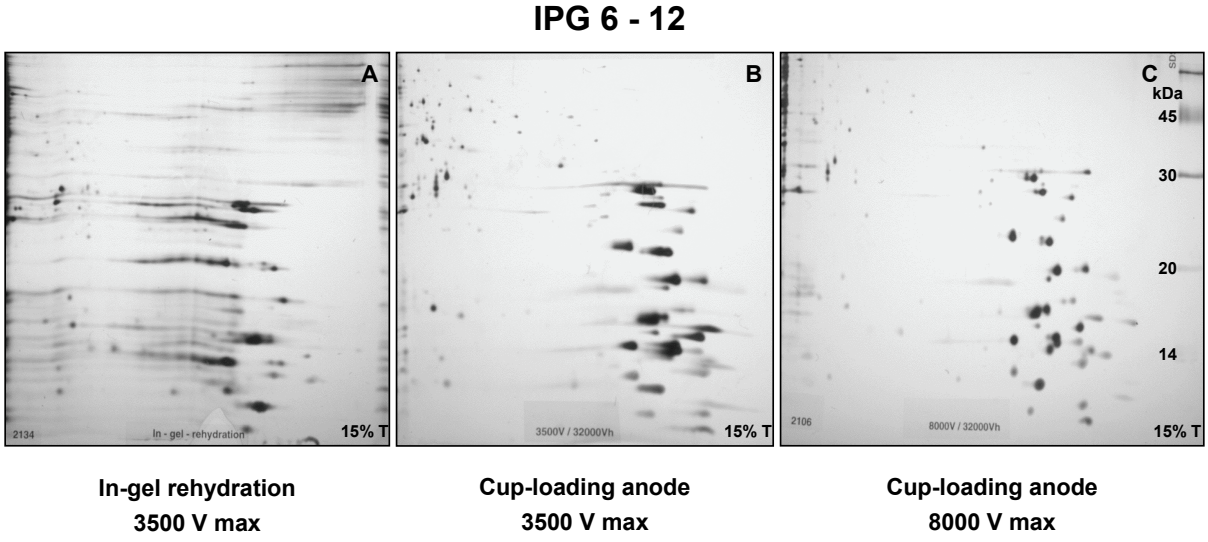

Fig. 27: Comparison of different IEF conditions for *Lactococcus lactis* proteins in IPG 6-12, silver stain. Same protein quantities were loaded by (A) in-gel rehydration or (B, C) cup-loading. For IEF (A, B) the Multiphor II or (C) IPGphor was used. The final applied voltage to complete IEF was (A, B) 3500 V or (C) 8000 V.

Finally, several different timeframes at 8000 V (kVh) were tested for the IEF under cup-loading conditions on the IPGphor. Fig. 28 shows the results of the IEF after 18 and 32 kVh. It is evident that the protein pattern has not changed within the chosen timeframe. Thus, times for the IEF between 18 and 32 kVh result in comparable patterns. Therefore, the time for IEF was set to 25 kVh for the IPG 6-12 to be in a clear margin of steady state.

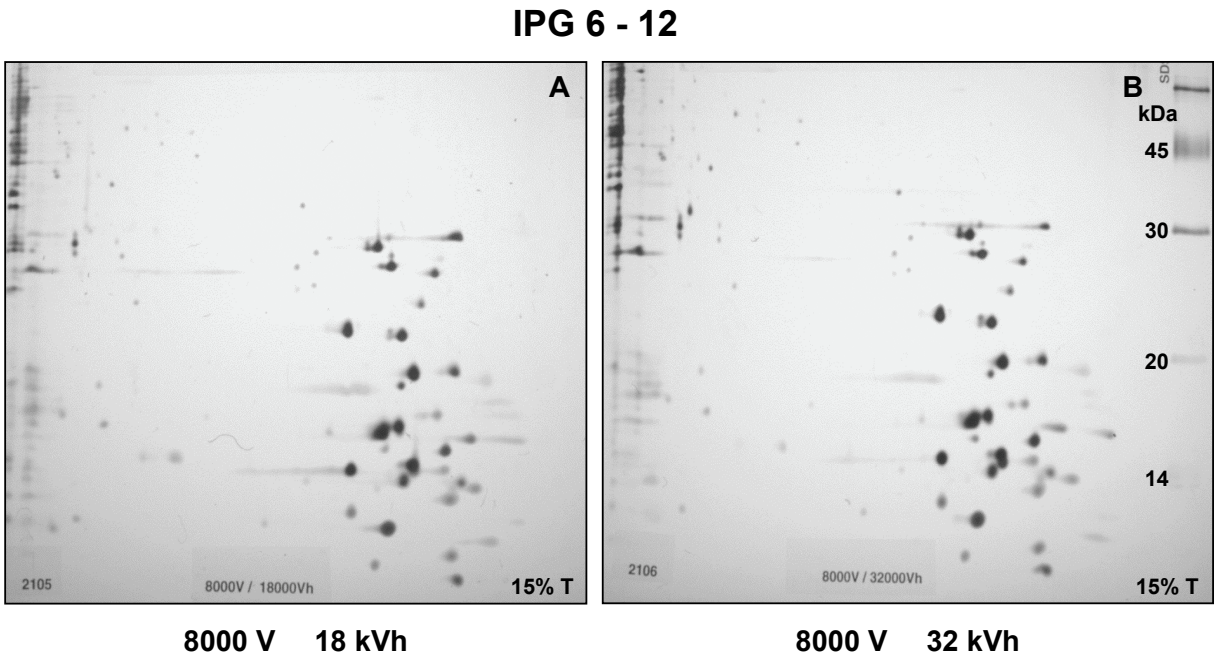

Fig. 28: Steady state IEF of *Lactococcus lactis* proteins in IPG 6-12, silver stain. For IEF, same protein quantities were loaded by cup-loading and utilizing the IPGphor. After sample entry, 18 kVh (A) or 32 kVh (B) at 8000 V were applied to reach steady state.

3.4.3 Mapping the alkaline proteome

The immobilized pH gradient from 6 to 12 facilitates an overview of the alkaline proteome of *Lactococcus lactis*. In this pH range, more than 200 protein spots were detected (Fig. 29). For improved visualization, additional gels were SYPRO RUBY™ stained (Fig. 30, p. 95). The spot pattern in Fig. 29 resembles the *in silico* proteome displayed in Fig. 24, p. 89. The majority of proteins is located near pH 6 at the anodic side and from the center of the IPG strip (≈pH 9) towards pH 12 at the cathodic side. Between pH 9 and 12 several proteins appear in clusters on the gel. For example, the cluster around Spot 51 consists of approximately 5 protein spots in IPG 6-12 and 8 in IPG 9-12. This complicates the image analysis of these proteins and the identification by MALDI-TOF MS. For improved resolution, the extract was applied on IPG 9-12 (Fig. 31, p. 96).

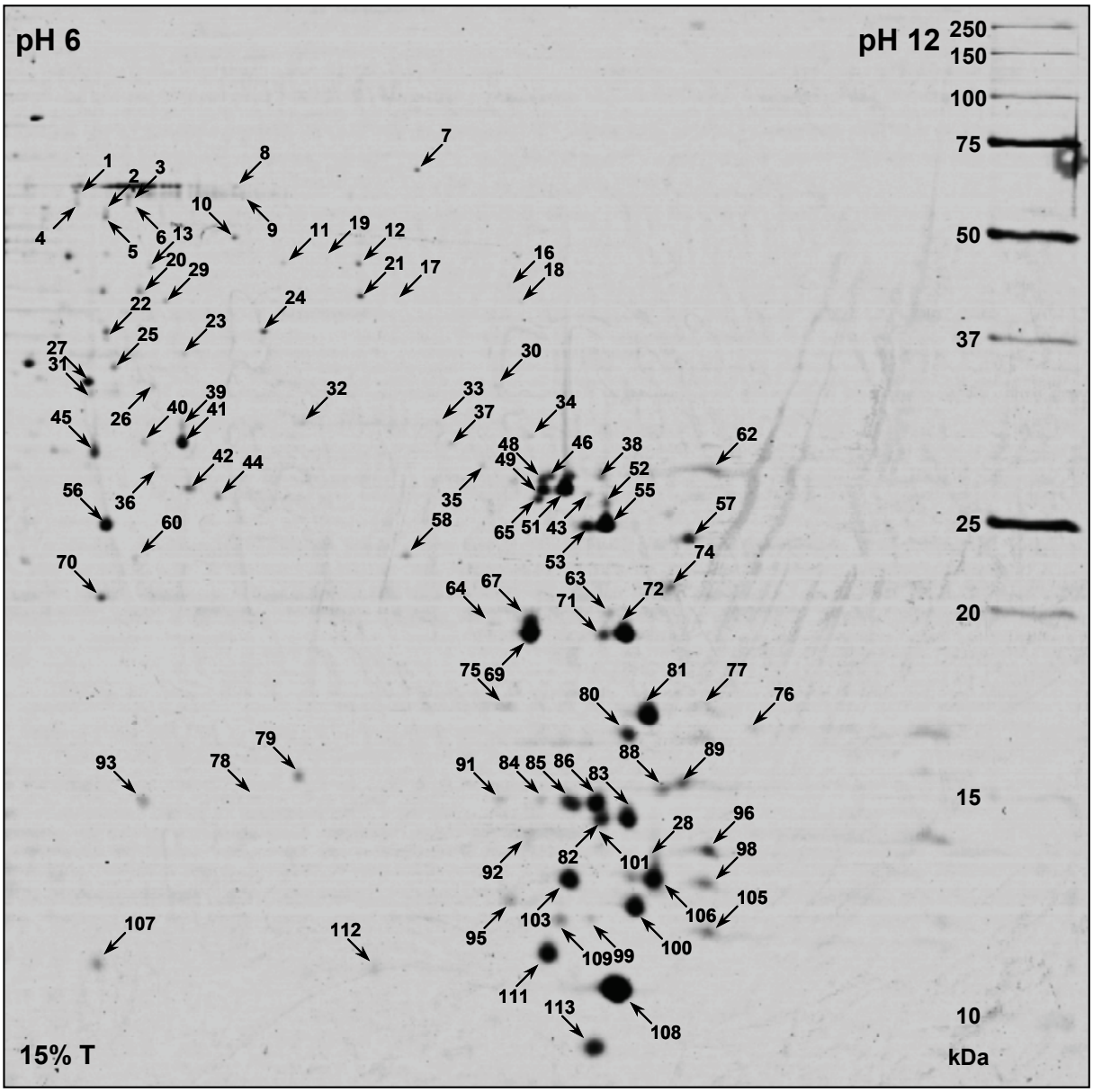

Fig. 29: Reference map (IPG 6-12) of the proteome of *Lactococcus lactis* IL 1403 grown in SA medium at 30°C, Coomassie stain. Approximately 500 µg of protein extract was applied by cup-loading and utilizing the IPGphor. After sample entry, 8000 V were applied to reach steady state. Numbered spots indicate identified proteins presented in Tab. 17, p. 97.

About 200 Spots excised from Coomassie stained gels covering pH 6-12 and 9-12 were submitted to MALDI-TOF MS to determine the peptide mass fingerprint. Spots close to pH 6 were sparsely picked, because the pH range from 4 to 7 was already focus of another study [24]. 153 of these spots were successfully identified and indicated as numbered spots in Fig. 29 and Fig. 31, p. 96. After connecting the spot identifications with the patterns of IPG-DALT 6-12 and 9-12, these gels were compared again. Now, 39 spots in the 6-12 range could be assigned to

corresponding spots in the 9-12 range. Therefore, these spots bear the same number in both gels in Fig. 29 and Fig. 31. Leaving out these redundantly picked spots, in total 114 spots permitted the identification of 85 different proteins, meaning several proteins are resembled by more than one spot on the gels.

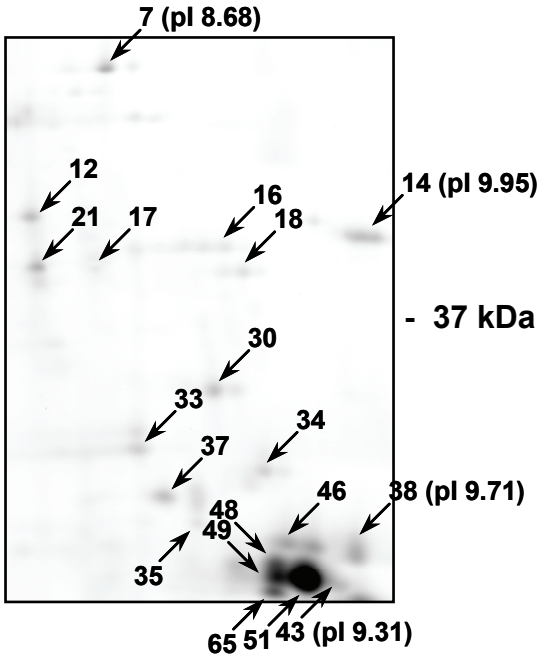

Fig. 30: Staining with SYPRO RUBY™ improves visualization of low abundant spots. Display detail of the reference map of the *Lactococcus lactis* IL 1403 proteome (IPG 6-12) is shown. For orientation, spot numbers are in agreement with Fig. 29 and the theoretical pIs of several proteins are indicated. Numbered spots indicate identified proteins presented in Tab. 17, p. 97.

After identification, the benefits of the narrow gradient from 9 to 12 are obvious. The previously mentioned cluster around spot 51 consists of 8 spots, which resemble three different proteins. Three of these spots could not be identified subsequent to IPG-DALT 6-12. This would leave these proteins undetected in this cluster. The application of IPG 9-12 therefore not only facilitated the detection and identification of further, possibly modified proteins, but also of proteins previously undetected in IPG 6-12 (e.g. Spot 61).

The predicted isoelectric points of the identified proteins range from 6.34 to 10.35 in IPG 6-12 and from 9.22 to 11.31 in IPG 9-12. The identified protein with lowest calculated molecular weight is the 50S ribosomal protein L30 (6.2 kDa) and with highest alcohol-acetaldehyde dehydrogenase (98.2 kDa). The latter enzyme and elongation factor Tu have predicted pIs of 5.71 and 4.89, respectively. Despite that,

these two proteins were detected in IPG 6- 12, alcohol-acetaldehyde dehydrogenase even in six distinct spots. Whether these spots represent modified or degraded proteins could not be analyzed by peptide mass fingerprinting.

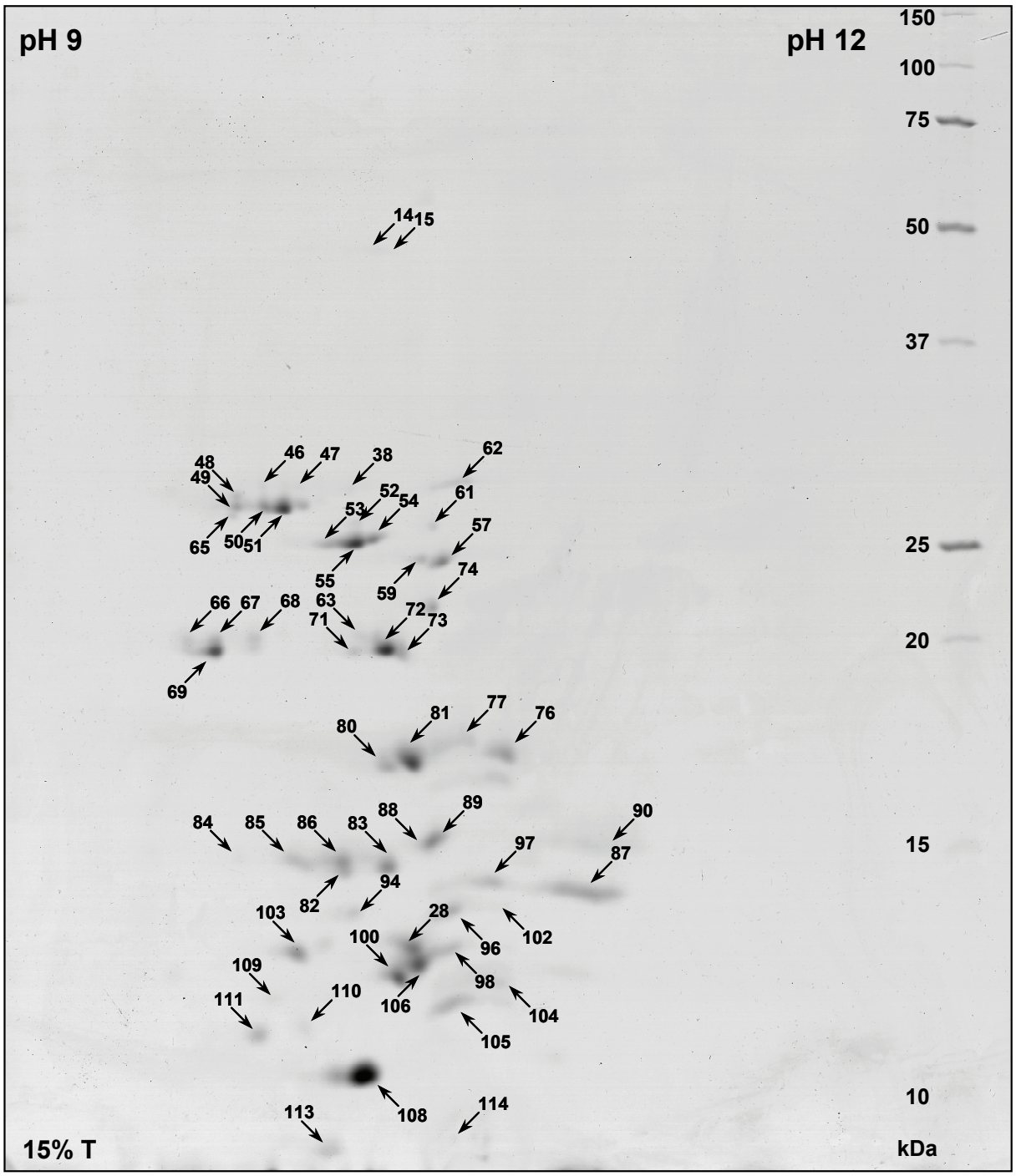

Fig. 31: Reference map (IPG 9-12) of the proteome of *Lactococcus lactis* IL 1403 grown in SA medium at 30°C, Coomassie stain. Approximately 500 mg of protein extract was applied by cup-loading and utilizing the IPGphor. After sample entry, 8000 V were applied to reach steady state. Numbered spots indicate identified proteins presented in Tab. 17.

A complete list of all identified proteins is displayed in Tab. 17. The table includes the codon adaptation index of the proteins, which was calculated for the *in silico* analysis. According to this, the CAI of the detected proteins range from as low as 0.313 for the manganese ABC transporter ATP binding protein (MtsB) up to 0.864 for the 30S ribosomal protein S15 (RpsO). Only 91 (10%) of lactococcal proteins with a predicted pI above 7 lie below a CAI of 0.313. The number of all identified proteins summarized in Tab. 17 against the CAI was included in Fig. 25, p. 90, comparing the frequency distribution of the CAI among all lactococcal proteins with those identified. The GRAVY value of all identified proteins is included in Tab. 17 too. Five of them have a value above zero and the highest is 0.26. For comparison with the predicted proteome, the result was included as individual series in the graph showing the number of proteins against the GRAVY value (Fig. 26, p. 91).

Tab. 17: Proteins of *Lactococcus lactis* IL1403 identified in the pH range between 6-12 and 9-12 after 2D electrophoresis. Identification was performed at least two times per spot by peptide mass fingerprint (e<0.01).

Spot no.	PID[a)]	Gene	Functional classification Protein name (EC number)	Theor. Mr	Theor. pI	CAI	GRAVY
			CELL ENVELOPE				
13	12725334	*dacA*	D-alanyl-D-alanine carboxypeptidase	46993.31	6.96	0.51	-0.125058
79	12723074	*tagD1*	glycerol-3-phosphate cytidiltransferase	16837.38	7.85	0.367	-0.595775
14, 15	12723133	*acmA*	N-acetylmuramidase (EC 3.5.1.28)	46592.06	9.95	0.475	-0.325285
21	12724594	*murG*	peptidoglycan synthesis protein MurG	39071.66	8.36	0.481	-0.211485
			CELL DIVISION				
46, 47	12723912	*ftsE*	cell-division ATP-binding protein FtsE	25799.76	9.45	0.452	-0.330435
35	12722941	*parA*	chromosome partitioning protein	29803.66	8.97	0.404	-0.409962
			ENERGY METABOLISM				
1-6	12725215	*adhE*	alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)	98220.83	5.71	0.685	-0.177962
31	12724787	*atpG*	ATP synthase gamma subunit (EC 3.6.1.34)	31997.39	6.34	0.567	-0.337716
9	12724085	*frdC*	fumarate reductase flavoprotein subunit (EC 1.3.99.1)	52852.69	8.77	0.555	-0.325299
22	12725003	*yudI*	oxidoreductase	36596.24	6.53	0.487	-0.156287
34	12722917	*yahI*	short-chain type dehydrogenase	30819.25	9.28	0.551	-0.269314
			FATTY ACID AND PHOSPHOLIPID METABOLISM				
32, 33	12723698	*accD*	acetyl-CoA carboxylase carboxyl transferase subunit beta (EC 6.4.1.2)	31769.89	8.54	0.471	-0.085764
26	12723278	*yeaG*	mevalonate kinase	34334.27	6.6	0.512	-0.165161

Spot no.	PID[a)]	Gene	Functional classification Protein name (EC number)	Theor. Mr	Theor. pI	CAI	GRAVY
45	12723453	*fabI*	NADH-dependent enoyl-ACP reductase	26382.42	6.44	0.695	0.156
			PROPHAGES				
63	12723315	*pi102*	prophage pi1 protein 02	21661.95	9.56	0.499	-0.598437
64	12723957	*pi202*	prophage pi2 protein 02	20505.88	8.91	0.372	-0.20221
			NUCLEOSIDES AND NUCLEOTIDES METABOLISM				
25	12724108	*guaC*	GMP reductase (EC 1.6.6.8)	35837.21	6.52	0.598	-0.106079
56	12724952	*upp*	uracil phosphoribosyltransferase (EC 2.4.2.9)	23230.28	6.54	0.64	0.006635
			REGULATORY FUNCTIONS				
78	12725088	*argR*	arginine catabolic regulator	16893.49	7.76	0.335	-0.057237
43	12724426	*rgrB*	GntR family transcriptional regulator	26731.61	9.31	0.427	-0.34766
58	12724115	*ysxL*	GTP-binding protein	22550.73	8.58	0.504	-0.518974
23	12724690	*rliA*	transcriptional regulator	36360.74	6.73	0.421	-0.259568
			REPLICATION				
20	12725291	*dnaJ*	DnaJ protein	40671.28	6.73	0.551	-0.682322
108	12723384	*hslA*	HU like DNA-binding protein	9676.15	9.52	0.854	-0.27033
11, 12	12723557	*hsdS*	type I restriction enzyme specificity protein (EC 3.1.21.3)	47068.62	8.28	0.411	-0.604423
			TRANSCRIPTION				
38	12724251	*rluB*	pseudouridine synthase	28722.13	9.71	0.469	-0.478988
29	12723945	*rluD*	pseudouridine synthase	33587.51	6.65	0.497	-0.355482
30	12725328	*rnhA*	ribonuclease HII (EC 3.1.26.4)	32205.11	9.06	0.435	-0.14726
			TRANSLATION				
52-55	12725147	*rpsC*	30S ribosomal protein S3	24033.74	9.52	0.744	-0.343318
57	12723147	*rpsD*	30S ribosomal protein S4	23164.56	10.07	0.846	-0.510345
77	12725134	*rpsE*	30S ribosomal protein S5	17595.27	10.2	0.697	-0.045833
76	12725332	*rpsG*	30S ribosomal protein S7	17683.39	10.35	0.794	-0.510968
84-86	12725137	*rpsH*	30S ribosomal protein S8	14685.11	9.55	0.817	-0.158333
90	12725323	*rpsI*	30S ribosomal protein S9	14098.24	11.31	0.671	-0.459231
100	12725154	*rpsJ*	30S ribosomal protein S10	11741.72	9.74	0.721	-0.5
105	12724920	*rpsO*	30S ribosomal protein S15	10342.97	10.19	0.864	-0.849438
106	12725144	*rpsQ*	30S ribosomal protein S17	10143.77	9.88	0.709	-0.843023
104	12725149	*rpsS*	30S ribosomal protein S19	10570.21	10.19	0.635	-0.698913
48-51	12725046	*rplA*	50S ribosomal protein L1	24049.75	9.34	0.784	-0.01048
62	12725153	*rplC*	50S ribosomal protein L3	21932.53	10.26	0.692	-0.293237
59	12725152	*rplD*	50S ribosomal protein L4	22305.44	9.94	0.722	-0.208173
66-69	12725140	*rplE*	50S ribosomal protein L5	20006.26	9.22	0.8	-0.177778
71-73	12725136	*rplF*	50S ribosomal protein L6	19257.22	9.69	0.758	-0.280337
80	12723661	*rplI*	50S ribosomal protein L9	16375.32	9.65	0.648	-0.281333
82, 83	12725047	*rplK*	50S ribosomal protein L11	14705.24	9.49	0.82	0.03617
81	12725324	*rplM*	50S ribosomal protein L13	16194.84	9.82	0.762	-0.256081
96	12725143	*rplN*	50S ribosomal protein L14	12922.08	10.11	0.68	-0.07623
88, 89	12725119	*rplQ*	50S ribosomal protein L17	14255.45	10.04	0.792	-0.542857
98	12725135	*rplR*	50S ribosomal protein L18	12390.15	10.11	0.74	-0.377391
87	12723803	*rplS*	50S ribosomal protein L19	14405.72	10.56	0.728	-0.5656
101	12724034	*rplU*	50S ribosomal protein L21	11437.21	9.66	0.782	-0.436539
97	12725148	*rplV*	50S ribosomal protein L22	12469.53	10.38	0.71	-0.132174
103	12725151	*rplW*	50S ribosomal protein L23	10743.51	9.46	0.724	-0.230928

Spot no.	PID[a)]	Gene	Functional classification Protein name (EC number)	Theor. Mr	Theor. pI	CAI	GRAVY
102	12725141	*rplX*	50S ribosomal protein L24	10876.75	9.84	0.757	-0.279208
113	12725145	*rpmC*	50S ribosomal protein L29	7859.16	9.4	0.711	-0.517391
114	12725133	*rpmD*	50S ribosomal protein L30	6193.4	10.29	0.723	0.261017
109-111	12724602	*rpmE*	50S ribosomal protein L31	9336.48	9.3	0.784	-0.883951
19	12724893	*tuf*	elongation factor Tu	43211.91	4.89	0.809	-0.24481
112	12725124	*infA*	translation initiation factor IF-1	8195.5	8.03	0.405	-0.266667
74	12724874	*infC*	translation initiation factor IF-3	18691.99	9.83	0.559	-0.472561
44	12724905	*trmH*	tRNA-guanosine methyltransferase (EC 2.1.1.34)	26514.55	7.07	0.447	-0.08595
37	12723367	*truA*	tRNA pseudouridine synthase A (EC 4.2.1.70)	29144.98	8.83	0.396	-0.596838
			TRANSPORT AND BINDING PROTEINS				
7	12724308	*yngB*	fibronectin-binding protein	61285.01	8.68	0.519	-0.466111
42	12724297	*mtsB*	manganese ABC transporter ATP binding protein	26754.04	6.97	0.313	-0.133755
8	12723212	*optA*	oligopeptide ABC transporter substrate binding protein	59697.78	8.54	0.648	-0.531743
39-41	12725169	*zitQ*	zinc ABC transporter ATP binding protein	27961.67	6.84	0.416	-0.637143
			UNKNOWN				
10	12724144	*yljE*	conserved hypothetical protein	52315.42	6.88	0.49	-0.175217
60	12724433	*yogG*	conserved hypothetical protein	22190.23	6.43	0.391	-0.298
27, 28	12722978	*ybdD*	HYPOTHETICAL PROTEIN	33748.1	6.4	0.526	-0.463816
17	12723116	*ycfD*	HYPOTHETICAL PROTEIN	43489.67	8.59	0.476	-0.20874
16	12723118	*ycfF*	HYPOTHETICAL PROTEIN	45553.39	9.06	0.467	-0.030733
70	12723370	*yeiF*	HYPOTHETICAL PROTEIN	19451.5	6.44	0.454	0.036667
107	12723663	*yhfC*	HYPOTHETICAL PROTEIN	9834.1	6.4	0.657	-0.627059
92	12723708	*yhjF*	HYPOTHETICAL PROTEIN	13571.73	8.98	0.442	-0.599138
24	12723794	*yiiH*	HYPOTHETICAL PROTEIN	36109.4	7.12	0.445	-0.475079
65	12724014	*ykhD*	HYPOTHETICAL PROTEIN	20115.95	9.34	0.436	-0.461453
18	12724662	*yqjE*	HYPOTHETICAL PROTEIN	43288.12	9.27	0.473	-0.257812
91	12725017	*yufA*	HYPOTHETICAL PROTEIN	13599.65	9.05	0.614	-0.691667
36	12723162	*ycjH*	UNKNOWN PROTEIN	29639.17	6.86	0.424	-0.190458
75	12723256	*ydiG*	UNKNOWN PROTEIN	18216.33	9.1	0.577	-0.825641
94	12723442	*yffA*	UNKNOWN PROTEIN	13221.24	9.63	0.424	-0.676786
95	12724892	*ytcE*	UNKNOWN PROTEIN	13154.27	9.13	0.617	-0.174561
93	12724933	*ytgE*	UNKNOWN PROTEIN	13434.47	6.78	0.672	-0.880342
99	12724949	*ytjA*	UNKNOWN PROTEIN	12180.11	9.52	0.502	-0.434286
61	12725091	*yvcA*	UNKNOWN PROTEIN	22109.95	9.9	0.374	-1.048223

a) PID, identifier according to GenBank (NCBI)

3.5 Features of the dynamic online database for the proteome of *L. lactis*

For quick access to the reference maps presented in section 3.4.3, p. 93, and the calculated data of the *in silico* proteome analysis of *L. lactis* (section 3.4.1, p. 89), a public database was constructed. The interface of the dynamic online database consists of three parts: the search including the resultset, the interactive reference maps and the summarized protein information table. The search and resultset serve as main interface for navigation and thus, are permanently available on the left side of the screen (Fig. 32).

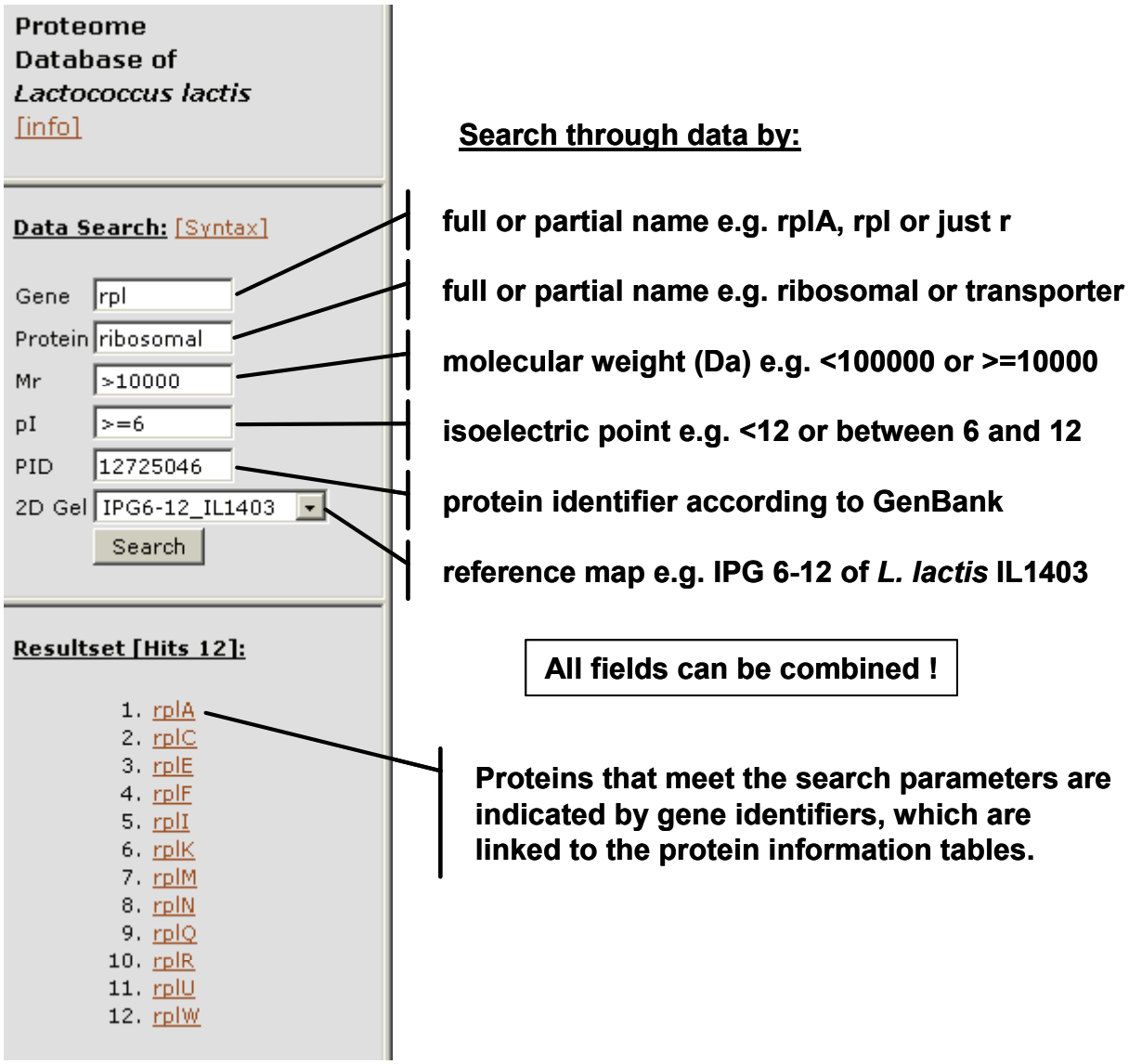

Fig. 32: Main navigation interface of the dynamic online database for the proteome of *L. lactis.*

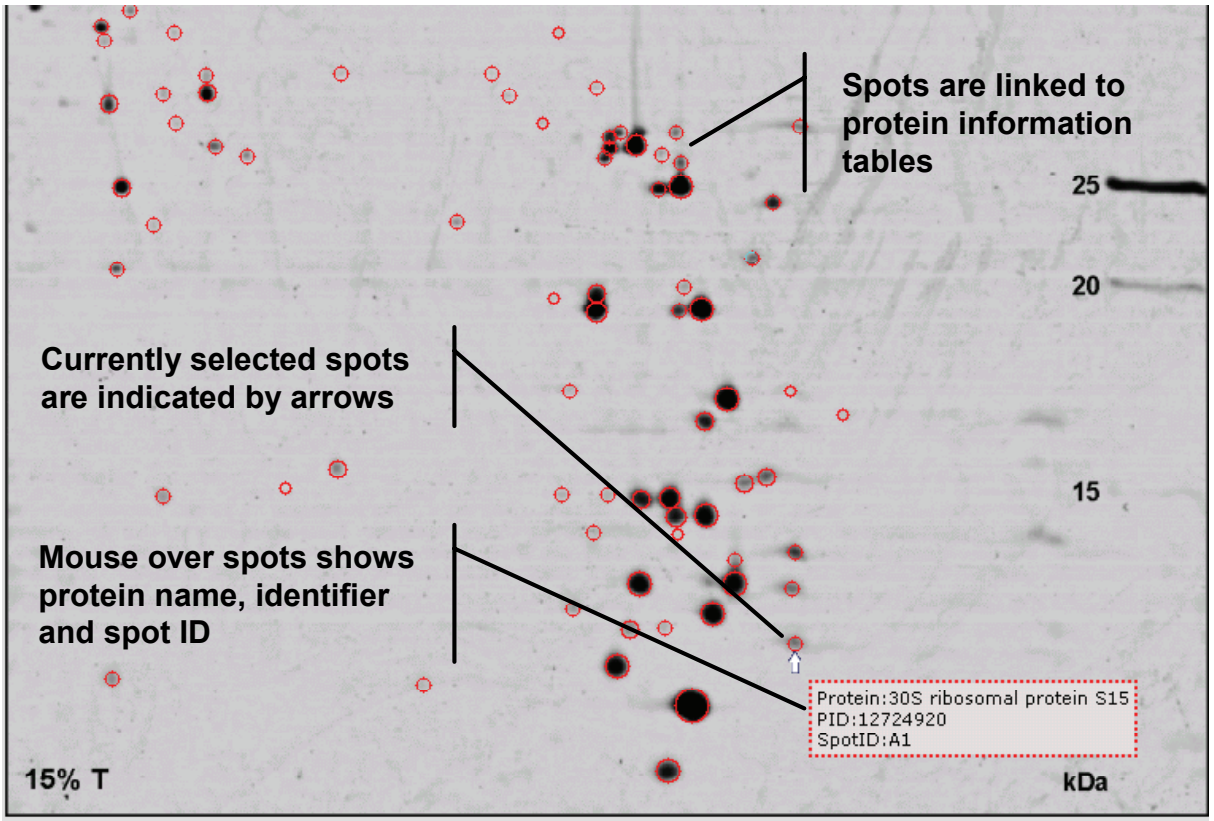

Fig. 33: Reference gels in the dynamic online database are linked to protein information tables and provide brief spot information by mouse over spot function. All identified proteins within the reference map are encircled in red. Information about growth or IPG-DALT conditions and references are also provided.

The interactive reference maps and the summarized protein information table both comprise manifold information. Therefore, the user can intuitively switch between these two pages, which make up most of the screen on the right side of the Internet browser (Fig. 33 and Fig. 34).

The search through the database is dominated either by six different parameters or by interactive exploration of the reference maps. As parameters, full or partial names of genes, proteins or identifiers are supported as well as ranges of molecular weights and isoelectric points (Fig. 32). Furthermore, the search can be limited to a selected 2D reference map too. After submission of the search request, all proteins, which fit the parameters, are indicated in the resultset. On the other hand, the interactive reference maps present all identified proteins on a 2D gel at one sight (Fig. 33). By positioning the mouse over the red encircled spots, information

about the spots, namely protein name, identifier and spot ID, are displayed. On clicking, the summarized protein information of the selected spot is presented in the Internet browser. Below the reference gel, detailed information about growth conditions, protein extraction, IPG-DALT conditions and references are provided.

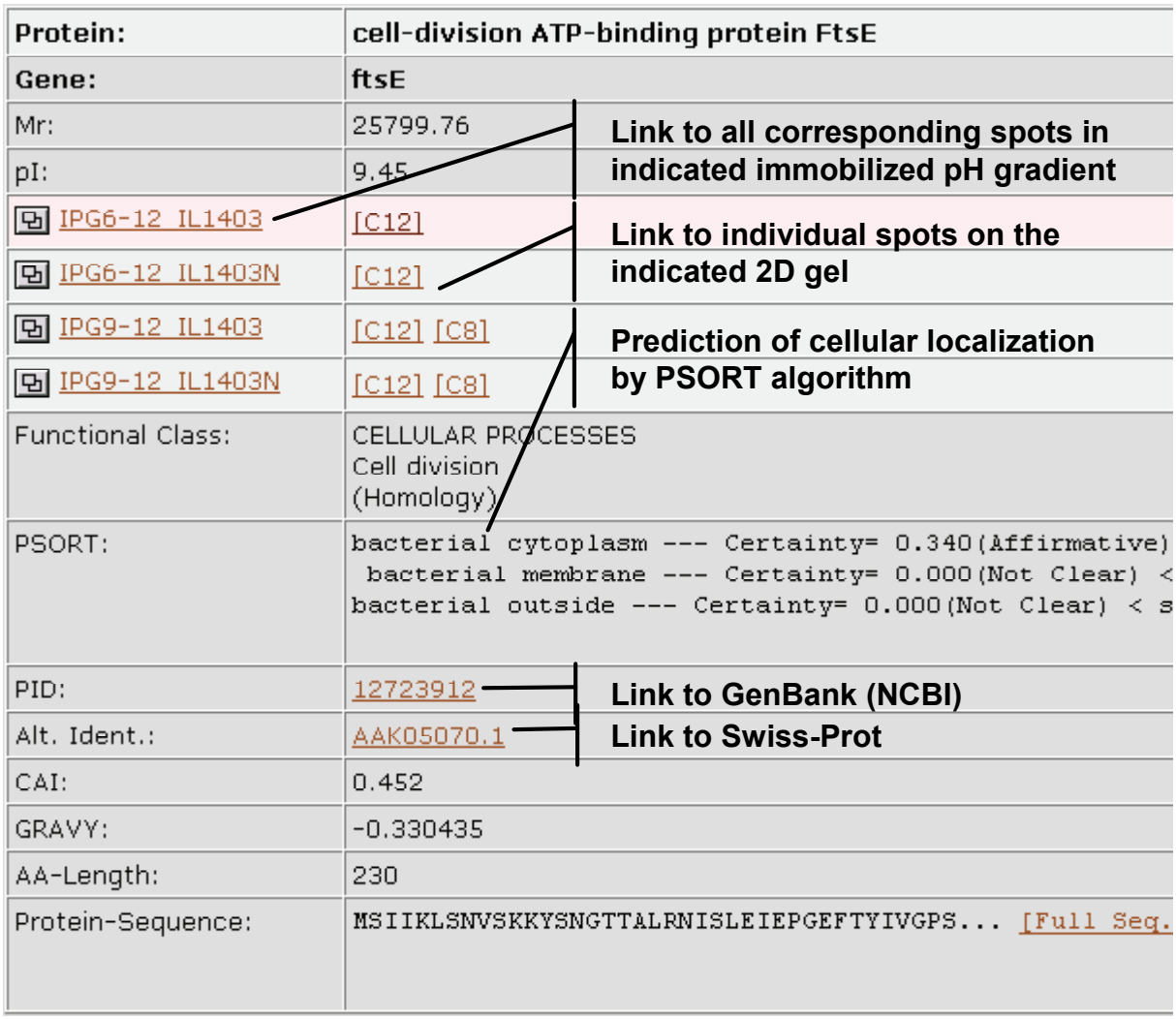

Fig. 34: The protein information table of the dynamic online database links spots on 2D gels with summarized protein characteristics, which are prevalently used in proteomics.

The protein information tables comprise the full length protein name, gene identifier, predicted molecular weight and isoelectric point as well as the functional classification according to the annotation on the MOLOKO website (http://spock.jouy.inra.fr/RL000801.html), and the predicted cellular localization calculated with the PSORT algorithm [156] (Fig. 34). Links to individual or all spots of the selected protein are implemented to switch from protein information to highlighted

spots on reference gels. Furthermore, two widely used identifiers link to the corresponding GenBank (NCBI) and Swiss-Prot database entries. The codon adaptation index and the grand average of hydropathicity value give information about expected expression [160] and solubility of the protein [161]. Finally, the complete protein sequence can be retrieved for further analyses and calculations.

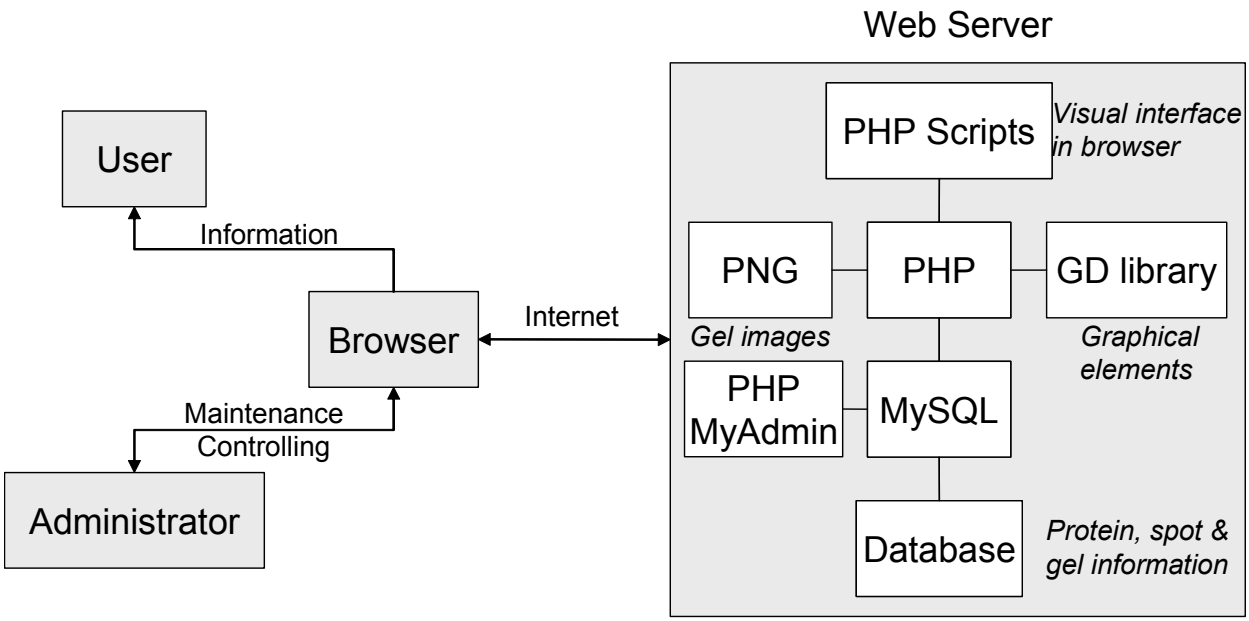

Fig. 35: The database architecture on the web server makes it independent from decentralized software. Frequently administered data, such as gel or experimental information, are controlled by embedded PHP scripts. Protein characteristics are in general only added once, and are therefore controlled by PHPMyAdmin. Both administrative tools are accessible by the Internet browser.

The sophisticated navigation and management by the embedded PHP scripts makes the database on the web server independent of any decentralized software (Fig. 35). Thus, the database can be accessed and maintained from every computer connected to the Internet, simply by using a web browser. For example, the spot summary in Fig. 34 is displayed by a PHP script and works like an empty table, which is filled with information on demand of the user. After submission of the search criteria, a PHP script displays the results in the resultset in the lower left of the Internet browser. By activating the link of one of these results (demand), another PHP script retrieves the information for this protein from the MySQL database in real-time and each field of the previously empty spot summary table is consecutively filled within parts of a second. In comparison with static HTML pages, in this database solution, additional information, such as reference maps or identified spots can be

added easily to the database instead of updating every HTML page of, for instance, all 2266 lactococcal proteins, which are currently part of the database. Even completely new protein characteristics can be added to the spot summary simply by uploading the additional information into reserved fields of the database. Then, slight modifications in the PHP script code are necessary to display the added data or extend the search options.

Protein information is administered online by PHPMyAdmin. Gel images are uploaded in PNG file format. The addition of gels, experimental data and spot coordinates to the database is supported by an administrative PHP script, which can be accessed only by collaborating laboratories e.g. mass spectrometry service stations or other registered users. This solution was chosen, because the latter data are more frequently added or changed. The administrative PHP script resembles a form in which the data and spot coordinates are copied. By submission of the form, the newly added data are immediately accessible online.

4 Discussion

4.1 High hydrostatic pressure effects step-wise altered protein expression in *Lactobacillus sanfranciscensis*

Life under high pressure up to 100 MPa has been proven to exist in the natural environment of the Mariana Trench and the Philippine Trench [162]. In such organisms, also called piezophiles, the occurrence of pressure regulated proteins has been reported [163, 164]. Their biotechnological potential is already evaluated [129] and resembles well known extremophiles like *Thermus aquaticus*, which revolutionized molecular biology. But also in non-piezophiles and even in higher eukaryotic cells the occurrence of pressure regulated proteins has been reported [21, 122, 123].

The pressure response of *Lactobacillus sanfranciscensis* emerges step-wise in the way that pressure dependent proteins are maximally induced or repressed at different pressures (Tab. 7, p. 51). Increasing the pressure beyond these maxima typically caused an attenuated effect on the regulation of these proteins and their spot intensity finally returned to the state of untreated cells. In contrast, in *E. coli* the induction of pressure dependent proteins increased with higher pressures and seemed to be uniformly maximal from 60 to 90 min upon a shift to 55 MPa [21]. A return to the original protein level could probably not be shown earlier, because pressures that exceed 100 MPa were not previously investigated in this way. Interestingly, in our study some protein levels were increased at pressures beyond 100 MPa. The greatest effect of pressure induction was observed in the acidic pH range. A protein of approximately 15kDa, pI ≈ 4.2 was strongly induced after 60 min at 150 MPa (spot B in Fig. 10c, p. 49). In *E. coli*, a protein with similar size was maximally induced at high pressure too, but it was focused in the alkaline pH range and could not be identified by database comparison [21].

Remarkably, some of the detected pressure dependent proteins were gradually increased with rising pressures, while only specific pressures caused an increase for other spots (e.g. spot M, Fig. 10d, p. 49). This observation is supported by the analysis of *Rhodosporidium sphaerocarpum*, where the variance of just 5 MPa resulted in the induction of three additional proteins [142].

Comparison of the protein levels of high pressure treated *Lactobacillus sanfranciscensis* with untreated ones revealed a possible relationship of the mentioned 15 kDa protein (spot B) and another protein in close vicinity on the 2D gel (spot A, Fig. 10c, p. 49). The intensities of spot B compared to those of spot A were inversely proportional influenced by pressures from 0.1 to 200 MPa. Spot A is of similar size, but its pI is approximately 0.2 pH units shifted towards the acidic end. In contrast to spot B, protein levels of spot A decreased at high pressure. By MALDI-TOF MS as well as LC-MS/MS, these two spots were identified as the same protein, which is obviously modified by pressure stimulation. Protein modifications upon stress induction have previously been shown to be part of signal cascades, which activate the expression of several proteins [165-167]. The fact that the pressure effect on spot A and B was maximal at 150 MPa for 60min, and was continuously decreasing at higher pressures, indicates that this process is not governed by the Le Chatelier's Principle (volume reduction). Otherwise, the effect would reach a steady state due to the preferred volume decrease under pressure.

Since the genome of *Lactobacillus sanfranciscensis* has not yet been sequenced, the sequence fragments generated for the 15 kDa proteins were compared with protein databases. Two (GYGFLTTDD, VTLDVED) of the five amino acid sequences matched to CSPE and the major cold shock protein of *Lactococcus lactis* subsp. *lactis* with 68% and 62% identity, respectively. *Lactobacillus sanfranciscensis* and *Lactococcus lactis* are both lactic acid bacteria. However, cold shock proteins are highly conserved and the major cold shock proteins of two other species of the genus *Lactobacillus* show higher sequence identity to that of other bacteria like *Bacillus subtilis* than to lactococcal ones [168]. Furthermore, the two sequences match to variable regions of the cold shock protein and therefore it is notable that the other three sequences do not match. Interestingly, one of these three together with one of the first (GYGFLTTDD, SLGSGGSL) show 64% sequence identity to a probable (CspA6) cold shock protein transcriptional regulator of the soil bacterium *Sinorhizobium meliloti*. Therefore, the unknown protein was first thought to be a paralogue of a cold shock protein. Recently, one cold shock protein in the genome of *Lactobacillus sanfranciscensis* was identified and it was sequenced [4]. The sequences GYGFLTTDD and VTLDVED proved to have 100% similarity to this cold shock protein, but the other sequences derived from spots A and B did not match. Regarding the size of the sequenced CSP (6.5 kDa), and the fact that not all

sequences derived from spots A and B match this protein, it cannot be ruled out that the spots A and B may represent more than one protein. The pI of the sequenced CSP in *Lactobacillus sanfranciscensis* was indicated as 4.26. This value matches to spot B, which has an experimental pI of approximately 4.2. Therefore, spot A (pI ≈ 4.0) seems to be the posttranslational modified form, which predominates at atmospheric condition.

Spots C and E seem to represent the same protein too. The amino acid sequences of these proteins derived by LC-MS/MS show clear homology to ribokinase RbsK of *Lactobacillus sakei* (Tab. 9, p. 54). Ribokinase has a calculated molecular mass of 32 kDa and primarily forms a dimer in solution [169]. Therefore, spots C and E (estimated mass 65 kDa) may represent ribokinase dimers. Ribokinase participates in the first step of ribose metabolism by phosphorylation of D-ribose to D-ribose-5-phosphate in the presence of ATP and magnesium. Since only hydrogen bonds seem to be involved during this catalysis [170], it is improbable that the two spots might be attributed to different states of catalysis. In contrast to spot A and B the intensity levels of both spots increased upon pressure treatment. In *Lactobacillus sakei*, it was supposed that the phosphotransferase system is involved in the negative regulation of ribose utilization [171]. Although no ribose was added to the Homohiochii medium, it may be part of the meat and yeast extracts, which are included in the medium. Disruption of the negative regulation of ribose utilization might lead to the observed increase. The phosphorylation of ribose prevents leakage of the neutral sugar across the cell membrane. Carbohydrates have a baroprotective effect on bacteria and proteins [17, 172, 173], thus the ability for intracellular ribose accumulation might have a similar baroprotective effect.

Several amino acid sequences deduced from spot F correspond with that of elongation factor Tu (EF-Tu; Tab. 9, p. 54). This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis. Additionally, a chaperone-like function in protein folding and protection against thermal denaturation for this protein was suggested [174]. Both properties would benefit *Lactobacillus sanfranciscensis* at high pressures, as protein biosynthesis gets increasingly inhibited [21, 141, 143] and protein denaturation accumulates with increasing pressure [22, 143].

GMP synthase (spot D, Tab. 9, p. 54) catalyzes the synthesis of GMP from xanthosine 5'-monophosphate (XMP) and is encoded by the guaA gene. It was shown that *Lactococcus lactis* double mutants of guaA and recA own multiple stress resistance [111]. Furthermore, mutation by insertion in the guaA gene in *Lactococcus lactis* conferred multiple stress resistance against carbon starvation and acid, heat, and oxidative stress [175]. The addition of guanine or guanosine suspended acid tolerance, whereas hypoxanthine sustained the resistance. Therefore, it was suggested that complementation of GMP biosynthesis suppressed the stress resistance phenotype. In the context of increased levels of elongation factor Tu at high pressure presented in our study, reduction of the GTP precursor GMP by decreased GMP synthase levels might act as control for translation.

The responsible mechanism for varied protein levels in particular at pressures exceeding 100 MPa remains unknown. On one hand, it was reported that protein synthesis is severely inhibited at high pressure [21, 141, 143]. On the other hand, for cold shock proteins such as spot A and B (Fig. 10c, p. 49) it has been shown that these proteins can be induced under conditions that completely block protein synthesis [176]. Investigation of human chondrocytic cells revealed that mRNA stabilization probably the reason for increased HSP70 levels at high pressure [144]. Since mRNA stabilization is highly selective, it provides one possible explanation for the step-wise altered protein expression in *Lactobacillus sanfranciscensis* at high pressures.

To our knowledge, the only other published stress analysis of *Lactobacillus sanfranciscensis* was dedicated to acid stress [88]. There, 30 proteins were listed and 63 proteins were shown on gels, which are presumably involved in acid stress response and adaptation, respectively. Furthermore, the positions of the stress proteins DnaK, DnaJ, GrpE and GroES in the 2D pattern of *Lactobacillus sanfranciscensis* were determined by immunoblotting. None of these proteins could be assigned to the presented pressure dependent proteins.

4.2 Heat shock analysis of *L. lactis* reveals several proteins previously not reported

In the most recent proteomic analysis of heat shock response in *L. lactis*, 17 proteins were reported to be induced more than twofold [7]. Three of these proteins were identified as the prominent chaperones DnaK, GroEL and GroES. One HrcA counterpart was also identified by Western Blotting, but its induction was not indicated. Later, Hsp23 was assigned to be ClpP [177] by comparison to the published reference gel in [7]. In the same way, ClpB and ClpE can probably be assigned to Hsp100 and Hsp84 (both ClpB) and Hsp85 (ClpE) [178]. Furthermore, proteins identified as GrpE and YkiE in a recent study on pH stress occur in the same positions as Hsp26 and Hsp17 proteins on 2D gels of the heat shock analysis [179]. In the present study using ^{35}S pulse labeling similar to the method in the former heat shock analysis [7], 21 proteins induced by heat shock were detected, of which 17 were identified. All previous spot identifications and in particular assignments besides YkiE (Hsp17) were confirmed, although here the strain IL1403 (subsp. *lactis)* and previously the strain MG 1363 (subsp. *cremoris*) was used. Remarkably, two isoforms of GroES in IL1403 and just one in MG1363 were detected. Both isoforms are increased in their spot intensity upon heat shock, but the more acidic form is less abundant. The origin of these two isoforms as well as those of others cannot be determined by MALDI-TOF analysis. Besides the known heat shock proteins, two peptidases (PepV, PepC), one hypothetical protein (YuiC), two regulators (PurR, LlrA), one uncharacterized oxidoreductase (YphC) and the superoxide dismutase (SodA) were identified by analysis of the temporal protein expression profile at heat shock with ^{35}S pulse labeling. Furthermore, three proteins (CysM, BmpA and DpsA) were determined with at least twofold increase upon heat shock in the total protein expression profile analyzed with the DIGE technique and four proteins (AcpD, PurN, YfeC and YifJ) with at least 1.5 fold induction. Note, that protein expression in the context of 2D electrophoresis and especially in case of analyses of total protein profile by DIGE, but also in case of pulse labeling is used for the sum of protein synthesis, processing and degradation. For example, proteins with relatively slow turn over might accumulate in the cells, although protein synthesis is low. At stress conditions, the protein degradation possibly increases while synthesis remains equivalent. Dependent on the methodical approach, unaltered or decreased protein

expression is visible. Therefore, analysis with DIGE complements ^{35}S pulse labeling and results occasionally diverge.

Referring to the reduction of stringency from twofold to 1.5-fold while remaining the same statistical significance ($p<0.05$) when filtering spots of DIGE experiments seemed to be appropriate, since only a small proportion of the in total analyzed spots met these criteria (41 in 45 min and 8 in 20 min heat shock analysis). Furthermore, 23 of these proteins were identified and assigned to corresponding spots on gels of the analysis by pulse labeling. In 9 cases, the included spots with 1.5 to 2-fold difference of the DIGE analysis indicated alteration in the same way as in pulse labeling (Tab. 13, p. 69). In general scheme of things, the DIGE analysis of 45 min was more coherent to pulse labeling results than that of 20 min, indicating protein alterations at total protein level are later detectable.

4.2.1 Differential expression of purine metabolism related proteins at heat shock

The increase of the regulator of purine biosynthetic genes (PurR) is of particular interest, because several proteins belonging to the purine metabolism were decreased upon heat shock (Tab. 13, p. 69), but besides of PurK, no protein with decreased expression was identified, which belongs to the *purDEK* and *purCSLQF* operons. The regulation of these two operons has shown to be dependent on an intact *purR* gene [180]. Thus, PurR possibly keeps proteins belonging to the two operons at a continual level, whereas other purine metabolism related proteins have decreased expression levels. The protein PurN, for which also regulation by PurR by means of a PurBox sequence was suggested [181], even seemed to be slightly induced in the DIGE analysis after 45 min heat shock (Tab. 10, p. 60). This protein for example escaped analysis by pulse labeling, because no corresponding spot was observed (for explanation see below). Activation of PurR was demonstrated to be induced under conditions of high 5-phosphoribosyl-1-pyrophosphate (PRPP) pools and abundance of PRPP was suggested to be dependent on PRPP consumption by phosphoribosyl transferases, which use PRPP to convert bases to corresponding monophosphates [180]. Reduced levels of phosphoribosyl transferases were not detected, but accumulation of phosphorylated bases due to reduced growth at heat shock might result in PRPP accumulation too.

Within the context of increased PurR and several decreased proteins of purine metabolism at heat shock, it is interesting that guanine nucleotide pools, notably (p)ppGpp (an alarmone) were proposed to act as signals that determine the level of stress response induction in *L. lactis* [175]. In another study, mutations in genes, implicated in purine metabolism, conferred multiple stress resistance to a thermosensitive *recA* strain [111]. Some of these mutations improved the heat stress resistance even in the mutated wildtype such as insertion into *deoB* or *guaA*. Notably, GuaA was also decreased in the present analysis (Tab. 13, p. 69). The decrease of purine nucleoside phosphorylase (DeoD) at heat shock also fits into the context of guanine pool involvement in regulation of stress response.

Recent investigations of the purine stimulon by 2D electrophoresis showed decreased abundance of proteins upon purine starvation that are related to energy metabolism, protein synthesis or are GTPases [181]. Five of six identified proteins in that study were also decreased upon heat shock in the present analysis (EF-TS, FtsZ, GapB, RpsB and trigger factor), indicating further similarities in purine regulation and heat shock response.

The repression of several kinases (PFK, PGK, PYK, CMK, PrsB and PyrH), the phosphocarrier protein HPr and further nucleoside triphosphate dependent proteins (Tab. 13, p. 69) is drawing attention. The repression of the kinases as well as the GTPases (FtsZ, EF-TS and EF-Tu) might serve as energy conservation in order to cope with the current stress response, but GTP is also the precursor of (p)ppGpp. Thus, our results support the suggestion of relevance of the guanine nucleotide pools in stress response of *L. lactis*, but also indicate the involvement of other nucleotide pools in heat shock response. On the other hand, the pool size of CTP and dCTP in *L. lactis* is under control of CTP synthase (PyrG) [182], which is also decreased at heat shock (Tab. 13, p. 69). In *L. lactis* as well as *B. subtilis*, *pyrG* expression is probably regulated by an attenuation mechanism responding to the CTP concentration in the cell [183, 184]. Thus, PyrG might simply be decreased, because CTP accumulates in the cell due to decreased transcription at heat shock.

HPr was decreased at heat shock in the present study and also at low pH published in a recent study [179]. This protein is part of the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS). After ATP-dependent phosphorylation of HPr by the bifunctional HPr kinase/P-Ser-HPr phosphatase, P-

Ser-HPr participates in carbon catabolite repression of an operon encoding a beta-glucoside-specific EII and a 6-P-beta-glucosidase, but also in inducer exclusion of the non-PTS carbohydrates maltose and ribose [185]. Furthermore, P-Ser-HPr functions as a coactivator in the CcpA-mediated transcriptional activation of the *las*-operon [186]. Since phosphofructokinase (PFK), pyruvate kinase (PYK) and L-lactate dehydrogenase (LDH) are part of the *las*-operon, their observed decrease in the present study is consistent to HPr repression.

4.2.2 Further proteins induced at heat shock and previously not reported

Superoxide dismutase (SodA) catalyzes the degradation of superoxide and was detected upon stress induction by low pH [179, 187]. There, it has been suggested that increased amounts of SodA at low pH might cope with greater concentrations of oxidizing intermediates. Since such intermediates accumulate during stress, this would explain, why SodA is within the first 20 min only visibly increased after pulse labeling and then, also after 45 min at total protein level (Tab. 13, p. 69). In *B. subtilis*, SodA is under the control of the σ^B factor and induced upon heat shock [93], but a counterpart to σ^B factor was not found in the genome of *L. lactis* [8].

The position of non-heme iron-binding ferritin (DpsA) in the high molecular range of 2D gels (Fig. 14, p. 57, spot 1) is unusual, since its calculated Mr is 17 kDa. Similar migration of DpsA at high Mr was reported in another 2D gel analysis, naming multimerization as reason for the detection at 141kDa [24]. MrgA, the ortholog to DpsA in *B. subtilis*, occurs in two positions on 2D gels and one of these seemed to be a dodecamer of MrgA [188] with similar Mr as the DpsA spot in the present analysis. MrgA is induced under oxidative stress and regulated by peroxide regulator (PerR). PerR is similar to Fur in *L. lactis*, but Fur has not been characterized yet [189].

The two-component system regulator LlrA belongs to the OmpR subfamily of bacterial response regulators [190]. Remarkably, insertional mutants of *llrA* were impaired in growth and arginine deiminase negative, a protein that was also repressed at heat shock in our study. Furthermore, it was suggested that the two component system KinA/LlrA might be involved in modulation of intracellular pH, because insertional mutants of this system were acid sensitive too.

The increase of dipeptidase (PepV) and aminopeptidase C (PepC) spots on gels representing heat shock might be explained by the necessity of the cell to cope with degradation of denatured proteins. However, the relevance is not discussed in detail, because these two increased spots occurring in ^{35}S pulse analysis could not definitely be assigned to corresponding spots on Coomassie stained gels by image overlay and might represent new spots.

Basic membrane protein A (BmpA) and cysteine synthase (CysM) are the only proteins in the present study, which were detected in two different analyses and had opposite spot ratios at heat shock, but both in a time dependent fashion. Furthermore, experimental Mr (approx. 23 kDa) and pI (approx. 5.5) of BmpA largely diverge from the theoretical values (Mr: 36.7 kDa, pI: 8.68), which means it is either processed or degraded. The reason for an increase within the first 20min at heat shock might be explained by increased degradation of BmpA and the decrease after 45 min by further degradation into smaller peptides. Missing of corresponding BmpA spots in pulse labeled extracts might be explained by altered protein degradation or processing, but on the other hand, Met/Cys content is especially low in BmpA (1.4%) while Lys content is clearly above the average of lactococcal proteins (11.1%). This problem also occurred in case of PurN (Met/Cys: 1.1%, Lys: 7.7%) and complicates comparison and interpretation of DIGE and ^{35}S pulse labeling analysis. Thus, absence of BmpA in pulse labeling might be a problem of detection limit. However, it should be mentioned that a PurBox sequence is also found in front of *bmpA* [189], but this must not necessarily mean that it is regulated by the PurR regulon.

In conclusion, several proteins implicated in energy and purine metabolism, translation, and in particular in regulation were identified, which are differentially expressed under heat shock conditions. Most of these proteins have not been reported before with respect to heat shock response in *L. lactis*. Comparison to other stress analyses revealed several similarities and suggests common mechanism, such as the influence of guanine nucleotide pools on heat and pH stress.

4.3 High pressure induced changes in the proteome of *L. lactis*

It has been shown that high pressure has various deleterious effects on cells and their components. Dependent on the level of pressure, these effects range from reversible processes like growth inhibition or quaternary structure dissociation of proteins to severe cell envelope damages and cell lysis (reviewed in [4, 22, 115]). Stress response analyses of different organisms to sublethal pressures have demonstrated that certain proteins are differentially expressed at high pressure [21, 127, 141, 142]. Similar to observations under other stress conditions, heat shock and/or cold shock proteins were among those, which showed elevated levels [16, 21] (see also induction of CSP in *Lactobacillus sanfranciscensis* at high pressure, section 3.1, p. 46). The present analysis was performed to identify components of the late stress response and recovery of *L. lactis* after high pressure treatment.

The comparative analysis showed that the response after pressure treatments at 60 or 90 MPa is more intense when 90 MPa were applied (Tab. 16, p. 86). The methodical approach by ^{35}S pulse labeling revealed 52 increased and 46 decreased spots compared to 19 increased and 17 decreased determined by the DIGE technique (90 MPa values in Tab. 15, p. 82, and Tab. 14, p. 77, respectively). The relatively short timeframe in which protein expression is monitored by pulse labeling makes the method more sensible to differences than comparison of total protein expression by DIGE. On the other hand, total protein concentrations are not reflected by pulse labeling. Therefore, DIGE complements pulse labeling, which is in particular demonstrated by the detection of some pressure dependent spots only in DIGE analyses. Complementing of DIGE and pulse labeling was also observed in heat shock experiments (discussed in section 4.2, p. 109).

Three proteins involved in cell envelope synthesis were increased subsequent to high pressure treatment. D-alanine-D-alanine ligase (DdL), UDP-N-acetylmuramate-alanine ligase (MurC) and D-Ala-D-Ala adding enzyme (MurF) are participating in the synthesis of UDP-N-actetylmuramyl pentapeptide, which is a cytoplasmatic precursor of the peptidoglycan [191]. Induction of those proteins indicates that cell envelope damages probably occur already at pressures below 100 MPa. Basic membrane protein A (BmpA) was decreased at total protein level after 60 MPa and after 45 min heat shock (see 4.2.2), but is otherwise uncharacterized. The experimental Mr (approx. 23 kDa) and pI (approx. 5.5) indicate

that this protein probably is either processed or partially degraded (theor. Mr: 36.7 kDa, pI: 8.68). BmpA was probably not detected with ^{35}S-Met/Cys labeling, because its Met/Cys content (1.4%) is relatively low compared to other lactococcal proteins.

Hydroxymyristoyl-acyl carrier protein dehydratase (FabZ1) and 3-oxoacyl-acyl carrier protein reductase (FabG1) are two decreased proteins after pressure treatments at 90 MPa. They are both implicated in elongation of fatty acids and it was suggested that FabZ1 (also named MabA) in *M. tuberculosis* preferably uses long-chain substrates [192]. On the other hand, fatty acid/phospholipid synthesis protein (PlsX) was increased under the same conditions. The function of PlsX in phospholipid synthesis is not completely solved. The differential expression of proteins participating in fatty acid and phospholipid synthesis indicates that a reorganization of fatty acids in the cell membrane induced by high pressure might take place. Reorganization of fatty acids in response to reduced membrane fluidity is observed under cold shock conditions [193]. Furthermore, the increased presence of unsaturated fatty acids in piezophiles or barotolerant bacteria is discussed in context with adaptation of these organisms to deep-sea environments (reviewed in [126]).

Thioredoxin (TrxA) was increased in the DIGE as well as in the pulse analysis in particular after treatment at 90 MPa. In addition, elevated levels of thioredoxin reductase (TrxB1) were detected by pulse labeling after 90 MPa. These results suggest that thioredoxin is increasingly needed in the reduced state after the pressure treatment at 90 MPa. Thioredoxin is participating in several different reactions including primarily ribonucleotide reduction, but serves also as activator of target proteins by reversible thiol-disulfide exchange reactions and as structural component in protein complexes [194]. One recent publication suggests that thioredoxin associates with NusG, FabG, FabZ, RecA and some other proteins [195], which were differentially expressed in the present differential analysis, but the nature of these associations needs to be further characterized.

Although the ribonucleotide reductase proteins NrdE, NrdF and NrdI are also increased subsequent to 90 MPa treatment, there seems to be no direct connection to TrxA, because it was suggested that the NrdEF complex uses the glutaredoxin-like protein NrdH as hydrogen donor [196, 197]. Differences in NrdH levels were not detected in the present study, but considering the small size of NrdH (theor. Mr: 6.7 kDa), it might have escaped 2D analysis. NrdI has a stimulatory effect on

ribonucleotide reduction and is part of the *nrdHIEF* operon [197]. It is generally supposed that for growth under anaerobic conditions class III ribonucleotide reductase such as NrdDG in *L. lactis* is necessary, but a *nrdD* negative mutant of *L. lactis* grew well under anaerobic conditions, because of active NrdEF [196]. Furthermore, wildtype *L. lactis* demonstrated the same kind of activity as *nrdD* mutant. Thus, it was suggested that NrdEF is the active ribonucleotide reductase in *L. lactis* and minimal oxygen concentrations are sufficient to activate the tyrosyl radical necessary for functional NrdEF. Stability of tyrosyl radical or NrdEF complex at high pressure is unknown. Reduction of ribonucleotides is the rate limiting step in DNA synthesis [198] and might be an important factor of cell growth control at high pressure besides the cell division protein FtsZ, for which decreased polymerization and ring formation at high pressure was reported [128, 136, 137].

Sequence comparison of oxidoreductase YudI with BLAST (data not shown) revealed that this protein shares high similarity with dihydrouridine tRNA synthases of diverse organisms. It was suggested that dihydrouridine may lend a certain degree of flexibility to RNA [199], but the cellular role has not been elucidated yet.

The GTPase Era is essential for growth and probably involved in 16S rRNA maturation and ribosome maturation in *E. coli* [200]. Conformational changes and dissociation of ribosomes were named for disruption of protein synthesis at high pressure [119, 143]. Therefore, increased levels of Era might cope with damaged ribosomes. Repression of Era resulted in elongated cells without constrictions and septum formation [201] similar to observations after pressurizing *E. coli* [136, 137], suggesting altered levels or limited functionality of Era at high pressure might also influence filament formation besides FtsZ. YqeL is another GTPase, which showed decreased protein levels after high pressure, but YqeL has not been characterized yet.

The expression of pyrimidine biosynthetic genes in *L. lactis* is probably dependent on an attenuator mechanism by way of pyrimidine operon regulator (PyrR) as it is in *B. subtilis* [202, 203]. Decreased levels of PyrR after high pressure treatments indicate that pyrimidine synthesis was probably repressed at high pressure. Aspartate carbamoyltransferase (PyrB) is part of the *pyrRPB-carA* operon and was likewise decreased. Regulation of the purine operons *purDEK* and *purCSLQF* is dependent on the activator PurR in *L. lactis* [180]. Therefore, increased

levels of PurR indicate that purine synthesis was probably repressed at high pressure too. Decreased levels of phosphoribosylaminoimidazole carboxylase (PurK) at total protein level but not detected in pulse labeling experiments support this assumption. In context of altered levels of seven proteins implicated in purine synthesis and salvage due to high pressure treatment, it is interesting that (i) under heat shock conditions similar differences were observed (see section 4.2.1, p. 110) and (ii) guanine nucleotide pools were proposed to be involved in regulation of stress response in *L. lactis* [111, 175]. A protein homolog to GMP synthase, one of the purine metabolism related proteins with decreased levels in *L. lactis*, was also decreased at high pressure in *Lactobacillus sanfranciscensis* (see section 3.1, p. 46).

YnaB was recently identified as transcriptional regulator with LexA-like features (also named HdiR in *L. lactis*) [112]. There, it was shown that self-cleavage of YnaB is mediated by activated RecA in presence of a DNA damaging agent. YnaB was then further degraded in dependence on ClpP, which resulted in induction of *ynaB* and *umuC* expression. In the present analysis, RecA was increased subsequent to high pressure treatments in addition to YnaB. RecA is activated in *E. coli* by conformational transition to RecA* upon binding to single-stranded DNA at stalled replication forks (reviewed in [204]). Stalling at high pressure was described for RNA polymerases [146], but was to our knowledge not investigated for DNA polymerases.

Besides ClpB no Clp protein was increased due to high pressure treatment and the chaperone GroEL was even decreased. The expression of heat shock proteins at high pressure in *E. coli* was reported to be time dependent in a similar way as upon heat shock and induction of e.g. GroEL was followed by repression [21]. In addition, the response was delayed. Therefore, increased levels of ClpB after stress treatment at 90 MPa are probably the single remnant of a weak heat shock response at high pressure.

Zinc transport transcriptional regulator (ZitR) was identified by sequence comparison [8], but is not further characterized. Metal regulation is often implicated in oxidative stress response, but within this context, it should be noticed that neither additional proteins of the *zitRSQP* operon nor proteins primarily necessary for detoxification, such as SodA, were detected in the present differential analysis.

For glucose inhibited division protein (GidC) in *L. lactis* no cellular function is known. In other organisms, GidA is often described as being involved in cell division, but for GidC no function is described.

Transcription antitermination protein (NusG) is involved in transcription and translation control [205], peptide deformylase (PDF) removes the N-terminal formyl group of nascent proteins and Glu-tRNA amidotransferase subunit A (GatA) is needed for Gln-tRNA formation [206]. Their expression was also sensible to pressure treatments and thus, they might be involved in reduced transcriptional and translational capacities at high pressure.

Phosphocarrier protein HPr was increased in DIGE but not in pulse labeling analyses, which indicates that HPr levels were elevated at high pressure. HPr is involved in regulation of glycolytic activity and differential expression was observed under cold shock, low pH and heat shock ([179, 186, 207] and section 4.2.2, p. 112). Altered glycolytic activity at high pressure was observed for example in *Lactobacillus sanfranciscensis* [208].

Finally, several uncharacterized proteins showed altered levels subsequent to high pressure treatment. Remarkably, four of them (YgdA, YuiC, YveC and YxdB) were also detected in the differential proteome analysis of heat shock in *L. lactis* (Tab. 13, p. 69), marking them as multi stress dependent proteins.

In conclusion, several proteins were identified in the present study, which are seemingly related to previously reported effects of high pressure onto cells, such as cell envelope damage, inhibited growth or disruption of protein synthesis. The mechanisms of pressure effects at molecular level were generally not recognized in these reports, but the way in which the discussed proteins are implicated in cellular networks might indicate the molecular causes for the deleterious effects.

4.4 The alkaline proteome of *L. lactis*

The distribution of the predicted isoelectric points among the proteome of *Lactococcus lactis* concludes in the presumption that up to 40% of proteins remained not investigated in previous studies, which mainly focus on pH 3 to 10 or even pH 4 to 7 (reviewed in [2, 23]). Indeed, this limitation excluded for a long time the stress protein DnaJ from 2-DE analysis, since its predicted pI is close to pH 7 and was therefore first detected in 1997 by Western blotting of an IPG-DALT covering pH 3 to 10 [7]. Furthermore, the present results emphasize this presumption.

Application of the protein extract via cup-loading at the anodic side in combination with increasing the final voltage during IEF up to 8000 V, enabled to separate more than 200 spots in the pH gradient from 6-12 (Fig. 27, p. 92). Key to successful IEF without spot streaking in IPG 6 to 12 and 9 to 12 is completing the IEF at 8000 V within one day. Over night IEF at low voltage and subsequently completing the IEF at 8000 V the next day, again resulted in spot streaking.

Separation of the proteins between 6 and 12 facilitates an overview of all predicted alkaline lactococcal proteins and provides an extension of the existing reference map between 4 and 7. In addition, the small overlapping range from pH 6 to 7 provides a connection and orientation between both reference maps. Eight proteins with experimentally determined pIs above 6 are integrated in the reference map covering pH 4 to 7 [24]. Five of these are ribosomal proteins and probably the result of precipitation at the cathode during IEF of IPG 4 to 7. These five ribosomal proteins were separated and identified in the pH range from 9 to 12 (Tab. 17, p. 97). The remaining three were not identified. AdhE and EF-Tu were mapped in the mentioned and in the present study, but evidently in different spots. Therefore, no example for a common protein focusing within the overlapping pH unit of IPG 4 to 7 and 6 to 12 can be named.

The narrow IPG from 9 to 12 increases the resolution and facilitates the detection and identification of further proteins as shown in Fig. 31, p. 96. The advantage of using narrow immobilized pH gradients was previously demonstrated for example with *S. cerevisiae* [42] or *L. lactis* [24]. Therefore, both types of gradients were applied for the set-up of an alkaline reference map. Several proteins appear in more than one spot on the gels in Fig. 29, p. 94, and Fig. 31, p. 96. In total, 114

different spots resulted in the identification of 85 proteins with 39 proteins appearing more than once. These spots might resemble modified forms of one protein. Posttranslational modification might also be the reason for the poor coverage in case of unidentified protein spots.

In organisms, codon usage and tRNA content are positively correlated, which relates to the protein production levels of individual genes [209]. The codon adaptation index (CAI) is an instrument to predict the expression level of proteins from the genome level via synonymous codon usage bias [160]. In case of *Lactococcus lactis* the highest CAIs for proteins with predicted pIs above 7 were calculated for ribosomal proteins, such as the 50S ribosomal proteins L5 (RplE, CAI 0.8). A view on the reference map in Fig. 29, p. 94, confirms spot 69, which was identified as RplE, is indeed highly expressed under the chosen growth conditions. Most of the proteins with high CAIs are ribosomal proteins (see section 3.4.1, p. 89) and they are highly expressed. Similar results for the correlation between CAI and protein expression in *Lactococcus lactis* were observed in the pH gradient from 4 to 7 [24]. At this point, it should be mentioned that the strict correlation between CAI and protein expression is questionable, but in general, the correlation seems to be appropriate for *L. lactis*.

Recent publications name low abundant proteins as critical for 2-DE [45, 210, 211]. In a comprehensive analysis covering the acidic pH range from 4 to 7 of *Lactococcus lactis*, only one of 238 identified protein bears a CAI below 0.4 (0.377) [24]. In the present analysis, seven proteins bearing a CAI below 0.4 were detected and successfully identified (Tab. 17, p. 97). Previously, it was indicated that 2-DE enables a poor access to proteins with low codon bias [210, 211]. The protein bearing the lowest CAI detected in this study is the manganese ABC transporter ATP binding protein (CAI 0.313). Only 91 (10%) of the alkaline lactococcal proteins have smaller CAIs. Among these 91 proteins are 16 (18%) transposases, 31 (34%) unknown, 22 (24%) prophage-encoded, 12 (13%) hypothetical and only 10 (11%) other proteins. As previously quoted, the correlation between CAI and protein expression might not always be strict. In case of MtsB for example, an increased expression due to manganese starvation as demonstrated in *B. subtilis* [212] is possible. If the cells already experience manganese starvation in the growth medium, which contains 0.08 µM $MnCl_2$, is not known.

Prophage-encoded proteins and transposases were excluded from a previous *in silico* proteome analysis [24]. The *in silico* proteome of *Lactococcus lactis* comprises 50 transposases and 219 prophage-encoded proteins, which means in total nearly 12%. Since in the present analysis at least two prophage-encoded proteins were detected and identified, all 2266 annotated lactococcal proteins were considered for the *in silico* analysis. To our knowledge, this is the first time prophage-encoded proteins were detected in 2-DE.

Besides low abundant proteins, another critical point in 2-DE is the separation of hydrophobic proteins. The grand average of hydropathicity (GRAVY) value is a measure for the hydrophobicity of a protein [161]. Values above zero indicate hydrophobic proteins. Compared to the predicted hydrophobic proteins in the alkaline range, a relatively small number of hydrophobic proteins was identified (Fig. 26, p. 91) and alternative extraction methods might be necessary for their analysis. Proteins with unknown function contribute 30% to the group of predicted alkaline and hydrophobic proteins. Whether *Lactococcus lactis* expresses all these and other proteins of this group in general or under the chosen growth condition is questionable.

For most of the proteins identified in the present set-up of an alkaline reference map, this is the first experimental evidence to be expressed in *Lactococcus lactis*. The largest group among the identified proteins is consisting of ribosomal proteins. They are thought to act as sensors of heat and cold shock [213] and therefore might be of special interest in proteome analyses with respect to stress response in microorganisms. Despite the fact that 29 ribosomal proteins were mapped in total in the present study, several more have high CAIs and theoretical pIs within the analyzed pH range, in particular between pH 9 and 12. All of them have calculated molecular weights of 15 kDa or smaller. On one hand, this size is close to the lower possible range of resolution of 2-DE and on the other hand, peptide mass finger printing gets increasingly difficult the smaller proteins are [214], especially if modifications are expected. Hence, in case that these proteins are focus of a study, a different buffer system in the second dimension and alternative digestion for peptide mass fingerprinting [214], or sequencing of the peptides is recommended.

Hypothetical or unknown proteins represent the second largest group of the identified proteins with 22%. The expression of those proteins is most interesting,

because up to now no function can be assigned to them. Proteome studies focused on the expression of these proteins in dependence on growth phase, stress, etc., might give insight into the cellular role of these proteins.

The reference maps as well as the complete data of the *in silico* analysis were included into the dynamic online database for proteomes, which was specially designed for easy distribution of the data via the Internet (see section 4.5). Clickable spots within the reference maps are linked with summarized protein information, such as molecular weight, pI, GRAVY, etc., and vice versa to permit a quick and intuitive search throughout the presented data. The database is available at www.wzw.tum.de/proteomik/lactis.

4.5 The dynamic online database for proteomes sets new standards for online 2D databases

The benefit of a proteome database comprising 2D reference maps is versatile. On one hand, the summarized protein information itself is useful for several types of proteomic applications, such as quick access to the isolelectric point of one or several proteins to choose a suitable buffer or immobilized pH gradient, or determination of a subset of proteins with a particular GRAVY value to estimate their solubility. In proteomics, characteristic values like the CAI or the GRAVY for example are compared and analyzed to estimate the part of a proteome, which probably can be covered by a particular methodical approach [211, 215] (see also section 4.4, p. 119). Therefore, these and other often used protein characteristics were computed for the complete theoretical proteome and implemented into the presented database.

The reference maps, on the other hand, are informative on various levels too. They deliver evidence for the expression of the identified proteins at the chosen growth condition. Furthermore, the deviation of the predicted molecular weight and/or isoelectric point indicates protein processing or posttranslational modification, in particular if one protein is represented by more than one spot. The spot intensity in comparison to other spots indicates the relative abundance of the protein in the cells. Unlike in other 2D databases, identified spots are marked by circles instead of crosses on gel images (Fig. 33, p. 101). Crosses cover up small spots or spots with low intensity and marked spots are barely visible. Increasing the contrast in general results in merging big spots. Circles leave the spots visible and indicate the spot area.

For following analysis at the proteome level, the reference maps give an overview, which proteins can be expected in the pH gradient and serve as orientation, especially if a particular set of proteins are of interest. The reference maps are no substitute for spot identification by peptide mass finger printing or protein sequencing and shall not serve as single source for protein information, but may support insignificant spot identifications obtained e.g. by poor sequence coverage.

In comparison to other 2D databases, no gels without identified spots were included in the database and the search for proteins of interest can be restricted to

reference gels, giving the user the advantage to know instantly, which protein can be found on what gel. *In silico* based 2D patterns were not included in the database, because only a part of the theoretical proteome is constitutively expressed and protein abundance is very different in cells [211]. For the selection of appropriate IPG strips or acrylamide content, proteins can be listed according to their isoelectric point and molecular weight. Finally, the database is simply navigated by the search and the resultset and results are interactively linked. Thus, the user needs only minimal time for acquiring the scope of the database and is not confused by multiple submenus. Furthermore, the precomputed protein characteristics instantly provide valuable information frequently used in proteomics and not listed in Swiss-Prot or GenBank [155, 157]. For quick access to data listed in the latter two databases, links were implemented.

One further aspect is covered by the presented proteome database: progress report or even quality assurance. Once the theoretical proteome of an organism is implemented, the database makes it easy to document 2D gels. The spots simply need to be marked and named with the corresponding protein identifier, which is in general indicated by each software used for protein identification. After uploading the gel and the spot coordinates, the experimental data are immediately accessible via the Internet or can be restricted to an Intranet. Thus, collaborating laboratories or e.g. proteomic service stations can easily share 2D related results even without the demand of experience in certain softwares for gel analysis.

5 Summary

The response of microorganisms to major environmental changes like temperature or pH shifts is well investigated and known as stress response. Heat shock response is characterized by the induction of a set of heat shock proteins. In *Lactococcus lactis*, the heat shock response has been previously analyzed by radioactive pulse labeling at proteome level, but besides prominent heat shock proteins, such as GroEL and DnaK, no further proteins have been identified. *L. lactis* has become model organism for lactic acid bacteria and was in the meantime completely sequenced. The fully sequenced genome of *L. lactis* and higher sensitivity in mass spectrometry promise higher identification rates. Furthermore, improved techniques in 2D electrophoresis, such as IPG technology and difference gel electrophoresis (DIGE), enhance the reliability and sensitivity for the detection of differences in comparative analyses. Therefore, the heat shock response of *L. lactis* was again analyzed to identify hitherto undetected and unidentified proteins involved in heat shock response.

The heat shock response was studied at the level of total protein expression with the DIGE technique and temporal protein expression analyzed with ^{35}S-Met/Cys pulse labeling. The analysis with DIGE represents to our knowledge the first analysis of total protein expression in *L. lactis* under heat shock conditions. Pulse labeling and DIGE revealed in total 21 proteins, which were induced more than twofold at heat shock. Besides the previously described heat shock proteins, further stress related proteins (e.g. DpsA, GrpE and SodA) as well as two regulators (LlrA and PurR) and some uncharacterized proteins (e.g. BmpA, YphC and YuiC) were among the proteins with increased abundance. In particular, proteins related to the purine metabolism presented one of the largest groups influenced by heat shock, supporting the suggestion that guanine nucleotide pools are involved in the regulation of stress response in *L. lactis*. Between the two analytical approaches (DIGE and pulse labeling), several similarities and differences in the differential analysis were determined. These results indicate the difference in the heat shock response at temporal and total protein level, and the capabilities of detecting stress proteins among total protein with the recently available DIGE technique.

The cellular response to the application of high hydrostatic pressure was analyzed in *Lactobacillus sanfranciscensis* and *L. lactis*, which are two lactic acid bacteria with importance for the food industry. High pressure is used for food preservation, and for some organisms, it has been shown that it induces stress response. In case of *Lactobacillus sanfranciscensis*, high resolution 2D electrophoresis with immobilized pH gradients (IPG-DALT) was utilized to compare protein patterns of atmospherically grown with pressure treated bacteria at pressures up to 200 MPa, applied for one hour. The comparative study was performed by using overlapping immobilized pH gradients (IPG) covering the pH range from 2.5 up to 12 in order to maximize the resolution for the detection of stress relevant proteins. For improved quantitative analysis, staining with SYPRO RUBY^{TM} was used in addition to silver staining. DIGE was not available at that time. By computer-aided image analysis, we detected more than a dozen spots within the pH range from 3.5 to 9 that were more than twofold increased or 50% decreased in their intensity upon high pressure treatment. Two of them (approx. pI 4.0 and 4.2; Mr ≈ 15 kDa) have almost identical MALDI-TOF MS spectra. They were identified by microsequencing with LC-MS/MS and homology to CSPE of *L. lactis* as the same cold shock protein. Since one of them is increased at nearly the same ratio as the other is decreased in its amount at pressure treatments up to 150 MPa, it possibly exemplifies a selective protein modification triggered by high pressure. The effect is probably not dependent on volume reduction, since it diminished at pressures above 150 MPa.

It was further remarkable that by monitoring the barosensitivity of the cells within 25 MPa steps, we observed a differential pressure induction or repression of the detected proteins. For example one of the cold shock proteins was maximally increased after 1h, 150 MPa while another protein (pI 7.5, Mr ≈ 25 kDa) was maximally increased after 1h, 50/75 MPa. This indicates a successive cell response and therefore different causes at the molecular level. Other proteins identified with LC-MS/MS, participate in translation, carbohydrate and purine metabolism.

Stress response of *L. lactis* IL1403 induced by high pressure was analyzed to identify proteins with altered expression during and in particular immediately after pressure treatment. The comparison of references and samples treated with 60 or 90 MPa was analyzed by DIGE and ^{35}S-Met/Cys pulse labeling. As in the heat shock analysis, DIGE represents total protein expression, whereas pulse labeling provides

a time dependent snapshot. Thus, pulse labeling experiments complement DIGE analyses.

Alterations in the protein pattern after high pressure treatment were detected to identify proteins, which are not only implicated in the immediate stress response, but also in the recovery of the bacteria. As a result, induced proteins might be used as indicators for cellular damages caused by high pressure. Indeed, elevated levels, for instance, of proteins involved in cell envelope synthesis like UDP-N-acetylmuramate-alanine ligase (MurC) and D-Ala-D-Ala adding enzyme (MurF) were detected after pressure treatment at 60 MPa. Even higher levels of MurC and MurF were detected when 90 MPa were applied. This indicates that cell envelope damage probably occurs already at pressures below 200 MPa, and thus far before cell inactivation.

Besides MurC and MurF, the expression of further proteins, which are related to previously reported effects of high pressure onto cells, such as inhibited growth (e.g. NrdEF, RecA, YnaB) or disruption of protein synthesis (e.g. Era), was influenced. Remarkably, as was observed in the analysis of heat stress, proteins implicated in the purine metabolism were again one of the largest goups, which were influenced. This once more indicates an interconnection of guanine nucleotide pools and regulation in stress response. The mechanisms of pressure effects at molecular level were generally not recognized in previous reports. However, comprehension of the cellular response to high pressure is of great interest, since the occurrence of pressure resistant strains and mutants of e.g. *E. coli* has already been reported. This study provides evidence of proteins, which are individually increased or decreased in their amount or even modified upon high hydrostatic pressure treatment. The way in which the identified proteins are implicated in cellular networks might indicate the molecular causes for deleterious effects of high pressure.

To our knowledge, the alkaline proteome of *L. lactis* has never been the subject of proteomic studies. Thus, several conditions for IEF were optimized and different electrophoresis systems were compared to finally set up a reference map for the alkaline proteome of *L. lactis*. With the optimized protocol, 96 spots in the pH gradient from 6-12 and 57 in another gradient from pH 9-12 were successfully identified by peptide mass fingerprinting (MALDI-TOF MS). In total, 85 different proteins were represented in those 153 spots with predicted pIs ranging from 6.34 to 11.31. Even

proteins considered as critical to analyze with the 2-DE such as hydrophobic, low molecular weight or low abundant proteins, were mapped and identified, e.g. NADH-dependent enoyl-ACP reductase (GRAVY: 0.156), translation initiation factor IF-1 (8 kDa) or manganese ABC transporter ATP binding protein (CAI: 0.313).

The complete *in silico* data of the proteome of *Lactococcus lactis* as well as clickable reference maps were included in a newly constructed database, which is available at www.wzw.tum.de/proteomik/lactis. The proteome database of *Lactococcus lactis* has special features, which were insufficiently or limited considered in existing databases. These features are simple database extension, extensive search functions and connections to predicted data, such as CAI and GRAVY. The integrated interface of the database supports detailed searching according to several protein characteristics through the experimental proteome presented as reference maps and the complete *in silico* proteome. The design of the database permits an extension without knowledge of any software language.

6 Zusammenfassung

Die Reaktion von Mikroorganismen auf größere Veränderungen in ihrer Umwelt, wie z.B. pH- oder Temperaturverschiebungen, ist gründlich untersucht und auch bekannt als Streßantwort. Charakteristisch für die Hitzeschockantwort ist die Induktion einer Gruppe von Hitzeschockproteinen. Bei *Lactococcus lactis* wurde die Hitzeschockantwort bisher auf Proteom-Ebene mit radioaktiver Pulsmarkierung analysiert, jedoch wurden bis auf bekannte Hitzeschockproteine wie z.B. GroEL und DnaK keine weiteren Proteine identifiziert. *L. lactis* wurde als Modellorganismus für Milchsäurebakterien mittlerweile vollständig sequenziert. Das vollständig sequenzierte Genom von *L. lactis* und eine höhere Sensitivität in der Massenspektrometrie versprechen bessere Erfolge bei der Proteinidentifizierung. Außerdem erhöhen Verbesserungen in der 2D Elektrophorese, wie z.B. die IPG Technologie und die Difference Gel Electrophoresis (DIGE), die Verläßlichkeit und die Sensitivität bei der Bestimmung von Unterschieden in vergleichenden Analysen. Deswegen wurde die Hitzeschockantwort von *L. lactis* nochmals untersucht, um an der Hitzeschockantwort beteiligte, jedoch bislang nichtdetektierte und nichtidentifizierte, Proteine zu identifizieren.

Die Hitzeschockantwort wurde auf der Ebene der Gesamtproteinexpression mit der DIGE Technik und der zeitweisen Proteinexpression mittels ^{35}S-Met/Cys Pulsmarkierung analysiert. Damit wurde nach unserem Wissenstand mit Hilfe von DIGE erstmals auch die Gesamtproteinexpression von *L. lactis* unter Hitzeschock untersucht. Durch Pulsmarkierung und DIGE wurden 21 Proteine nachgewiesen, die unter Hitzeschock induziert wurden. Neben den bisher aus Publikationen über *L. lactis* bekannten Hitzeschockproteinen waren weitere Streßproteine (z.B. DpsA, GrpE und SodA), sowie zwei Regulatoren (LlrA und PurR) und einige nichtcharakterisierte (z.B. BmpA, YphC und YuiC) unter den Proteinen, die in höheren Mengen auftraten. Proteine des Purin-Metabolismus stellten eine der größten Gruppen dar, die unter Hitzeschock beeinflußt wurden. Dies stützt den Hinweis, daß Guanin-Nukleotid Reservoirs an der Regulation der Streßantwort in *L. lactis* beteiligt sind. Zwischen den zwei analytischen Verfahren (DIGE und Pulsmarkierung) wurden sowohl Gemeinsamkeiten als auch Unterschiede in der differentiellen Analyse festgestellt. Diese Ergebnisse zeigen den Unterschied der Hitzeschockantwort auf zeitabhängiger und gesamter Proteinebene, und die

Leistungsfähigkeit der neu verfügbaren DIGE Technik Streßproteine im Gesamtprotein nachzuweisen.

Die zelluläre Antwort auf die Anwendung von hydrostatischem Hochdruck wurde bei *Lactobacillus sanfranciscensis* und *L. lactis*, zwei Milchsäurebakterien mit Bedeutung für die Lebensmittelindustrie, untersucht. Hochdruck wird zur Konservierung von Lebensmitteln benutzt und es wurde bei einigen Organismen bereits gezeigt, daß dadurch eine Streßantwort hervorgerufen wird. Im Fall von *Lactobacillus sanfranciscensis* wurde die hochauflösende 2D Elektrophorese benutzt, um Proteinmuster von atmosphärisch gewachsenen mit denen von druckbehandelten Bakterien bei Druckanwendung bis zu 200 MPa für 1h zu vergleichen. Die Studie wurde mit Hilfe von überlappenden immobilisierten pH Gradienten (IPG) im pH Bereich von 2,5 bis 12 durchgeführt, um ein maximales Auflösungsvermögen zum Auffinden von streßrelevanten Proteinen zu erzielen. Zur verbesserten quantitativen Analyse wurde zusätzlich zur Silberfärbung auch die Fluoreszenzfärbung mit SYPRO RUBY™ eingesetzt. Die DIGE Technik war zum Zeitpunkt dieser Analyse noch nicht verfügbar. Mit Hilfe von computergestützter Bildanalyse wurden 13 Spots im pH Bereich von 3.5 bis 9 nachgewiesen, deren Intensität nach Hochdruckbehandlung mehr als zweifach erhöht oder um 50% verringert war. Davon wiesen zwei Spots (pI ≈ 4,0 und pI ≈ 4,2; Mr ≈ 15 kDa) fast identische MALDI-TOF MS Spektren auf. Durch Mikrosequenzierung mit Hilfe von LC-MS/MS und Homologie zu CSPE von *L. lactis* wurden beide als das gleiche Kälteschockprotein identifiziert. Da eines der beiden Proteine bei Hochdruckbehandlungen bis zu 150 MPa etwa im gleichen Verhältnis an Menge zunimmt wie das andere abnimmt, könnte es sich hierbei um ein Beispiel einer selektiven Proteinmodifikation handeln, die durch Hochdruck ausgelöst wird. Dieser Effekt ist wahrscheinlich nicht von einer Volumenreduktion abhängig, da er bei Drücken über 150 MPa wieder abnimmt.

Außerdem war bemerkenswert, daß durch die Analyse der Barosensitivität der Zellen im Abstand von 25 MPa Schritten, eine abgestufte Druckinduktion bzw. -repression der Proteine beobachtet werden konnte. So war z.B. eines der Kälteschockproteine nach 1h, 150 MPa maximal induziert, während ein anderes Protein (pI 7,5, Mr ≈ 25 kDa) nach 1h, 50/75 MPa maximal induziert war. Dies deutet auf eine stufenweise Zellantwort und somit unterschiedliche Ursachen auf

molekularer Ebene. Weitere durch LC-MS/MS identifizierte Proteine sind beteiligt an der Translation, dem Kohlenhydrat- und dem Purin-Metabolismus.

Die Streßantwort von *L. lactis* IL1403 auf Hochdruck wurde analysiert, um Proteine mit veränderter Expression während und nach der Hochdruckbehandlung zu identifizieren. Der Vergleich von Referenzen und Proben, die mit 60 bzw. 90 MPa behandelt wurden, erfolgte durch Analyse mit DIGE und ^{35}S-Met/Cys Pulsmarkierung. Wie in der Hitzeschockanalyse veranschaulicht DIGE die Gesamtproteinexpression und Pulsmarkierung die zeitweise Proteinexpression. Somit werden Pulsmarkierungsexperimente durch DIGE Analysen ergänzt.

Änderungen im Proteinmuster nach Hochdruckbehandlung wurden untersucht, um Proteine zu identifizieren, die nicht nur an der unmittelbaren Streßantwort beteiligt sind, sondern auch an der Erholungsphase der Bakterien. Dadurch könnten induzierte Proteine als Indikatoren für zelluläre Schäden dienen, die durch Hochdruck verursacht wurden. Tatsächlich wurden nach Hochdruckbehandlung bei 60 MPa erhöhte Mengen von Proteinen festgestellt, die beispielsweise an der Zellhüllsynthese beteiligt sind, z.B. UDP-N-acetylmuramate-alanine ligase (MurC) und D-Ala-D-Ala adding enzyme (MurF). Nach Behandlung bei 90 MPa wurden von MurC und MurF sogar noch höhere Mengen festgestellt. Dies deutet darauf hin, daß die Zellhülle wahrscheinlich bereits bei einem niedrigeren Hochdruck als 200 MPa geschädigt wird, und damit weit unterhalb des Druckbereichs, bei dem eine Zellinaktivierung stattfindet.

Außer MurC und MurF war die Expression weiterer Proteine beeinflußt, die mit vorherig beschriebenen Hochdruckeffekten auf Zellen, wie gehemmtes Wachstum (z.B. NrdEF, RecA, YnaB) oder Störung der Proteinsynthese (z.B. Era), im Zusammenhang stehen. Bemerkenswert war, daß genau wie unter Hitzeschock auch hier Proteine des Purin-Metabolismus zu einer der größten Gruppen gehörten, die durch Hochdruck beeinflußt wurden. Dies deutet einmal mehr auf einen Zusammenhang des Guanin-Nukleotid Reservoirs der Zellen und ihrer Streßantwort. Die Mechanismen von Druckeffekten auf molekularer Ebene wurden in bisherigen Studien im Allgemeinen nicht identifiziert. Das Verstehen der Zellantwort auf Hochdruck ist jedoch von großem Interesse, da bereits hochdruckresistente Stämme und Mutanten von z.B. *E. coli* in der Fachliteratur beschrieben wurden. In der vorliegenden Studie gelang es Proteine nachzuweisen, deren Menge nach

Hochdruckbehandlung individuell erhöht oder vermindert ist, und die durch Hochdruck wahrscheinlich posttranslational modifiziert werden. Die Art und Weise in der die identifizierten Proteine in zelluläre Netzwerke eingebunden sind, könnte nun Aufschluß über die molekularen Ursachen der schädigenden Wirkung von Hochdruck geben.

Das basische Proteom von *L. lactis* wurde nach unserem Wissensstand in bisherigen Studien nicht berücksichtigt. Daher wurden in dieser Studie verschiedene Parameter für die IEF optimiert und dabei mehrere elektrophoretische Systeme verglichen, um letztendlich eine Referenzkarte des basischen Proteoms von *L. lactis* zu erstellen. Mit dem optimierten Protokoll wurden 96 Spots bei Verwendung von IPG 6-12 und 57 Spots in einem weiteren Gradienten von 9-12 durch Peptide Mass Fingerprinting (MALDI-TOF MS) identifiziert. Die 153 identifizierten Spots repräsentieren insgesamt 85 unterschiedliche Proteine, die theoretische isoelektrische Punkte von 6,34 bis 11,31 aufweisen. Darunter befinden sich auch Proteine, die man als problematisch für die 2-DE Analyse betrachtet, nämlich hydrophobe, niedermolekulare oder schwach expremierte Proteine, wie z.B. NADH-dependent enoyl-ACP reductase (GRAVY: 0.156), Translation initiation factor IF-1 (8 kDa) oder Manganese ABC transporter ATP binding protein (CAI: 0.313).

Die gesamten *in silico* Daten des Proteoms von *L. lactis* ebenso wie interaktive Referenzkarten wurden in eine dafür eigens konstruierte Datenbank gefügt, welche unter www.wzw.tum.de/proteomik/lactis erreichbar ist. Die Proteomdatenbank für *L. lactis* wurde mit bestimmten Eigenschaften versehen, die in bisherigen 2D Datenbanken unzureichend oder nur eingeschränkt berücksichtigt wurden. Dazu zählen eine unproblematische Datenbankerweiterung, umfassende Suchfunktionen und Einbindung von vorberechneten, im Gebiet Proteomik oft verwendeten Proteineigenschaften, wie z.B. CAI oder GRAVY. Die integrierte Benutzeroberfläche der konstruierten Internetdatenbank unterstützt eine detaillierte Suche bezüglich mehrerer Proteineigenschaften über das experimentelle Proteom in Form von Referenzkarten und dem vollständigen *in silico* Proteom. Die Struktur der Datenbank ermöglicht außerdem Datenerweiterungen ohne die Kenntnis einer Programmiersprache.

7 References

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8 Table of figures

9 Appendix

Tab. 18: Proteins identified by peptide mass fingerprint with MALDI-TOF. Listed are experimental isoelectric point, molecular weight, sequence coverage and number of peptides, which resulted in the identification of proteins.

PID[a)]	Gene	Protein (EC number)	Theor. Mr	Theor. pI	Exp. Mr[b)]	Exp. pI[b)]	No. Pep.[c)]	Cov.[c)]	GRAVY	CAI
		AMINO-ACID BIOSYNTHESIS								
12724226	*aroH*	Trp-sensitive phospho-2-dehydro-deoxyheptonate aldolase (EC 4.1.2.15)	38842.7	5.08	36	5.05	22	55	-0.649	0.48
12723425	*cysM*	cysteine synthase (EC 4.2.99.8)	32358.02	5.76	32	5.75	5	24	0.022	0.486
12724564	*dapB*	dihydrodipicolinate reductase (EC 1.3.1.26)	28490.82	5.8	28	5.7	30	87	-0.052	0.448
12723496	*glyA*	serine hydroxymethyltransferase (EC 2.1.2.1)	44789.99	5.45	46	5.5	14	45	-0.105	0.541
12724918	*proC*	pyrroline-5-carboxylate reductase (EC 1.5.1.2)	27972.09	5.8	28	5.8	19	54	0.126	0.552
12723499	*serA*	D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	43637.68	5.97	40	5.95	5	13	-0.137	0.435
		BIOSYNTHESIS OF COFACTORS, PROSTHETIC GROUPS, AND CARRIERS								
12723642	*menB*	dihydroxynaphthonic acid synthase (EC 4.1.3.36)	30880.28	5.5	32	5.5	8	38	-0.221	0.604
12724655	*trxA*	thioredoxin	11672.41	4.32	12	4.3	11	73	-0.161	0.696
12723876	*trxB1*	thioredoxin reductase (EC 1.6.4.5)	33894.43	4.76	32	4.7	5	20	-0.226	0.619
		CELL ENVELOPE								
12723133	*acmA*	N-acetylmuramidase (EC 3.5.1.28)	46592.06	9.95	47	9.8	13	37	-0.325	0.475
12724428	*bmpA*	basic membrane protein A	36652.03	8.68	23	5.5	20	60	-0.298	0.68
12725334	*dacA*	D-alanyl-D-alanine carboxypeptidase	46993.31	6.96	47	6.8	13	27	-0.125	0.51
12723209	*ddl*	D-alanine-D-alanine ligase (EC 6.3.2.4)	38693.07	4.66	40	4.65	34	72	-0.108	0.534
12724321	*hasC*	UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	34873.02	5.5	34	5.65	9	54	-0.33	0.677
12725089	*murC*	UDP-N-acetylmuramate-alanine ligase (EC 6.3.2.8)	51740.1	5.14	53	5.25	7	28	-0.316	0.578
12723210	*murF*	D-Ala-D-Ala adding enzyme (EC 6.3.2.15)	48823.3	5.42	49	5.4	42	75	-0.164	0.495
12724594	*murG*	peptidoglycan synthesis protein MurG	39071.66	8.36	43	8.3	11	31	-0.211	0.481
12723074	*tagD1*	glycerol-3-phosphate cytidiltransferase	16837.38	7.85	14	7.8	5	35	-0.596	0.367
		CELL DIVISION								
12723912	*ftsE*	cell-division ATP-binding protein FtsE	25799.76	9.45	26	9.35	21	64	-0.33	0.452
12722941	*parA*	chromosome partitioning protein	29803.66	8.97	27	8.9	13	49	-0.41	0.404
		ENERGY METABOLISM								
12725215	*adhE*	alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)	98220.83	5.71	100	5.7	11	17	-0.178	0.685

PID[a)]	Gene	Protein (EC number)	Theor. Mr	Theor. pI	Exp. Mr[b)]	Exp. pI[b)]	No. Pep.[c)]	Cov.[c)]	GRAVY	CAI
12725086	*arcA*	arginine deiminase (EC 3.5.3.6)	46043.16	5.15	27	5.15	10	56	-0.383	0.665
12724785	*atpE*	ATP synthase epsilon subunit (EC 3.6.1.34)	15669.77	5.73	15	5.7	6	53	-0.334	0.496
12724787	*atpG*	ATP synthase gamma subunit (EC 3.6.1.34)	31997.39	6.34	33	6.4	21	60	-0.338	0.567
12724157	*citE*	citrate lyase beta chain (EC 4.1.3.6)	33307.51	4.9	33	4.9	36	69	0.001	0.39
12723548	*enoA*	enolase (EC 4.2.1.11)	46911.77	4.68	47	4.7	11	33	-0.235	0.85
12724945	*fbaA*	fructose-bisphosphate aldolase (EC 4.1.2.13)	31989.56	5.04	31	5.05	8	30	-0.004	0.829
12724085	*frdC*	fumarate reductase flavoprotein subunit (EC 1.3.99.1)	52852.69	8.77	53	7.5	9	24	-0.325	0.555
12725023	*galE*	UDP-glucose 4-epimerase (EC 5.1.3.2)	36229.98	5.18	34	5.15	28	74	-0.34	0.55
12725315	*gapB*	glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	35819.46	5.57	36	5.6	8	33	-0.11	0.897
12724312	*ldh*	L-lactate dehydrogenase (EC 1.1.1.27)	35050.69	4.97	34	4.95	11	27	-0.018	0.804
12724153	*mae*	malate oxidoreductase (EC 1.1.1.38)	40475.63	5.21	38	5.15	6	32	0.114	0.475
12722901	*pdhC*	dihydrolipoamide acetyltransferase component of PDH complex (EC 2.3.1.12)	56316.88	4.95	80	4.9	29	45	-0.147	0.651
12722900	*pdhD*	lipoamide dehydrogenase component of PDH complex (EC 1.8.1.4)	49866.82	4.9	53	4.85	10	30	0.039	0.644
12724314	*pfk*	6-phosphofructokinase (EC 2.7.1.11)	35805.61	5.68	32	5.65	7	24	-0.027	0.704
12723104	*pgk*	phosphoglycerate kinase (EC 2.7.2.3)	42070.09	5.06	40	5	20	73	-0.016	0.821
12723203	*pmg*	phosphoglycerate mutase (EC 5.4.2.1)	26329.75	5.3	26	5.35	11	61	-0.455	0.843
12724313	*pyk*	pyruvate kinase (EC 2.7.1.40)	54254.84	5.27	54	5.25	13	32	-0.172	0.816
12724634	*tkt*	transketolase (EC 2.2.1.1)	71725.86	5.06	75	5	31	43	-0.241	0.59
12724096	*tpiA*	triosephosphate isomerase (EC 5.3.1.1)	26905.42	4.63	26	4.6	11	72	-0.041	0.816
12722917	*yahI*	short-chain type dehydrogenase	30819.25	9.28	30	9.2	20	58	-0.269	0.551
12723032	*ybiE*	oxidoreductase	23260.49	5.8	22	5.8	9	48	-0.465	0.515
12724215	*ymgK*	oxidoreductase	32156.52	5.88	32	5.9	15	62	-0.511	0.35
12724529	*yphC*	oxidoreductase	31519.72	5.78	31	5.75	8	46	-0.427	0.433
12725003	*yudI*	oxidoreductase	36596.24	6.53	34	6.55	23	66	-0.156	0.487
		FATTY ACID AND PHOSPHOLIPID METABOLISM								
12723698	*accD*	acetyl-CoA carboxylase carboxyl transferase subunit betta (EC 6.4.1.2)	31769.89	8.54	32	8.7	8	26	-0.086	0.471
12722960	*acpD*	acyl carrier protein phosphodiesterase	24662.79	5.28	23	5.2	32	69	-0.517	0.525
12723693	*fabG1*	3-oxoacyl-acyl carrier protein reductase (EC 1.1.1.100)	25598.5	5.72	26	5.7	6	33	0.106	0.609
12723453	*fabI*	NADH-dependent enoyl-ACP reductase	26382.42	6.44	27	6.5	6	34	0.156	0.695
12723452	*fabZ1*	hydroxymyristoyl-acyl carrier protein dehydratase	16796.42	5.51	16	5.5	17	64	-0.119	0.7
12722912	*plsX*	fatty acid/phospholipid synthesis protein	34786.01	5.72	35	5.75	27	67	0.061	0.526
12723278	*yeaG*	mevalonate kinase	34334.27	6.6	34	6.75	11	22	-0.165	0.512

PID[a)]	Gene	Protein (EC number)	Theor. Mr	Theor. pI	Exp. Mr[b)]	Exp. pI[b)]	No. Pep.[c)]	Cov.[c)]	GRAVY	CAI
		PROPHAGES								
12723315	*pi102*	prophage pi1 protein 02	21661.95	9.56	21	9.55	21	77	-0.598	0.499
12723957	*pi202*	prophage pi2 protein 02	20505.88	8.91	20	8.9	18	67	-0.202	0.372
		PURINES, PYRIMIDINES, NUCLEOSIDES AND NUCLEOTIDES								
12724720	*cmk*	cytidine monophosphate kinase (EC 2.7.4.14)	24548.3	5.36	24	5.3	21	81	-0.28	0.539
12723866	*deoD*	purine nucleoside phosphorylase (EC 2.4.2.1)	25427.15	5.15	22	5.1	8	49	0.035	0.644
12724481	*guaA*	GMP synthase (EC 6.3.5.2)	56780.71	4.86	54	4.8	45	64	-0.13	0.654
12723077	*guaB*	IMP dehydrogenase (EC 1.1.1.205)	52826.54	5.95	53	6	22	67	-0.058	0.62
12724108	*guaC*	GMP reductase (EC 1.6.6.8)	35837.21	6.52	35	6.6	27	66	-0.106	0.598
12723918	*nrdE*	ribonucleoside-diphosphate reductase alpha chain (EC 1.17.4.1)	81495.45	5.22	80	5.2	19	33	-0.293	0.603
12723917	*nrdF*	ribonucleoside-diphosphate reductase beta chain (EC 1.17.4.1)	37615.07	4.45	34	4.45	9	44	-0.394	0.611
12723919	*nrdI*	ribonucleotide reductase	15660.84	4.8	18	4.9	9	32	-0.16	0.469
12724888	*prsB*	ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	35278.25	5.34	34	5.35	5	28	0.013	0.573
12724993	*purA*	adenylosuccinate synthase (EC 6.3.4.4)	47325.72	5.41	47	5.4	11	30	-0.232	0.595
12724651	*purB*	adenylosuccinate lyase (EC 4.3.2.2)	49687.41	5.37	48	5.35	46	79	-0.438	0.568
12724509	*purK*	phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21)	39159.49	5.22	38	5.25	8	31	-0.255	0.472
12724522	*purN*	phosphoribosylglycinamide formyltransferase (EC 2.1.2.2)	20482.26	5.32	20	5.35	7	45	-0.175	0.411
12724608	*pyrB*	aspartate carbamoyltransferase (EC 2.1.3.2)	34558.26	5.52	33	5.6	15	50	-0.225	0.496
12723372	*pyrG*	CTP synthetase	59552.1	5.57	62	5.7	16	30	-0.249	0.65
12725056	*pyrH*	UMP-kinase (EC 2.7.4.-)	25677.42	5.25	23	5.1	5	27	-0.045	0.551
12723048	*rmlA*	glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24)	32130.68	4.66	32	4.65	5	21	-0.211	0.606
12724952	*upp*	uracil phosphoribosyltransferase (EC 2.4.2.9)	23230.28	6.54	23	6.55	7	31	0.007	0.64
12724109	*xpt*	xanthine phosphoribosyltransferase (EC 2.4.2.-)	21806.28	5.3	22	5.3	28	76	-0.017	0.566
		REGULATORY FUNCTIONS								
12725088	*argR*	arginine catabolic regulator	16893.49	7.76	14	7.6	13	76	-0.057	0.335
12723011	*codY*	transcriptional regulator	29128.45	4.79	27	4.8	10	46	-0.068	0.627
12723220	*eraL*	GTP-binding protein Era	34737.04	5.88	34	5.85	33	55	-0.409	0.556
12724600	*llrA*	two-component system regulator	26684.36	5.32	26	5.25	25	75	-0.492	0.559
12725330	*purR*	regulator of purine biosynthetic genes	30360.87	5.49	31	5.5	28	77	-0.09	0.459
12724611	*pyrR*	pyrimidine operon regulator	19830.79	5.26	22	5.3	5	35	-0.421	0.606
12724426	*rgrB*	GntR family transcriptional regulator	26731.61	9.31	25	9.45	24	78	-0.348	0.427
12724690	*rliA*	transcriptional regulator	36360.74	6.73	36	6.85	16	33	-0.26	0.421
12724246	*ynaB*	transcriptional regulator (HdiR)	28894.04	4.6	30	4.65	13	59	-0.689	0.447

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12723078	*yqeL*	GTP-binding protein	42247.53	6.43	43	5.75	8	31	-0.201	0.5
12724115	*ysxL*	GTP-binding protein	22550.73	8.58	22	8.6	5	29	-0.519	0.504
12725171	*zitR*	zinc transport transcriptional regulator	16366.72	5.56	13	5.6	18	71	-0.365	0.324
		REPLICATION								
12725291	*dnaJ*	DnaJ protein	40671.28	6.73	41	6.7	25	54	-0.682	0.551
12723557	*hsdS*	type I restriction enzyme specificity protein (EC 3.1.21.3)	47068.62	8.28	47	8.3	25	45	-0.604	0.411
12723384	*hslA*	HU like DNA-binding protein	9676.15	9.52	9	9.6	6	57	-0.27	0.854
		STRESS RELATED PROTEINS								
12724524	*clpB*	ClpB protein	97335.08	5.09	95	5.05	52	60	-0.37	0.551
12723443	*clpE*	ATP-dependent protease ATP-binding subunit	83144.86	5.02	85	4.95	5	7	-0.491	0.554
12723580	*clpP*	ATP-dependent Clp protease proteolytic subunit	22082.23	4.98	21	4.85	8	23	-0.23	0.644
12723891	*dnaK*	DnaK protein	64987.16	4.62	65	4.55	19	49	-0.342	0.788
12725073	*dpsA*	non-heme iron-binding ferritin	16652.83	4.69	165	4.65	6	40	-0.297	0.683
12724901	*ftsZ*	cell division protein FtsZ	43988.22	4.54	46	4.5	8	33	-0.216	0.709
12724202	*gidC*	glucose inhibited division protein GidC	49991.91	5.43	51	5.4	25	55	-0.343	0.49
12723267	*groEL*	60 KD chaperonin	57201.42	4.75	58	4.7	8	23	-0.006	0.677
12723266	*groES*	10 KD chaperonin	10220.82	5.03	10	5	5	63	-0.036	0.473
12723890	*grpE*	stress responce protein GrpE	20580.95	4.43	22	4.45	12	40	-0.645	0.597
12723223	*recA*	RecA protein	41477.3	5.39	40	5.45	7	28	-0.252	0.647
12723282	*sodA*	superoxide dismutase	23253.88	5.03	22	4.95	6	42	-0.446	0.711
12723436	*tig*	trigger factor	46930.55	4.43	55	4.4	6	28	-0.401	0.8
		TRANSCRIPTION								
12725158	*nusG*	transcription antitermination protein	21110.02	4.55	22	4.5	10	47	-0.243	0.684
12724251	*rluB*	pseudouridine synthase	28722.13	9.71	26	9.55	27	68	-0.479	0.469
12723945	*rluD*	pseudouridine synthase	33587.51	6.65	40	6.8	13	42	-0.355	0.497
12725328	*rnhA*	ribonuclease HII (EC 3.1.26.4)	32205.11	9.06	33	9	13	56	-0.147	0.435
		TRANSLATION								
12723447	*def*	polypeptide deformylase	23769.28	5.03	24	5	14	61	-0.337	0.642
12723014	*gatA*	Glu-tRNA amidotransferase subunit A	52071.24	5.51	53	5.55	17	44	-0.119	0.61
12723016	*gatB*	Glu-tRNA amidotransferase subunit B	54730.99	5.32	53	5.2	6	20	-0.451	0.517
12725124	*infA*	translation initiation factor IF-1	8195.5	8.03	9	8.2	5	25	-0.267	0.405
12724874	*infC*	translation initiation factor IF-3	18691.99	9.83	20	9.9	6	31	-0.473	0.559
12724911	*pepC*	aminopeptidase C	49914.1	4.74	49	4.7	35	54	-0.48	0.639

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12723768	*pepV*	dipeptidase	51943.44	4.75	53	4.7	6	20	-0.301	0.729
12725046	*rplA*	50S ribosomal protein L1	24049.75	9.34	25	9.35	11	39	-0.01	0.784
12725153	*rplC*	50S ribosomal protein L3	21932.53	10.26	26	10.25	6	22	-0.293	0.692
12725152	*rplD*	50S ribosomal protein L4	22305.44	9.94	23	9.95	5	25	-0.208	0.722
12725140	*rplE*	50S ribosomal protein L5	20006.26	9.22	20	9.2	12	66	-0.178	0.8
12725136	*rplF*	50S ribosomal protein L6	19257.22	9.69	19	9.7	11	56	-0.28	0.758
12723661	*rplI*	50S ribosomal protein L9	16375.32	9.65	16	9.7	5	39	-0.281	0.648
12725047	*rplK*	50S ribosomal protein L11	14705.24	9.49	13	9.5	5	33	0.036	0.82
12725324	*rplM*	50S ribosomal protein L13	16194.84	9.82	16	9.8	10	45	-0.256	0.762
12725143	*rplN*	50S ribosomal protein L14	12922.08	10.11	12	10.15	6	34	-0.076	0.68
12725119	*rplQ*	50S ribosomal protein L17	14255.45	10.04	14	10.05	6	47	-0.543	0.792
12725135	*rplR*	50S ribosomal protein L18	12390.15	10.11	11	10.1	5	43	-0.377	0.74
12723803	*rplS*	50S ribosomal protein L19	14405.72	10.56	12.5	10.7	7	36	-0.566	0.728
12724034	*rplU*	50S ribosomal protein L21	11437.21	9.66	12.5	9.5	5	39	-0.437	0.782
12725148	*rplV*	50S ribosomal protein L22	12469.53	10.38	12.5	10.3	12	69	-0.132	0.71
12725151	*rplW*	50S ribosomal protein L23	10743.51	9.46	11	9.4	10	61	-0.231	0.724
12725141	*rplX*	50S ribosomal protein L24	10876.75	9.84	12	10.3	15	52	-0.279	0.757
12725145	*rpmC*	50S ribosomal protein L29	7859.16	9.4	8	9.45	5	42	-0.517	0.711
12725133	*rpmD*	50S ribosomal protein L30	6193.4	10.29	8	10.2	5	47	0.261	0.723
12724602	*rpmE*	50S ribosomal protein L31	9336.48	9.3	9.5	9.3	5	57	-0.884	0.784
12725213	*rpsB*	30S ribosomal protein S2	28538.73	5.08	32	5.1	6	29	-0.281	0.83
12725147	*rpsC*	30S ribosomal protein S3	24033.74	9.52	24	9.55	14	61	-0.343	0.744
12723147	*rpsD*	30S ribosomal protein S4	23164.56	10.07	23	10.1	11	67	-0.51	0.846
12725134	*rpsE*	30S ribosomal protein S5	17595.27	10.2	17	10.2	7	38	-0.046	0.697
12725332	*rpsG*	30S ribosomal protein S7	17683.39	10.35	16	10.4	7	40	-0.511	0.794
12725137	*rpsH*	30S ribosomal protein S8	14685.11	9.55	13.5	9.5	6	34	-0.158	0.817
12725323	*rpsI*	30S ribosomal protein S9	14098.24	11.31	14	11.2	5	20	-0.459	0.671
12725154	*rpsJ*	30S ribosomal protein S10	11741.72	9.74	10.5	9.75	6	39	-0.5	0.721
12724920	*rpsO*	30S ribosomal protein S15	10342.97	10.19	10	10.2	5	46	-0.849	0.864
12725144	*rpsQ*	30S ribosomal protein S17	10143.77	9.88	11	9.9	7	37	-0.843	0.709
12725149	*rpsS*	30S ribosomal protein S19	10570.21	10.19	10.5	10.3	16	70	-0.699	0.635
12724905	*trmH*	tRNA-guanosine methyltransferase (EC 2.1.1.34)	26514.55	7.07	26	7.1	21	78	-0.086	0.447
12723367	*truA*	tRNA pseudouridine synthase A (EC 4.2.1.70)	29144.98	8.83	28	8.8	24	84	-0.597	0.396

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12725212	*tsf*	elongation factor Ts	36669.66	4.92	37	4.9	8	43	-0.157	0.758
12724893	*tuf*	elongation factor Tu	43211.91	4.89	45	4.9	12	49	-0.245	0.809
		TRANSPORT AND BINDING PROTEINS								
12724782	*glnQ*	glutamine ABC transporter ATP-binding protein	27071.34	5.19	25	5.15	26	63	-0.09	0.623
12724297	*mtsB*	manganese ABC transporter ATP binding protein	26754.04	6.97	26	6.9	6	27	-0.134	0.313
12723212	*optA*	oligopeptide ABC transporter substrate binding protein	59697.78	8.54	58	7.4	26	46	-0.532	0.648
12723216	*optF*	oligopeptide ABC transporter ATP binding protein	35014.99	6.04	33	6	10	48	-0.36	0.527
12724723	*ptnAB*	mannose-specific PTS system component IIAB (EC 2.7.1.69)	35064.49	5.14	35	5.1	5	23	0.022	0.611
12722964	*ptsH*	phosphocarrier protein HPr	9179.45	5.05	11	4.9	5	57	0.13	0.819
12722914	*yahG*	ABC transporter ATP binding protein	61381.42	4.71	62	4.7	20	50	-0.323	0.632
12723113	*ycfB*	ABC transporter ATP binding protein	27287.6	7.67	12	4.75	6	26	-0.24	0.353
12724308	*yngB*	fibronectin-binding protein	61285.01	8.68	61	8.7	44	58	-0.466	0.519
12725169	*zitQ*	zinc ABC transporter ATP binding protein	27961.67	6.84	28	6.9	14	37	-0.637	0.416
		UNKNOWN								
12722978	*ybdD*	HYPOTHETICAL PROTEIN	33748.1	6.4	34	6.4	8	29	-0.464	0.526
12723005	*ybfE*	HYPOTHETICAL PROTEIN	16615.02	5.25	16	5.25	13	75	-0.208	0.508
12723116	*ycfD*	HYPOTHETICAL PROTEIN	43489.67	8.59	43	8.55	20	43	-0.209	0.476
12723118	*ycfF*	HYPOTHETICAL PROTEIN	45553.39	9.06	46	9.1	22	40	-0.031	0.467
12723142	*ychG*	HYPOTHETICAL PROTEIN	23316.58	4.55	23	4.55	14	50	-0.229	0.486
12723143	*ychH*	acetyltransferase	26776.81	5.34	27	5.3	5	20	0.161	0.459
12723148	*yciC*	HYPOTETICAL PROTEIN	78771.34	5.63	80	5.5	28	43	-0.242	0.504
12723162	*ycjH*	UNKNOWN PROTEIN	29639.17	6.86	28	6.8	6	24	-0.19	0.424
12723256	*ydiG*	UNKNOWN PROTEIN	18216.33	9.1	17	9.1	23	76	-0.826	0.577
12723370	*yeiF*	HYPOTHETICAL PROTEIN	19451.5	6.44	6.5	20	5	37	0.037	0.454
12723433	*yfdE*	conserved hypothetical protein	29070.21	5.14	28	5	12	53	-0.187	0.346
12723442	*yffA*	UNKNOWN PROTEIN	13221.24	9.63	12	9.55	11	72	-0.677	0.424
12723518	*ygaJ*	UNKNOWN PROTEIN	20659.97	4.49	20	4.5	11	52	-0.667	0.619
12723547	*ygdA*	HYPOTHETICAL PROTEIN	21282.95	5.36	23	5.35	5	31	-0.656	0.616
12723663	*yhfC*	HYPOTHETICAL PROTEIN	9834.1	6.4	9.5	6.5	12	94	-0.627	0.657
12723708	*yhjF*	HYPOTHETICAL PROTEIN	13571.73	8.98	12.5	9.1	12	86	-0.599	0.442
12723794	*yiiH*	HYPOTHETICAL PROTEIN	36109.4	7.12	37	7.7	21	57	-0.475	0.445
12723869	*yjfJ*	conserved hypothetical protein	11941.49	5.84	11	5.75	8	47	-0.281	0.455
12723900	*yjiF*	conserved hypothetical protein	35697.64	5.21	34	5.25	31	74	-0.002	0.453

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12724014	*ykhD*	HYPOTHETICAL PROTEIN	20115.95	9.34	24	9.25	5	37	-0.461	0.436
12724030	*ykiF*	conserved hypothetical protein	30048.58	4.99	30	5	5	23	-0.14	0.533
12724144	*yljE*	conserved hypothetical protein	52315.42	6.88	51	7.1	7	20	-0.175	0.49
12724433	*yogG*	conserved hypothetical protein	22190.23	6.43	21	6.65	9	32	-0.298	0.391
12724662	*yqjE*	HYPOTHETICAL PROTEIN	43288.12	9.27	43	9.2	15	32	-0.258	0.473
12724665	*yraB*	HYPOTHETICAL PROTEIN	14979.72	4.71	28	4.9	15	72	-0.98	0.56
12724703	*yreC*	UNKNOWN PROTEIN	21670.31	9.14	21	4.4	5	49	-0.274	0.378
12724862	*ytaA*	conserved hypothetical protein	15700.1	5.2	16	5.1	16	96	0.114	0.698
12724892	*ytcE*	UNKNOWN PROTEIN	13154.27	9.13	11	9.15	18	96	-0.175	0.617
12724933	*ytgE*	UNKNOWN PROTEIN	13434.47	6.78	13	6.75	11	68	-0.88	0.672
12724936	*ytgG*	HYPOTHETICAL PROTEIN	26545.46	5.02	27	5.05	8	53	-0.192	0.465
12724949	*ytjA*	UNKNOWN PROTEIN	12180.11	9.52	10.5	9.5	13	82	-0.434	0.502
12724959	*ytjH*	HYPOTHETICAL PROTEIN	27295.82	5.85	32	5.15	5	23	-0.584	0.515
12725017	*yufA*	HYPOTHETICAL PROTEIN	13599.65	9.05	13.5	9	10	68	-0.692	0.614
12725053	*yuiC*	HYPOTHETICAL PROTEIN	32312.76	5.78	32	5.8	19	58	-0.486	0.517
12725091	*yvcA*	UNKNOWN PROTEIN	22109.95	9.9	23	9.95	7	36	-1.048	0.374
12725101	*yvdE*	HYPOTHETICAL PROTEIN	26511.12	5.41	27	5.4	8	41	-0.197	0.428
12725108	*yveC*	UNKNOWN PROTEIN	18490.78	4.56	18	4.55	13	72	-0.341	0.488
12725214	*ywcC*	conserved hypothetical protein	37287.83	5.74	37	5.8	5	28	-0.313	0.538
12725316	*yxdB*	HYPOTHETICAL PROTEIN	19508.19	5.43	20	5.65	17	78	-0.672	0.411

a) PID, identifier according to GenBank (NCBI)
b) Experimental Mr and pI as derived from 2D gels
c) Number of peptides and sequence coverage determined with peptide mass fingerprinting (MALDI-TOF-MS)