Microbial Proteomics

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Academic Dissertation

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I don’t have any solution,
but I certainly admire the problem.
-Asleigh Brilliant

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Abstract

Proteomics, the study of the proteome, was first introduced in the 1990s, referring to the protein complement expressed by the genome or by the cell or tissue at a certain time. Proteomics provides detailed descriptions of the structure and function of biological systems in different biological conditions. Although proteomics as a field is relatively new, many methodologies in proteomics have been under development for decades. Proteome analysis is often accomplished by a combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Different 2-DE-based methodologies are used for protein separation and visualization, whereas protein identifications are performed by mass spectrometric methods.

A proteomic experiment often aims to analyse as many proteins as possible in a proteome and thus usually consists of detecting up to several thousands of proteins. The separation of proteins and peptides is therefore a key element in proteomic analyses. As 2-DE has been a core component of the work presented here, it is important to specifically highlight the advances in gel electrophoretic separation methods, such as difference gel electrophoresis (DIGE), which completes traditional 2-DE with superior sensitivity, dynamic range and quantitation. The development of MS-based proteomics, including the instrumentation and methods for data acquisition, has dramatically enhanced the sensitivity and throughput of protein identification. The sensitivity of current instruments has now reached levels that allow the identification of virtually any protein visible in a 2-DE gel.

In this thesis, proteomic methods were set up and adapted for baker's yeast, *Saccharomyces cerevisiae,* the probiotic bacterium *Lactobacillus gasseri* and for the mastitis pathogen *Streptococcus uberis*.

Increasing concern about global climate warming has accelerated the development of alternative energy sources. Bioethanol production from cellulosic biomass by fermentation with baker's yeast *S. cerevisiae* is one of the most studied areas in this field. D-xylose is abundant in the biomass, thus the metabolic engineering of *S. cerevisiae* for utilisation of pentose sugars is of particular interest. In the first part of this thesis, in studies I and II, 2-DE separation and mass spectrometric protein identification techniques were applied for recombinant xylose-fermenting *S. cerevisiae*. Yeast proteins were identified by peptide mass fingerprinting (PMF) and peptide sequence tag analysis. For PMF, peptides from proteins of interest were analysed by MALDI-TOF MS. Peptide sequence tag analysis was carried out by ESI-MS/MS, after sample concentration in nano flow-rate liquid chromatography.

In the second part of this work, proteomic methods were applied to investigate stress responses of bacteria. In study III, 2-D DIGE was adopted and protein identification with PMF was applied to study the overall heat shock response of a probiotic bacterium *L. gasseri*. Probiotics are microbes that are known to confer health benefits in the host. The use of food products containing probiotic microorganisms is of increasing economic importance and during the manufacturing processes and digestion these microorganisms are exposed to technological and digestive stresses. The study of heat shock response of *L. gasseri* revealed, in addition to classical heat shock proteins, an increased abundance of four Clp ATPases. Clp ATPases are a family of stress proteins that are known as regu-

lators of several biological processes and virulence factors in a number of pathogenic bacteria. We also showed that a functional *clpL* gene is essential for the development of constitutive and induced thermotolerance in *L. gasseri.*

In study IV, previously adapted 2-D DIGE and PMF-based protein identification methods were applied to investigate the global changes in the proteomes of *S. uberis* in response to mutagenesis-inducing ciprofloxacin challenge, and to elucidate the mechanisms by which resistance to ciprofloxacin is developed. *S. uberis* is an environmental mastitis pathogen and it is known that the fluoroquinolone antibiotic ciprofloxacin induces a mutagenic response in *S. uberis*. This proteomic study revealed activation of the oxidative damage response, reduction in NADH generation and changes in the pool of deoxyribonucleotides, potentially providing *S. uberis* time to stimulate mutagenesis and adapt to changes in its environment.

In proteomic studies, developments and improvements in MS-based techniques have revolutionized the identification of proteins. Instrumentation, software and data management capabilities enable proteomics to be widely applied in biological research as well as in the medical and food industry. However, the identification of the proteins present in a sample is only the first step in the process of understanding their functions. Thus, a successful model of protein function and regulation pathways in the cell requires a broad understanding of protein interaction with other proteins and a comprehensive understanding of cellular metabolism.

List of abbreviations

ATCC	American type culture collection
1-DE	One-dimensional gel electrophoresis
2-DE	Two-dimensional gel electrophoresis
DIGE	Difference gel electrophoresis
CBB	Coomassie brilliant blue
CF	Ciprofloxacin
CI	Chemical ionization
CID	Collision induced dissociation
CIRCE	Controlling inverted repeat of chaperone expression
DNA	Deoxyribonucleic acid
EMSA	Electrophoretic mobile shift assay
ESI	Electrospray ionization
FAB	Fast atom bombardment
FTICR	Fourier transform ionization cyclotron resonance
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
IAA	Iodoacetamide
ICAT	Isotope coded affinity tag
IEF	Isoelectric focusing
ITRAQ	Isotope coded tag for relative and absolute quantification
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry / Mass spectrometer
MS/MS	Tandem mass spectrometry
ORF	Open reading frame
PD	Plasma desorption
PMF	Peptide mass fingerprinting
PSD	Post source decay
pI	Isoelectric point
RNA	Ribonucleic acid
TCA	Tricarboxylic acid cycle, citric acid cycle
TOF	Time-of-flight
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SILAC	Stable isotope labelling with amino acids in cell culture

List of publications

This thesis is based on the following original articles, which are referred to in the text by the Roman numerals I-IV.

I. Poutanen M, Salusjärvi L, Ruohonen L, Penttilä M and Kalkkinen N. 2001. Use of matrix-assisted laser desorption/ionization time-of-flight mass mapping and nanospray liquid chromatography/electrospray ionization tandem mass spectrometry sequence tag analysis for high sensitivity identification of yeast proteins separated by two-dimensional gel electrophoresis.
Rapid Communications in Mass Spectrometry. 15(18):1685-1692.

II. Salusjarvi L, Poutanen M, Pitkanen J-P, Koivistoinen H, Aristidou A, Kalkkinen N, Ruohonen L and Penttilä M. 2003. Proteome analysis of recombinant xylose-fermenting *Saccharomyces cerevisiae*.
Yeast. 20:295-314.

III. Suokko A, Poutanen M, Savijoki K, Kalkkinen N and Varmanen P. 2008. ClpL is essential for induction of thermotolerance and is potentially part of the HrcA regulon in *Lactobacillus gasseri*.
Proteomics. 8(5):1029-41.

IV. Poutanen M, Varhimo E, Kalkkinen N, Sukura A, Varmanen P and Savijoki K. 2008. Two-dimensional difference gel electrophoresis analysis of *Streptococcus uberis* in response to mutagenesis-inducing ciprofloxacin challenge.
Submitted to *Journal of Proteome Research.*

1. Introduction

Proteomics, the study of a proteome, is the systematic and simultaneous study of the many and diverse properties of proteins. It aims to provide detailed descriptions of the structure and function of biological systems in different biological conditions. A proteome is the entire complement of proteins expressed by a genome. Proteome analysis is most often accomplished by a combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). With 2-DE the complex and variable protein mixture is separated and visualized and MS is applied to identify the proteins of interest. In this thesis, proteomics is used to study yeast metabolism, the stress responses of a probiotic bacterium and the potential mutagenesis mechanism of a pathogenic bacterium.

In 2-DE, proteins are separated in a polyacrylamide gel matrix in two dimensions, first according to their isoelectric point (charge) and then based on their molecular weight. The basis of 2-DE was already presented decades ago, but the methods for sensitive protein identification took time to develop. MS measures the molecular masses of charged molecules (analytes), which in proteomics are usually peptides and less frequently charged proteins. MS measures the mass-to-charge ratios of charged molecules, producing mass spectra that essentially provide mass information on all of the ionisable components in a sample.

The availability of complete genome sequences has made it possible to study the expression of all the genes at once, where before only one gene could be studied at a time. Baker's yeast, *Saccharomyces cerevisiae*, is one of the simplest eukaryotes and an effective model system for understanding basic cellular processes (Kumar and Snyder 2001). The yeast genome was the first eukaryotic genome for which the genome sequence was reported (Goffeau *et al.,* 1996). Yeast-based functional genomics and proteomics technologies have contributed greatly to our understanding of bacterial, yeast and human gene functions (Brown and Botstein 1999, Dolinski and Botstein 2005, Hartwell *et al.,* 1999, Botstein and Fink 1988).

Systems biology is the integrated study of a functional organism. Instead of focusing on individual components, such as on genes, proteins and biochemical reactions, systems biology focuses on all the components and the interactions between them. In the future, *S. cerevisiae* will continue to have a key role as an important model organism in the development of many current and future proteomics technologies and systems biology (Hartwell *et al.,* 1999, Ideker *et al.,* 2001).

Due to the global concern about climate warming, alternatives to petroleum-derived fuels are being developed, bioethanol being the most common replacement product (Gray *et al.,* 2006). Current ethanol production processes using crops such as sugar cane and corn are well established (Torney 2007, Wyman 2007). However, cheaper substrates such as lignocelluloses, could make bioethanol more competitive with fossil fuels (Zaldivar *et al.,* 2001). One important requirement for this to happen is the ability of a micro-organisms to efficiently ferment a variety of sugars (pentoses and hexoses) and to tolerate stressful conditions. Bioethanol production from cellulosic biomass by fermentation with *S. cerevisiae* is one of the most studied areas (Cardona and Sanchez 2007, Chu and Lee 2007, Hahn-Hagerdal *et al.,* 2007). A significant portion of the hemi-

cellulose fraction of biomass is comprised of xylans, making its constituent D-xylose the second most abundant sugar in nature. Because *S. cerevisiae* does not naturally utilize xylose or other pentose sugars, the introduction of an active xylose utilization pathway into *S. cerevisiae* is likely to have a major effect on cellular metabolism.

Probiotics are microbes that are potentially beneficial to the host and food products containing probiotic microorganisms are of increasing economic importance. However, during the manufacturing processes and digestion these microorganisms are exposed to technological and digestive stresses, such as bile and digestion enzymes (Corcoran *et al.,* 2008). These exposures influence the regulated expression of stress-related proteins, such as caseino lytic proteins (Clps), which are also important in various cellular processes during normal physiological conditions (Frees *et al.,* 2007). *Lactobacillus gasseri* is a probiotic, Gram-positive bacterium, whose recently published genome sequence suggests the presence of several Clp proteins from the ClpATPase family of proteins. ATPases are known to be essential in different stress responses in numerous Gram-positive bacteria functioning as proteases and as chaperones (Frees *et al.,* 2007).

Streptococcus uberis is an environmental mastitis pathogen (Hill 1988, Leigh 1999). Mastitis is the most common and costly disease of dairy cows and the most common reason for antibiotic treatments. The use of antimicrobials against

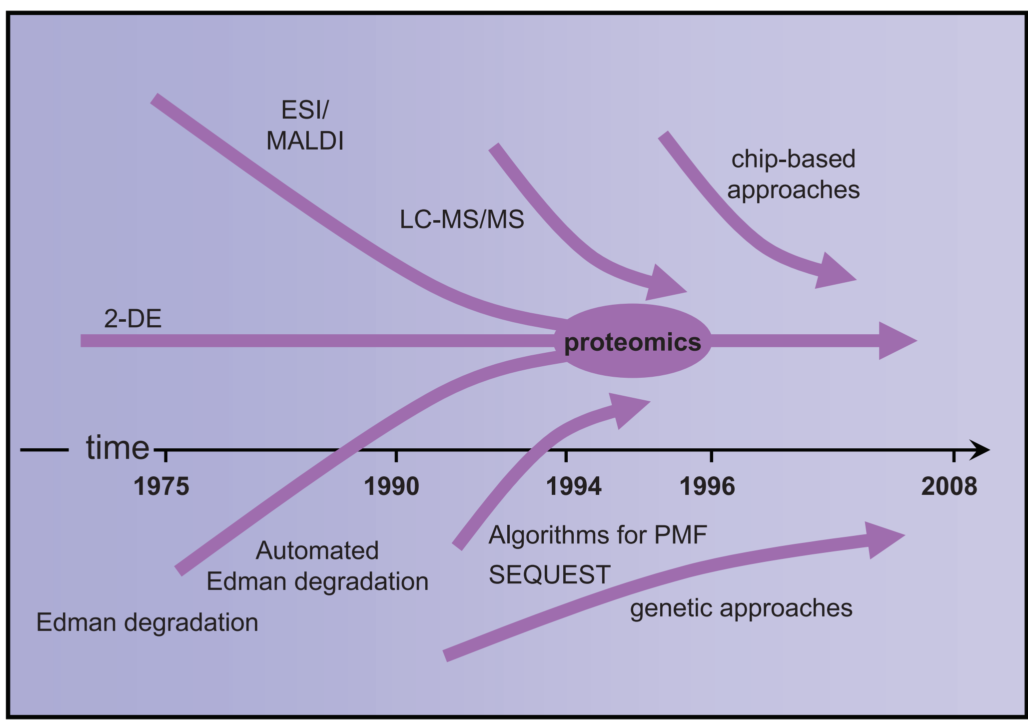

Figure 1. *Time-line indicating the convergence of different technologies and resources into the proteomic process. Advances in mass spectrometry and the generation of large quantities of nucleotide sequence information, combined with computational algorithms that could correlate the two, led to emergence of proteomics as a field. The figure is adapted from Patterson & Aebersold (2003).*

human and veterinary diseases has been extensive during recent decades. Thus, pathogenic bacteria are being repeatedly exposed to antimicrobials, which is likely to induce a variety of survival strategies to enhance viability and successful virulence. It has been suggested that *S. uberis* is able to adapt to antibiotic treatment (Milne *et al.,* 2005), but the mechanisms underlying this adaptive mutagenesis are largely unknown. The use of fluoroquinolones, such as ciprofloxacin, is gaining wider attention, since exposure to these antibiotics has been shown to increase the genetic variation and mutation rate in bacteria (Cirz *et al.,* 2006, Cirz and Romesberg 2006, Lopez *et al.,* 2007).

In this thesis, proteomic methods including different forms of 2-DE based protein separation and mass spectrometric identification techniques are adapted and validated. These techniques were used to reveal the metabolic responses of recombinant xylose-fermenting *Saccharomyces cerevisiae*, the heat-shock response of probiotic bacterium *Lactobacillus gasseri* and provide an overview of the mastitis pathogen *Streptococcus uberis* when exposed to the mutagenesis-inducing antibiotic ciprofloxacin.

2. Review of the literature

2.1. Proteomics

The term proteome, the protein complement expressed by the genome in a cell or tissue at a certain time, was first introduced in the 1990s (Wilkins *et al.,* 1996, Wasinger *et al.,* 1995). The proteome differs between organisms depending on the genome and on external and internal conditions such as the physiological state, health, disease, drugs and stress. Compared with the genome, the complexity of the proteome is far greater due to protein processing and modification. Proteomics focuses on studying the many and diverse properties of proteins in order to provide detailed descriptions of a variety of biological systems (Patterson and Aebersold 2003). Although proteomics as a field is relatively new, the methodologies in proteomics have been under development for decades.

The proteome is highly dynamic, changing constantly with time and conditions. In one genome the proteomes of different tissues and cell compartments differ: for instance, the proteome of mitochondria differs from that of the proteome of cell surface in the same organism. Proteins interact to form functional networks which are made up of various species of interacting molecules. The diversity of the proteome is also increased by structural and dynamic protein modifications.

2.1.1. From genomics to bioinformatics

Genomics is the study of an organism's entire genome. Genomics was in practise founded in the 1970s, but took off with the initiation of genome projects on several organisms (NCBI Genome projects, www.ncbi.nlm.nih.gov). Among others, the human genome was announced in 2003 by the Human Genome Project, which was coordinated by the U.S. Department of Energy and the National Institutes of Health (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml). The knowledge and availability of full genomes has created the need for functional descriptions of genomes. Functional genomics responds to this need and describes the dynamic properties of the genome, gene transcription and translation and gene and protein functions and interactions. Comparative genomics, on the other hand, focuses on the relationships between the genomes of different strains or species (Hardison 2003).

Together with the growing number of sequenced genomes, new questions are being raised about the functional roles of genes and proteins and their regulation and interaction. Transcriptomics, a genome-wide measurement of messenger RNA (mRNA) expression levels based on DNA microarray technology, is one of the tools for understanding the genes and pathways involved in different biological processes (Gomase and Tagore 2008). Although mRNA is not the ultimate product of a gene, transcription is the first step in gene regulation, and information about transcription levels is needed to understanding gene regulatory networks.

Metabolomics is the systematic study of metabolites, the low molecular weight molecules produced by active, living cells under different conditions and times in their life cycles (Issaq *et al.,* 2008). Unlike proteomics, where the number of proteins in a biological system is in the thousands, in metabolomics the number of metabolites is in the hundreds, but due to

their large chemical and physical diversity, the analysis of metabolomes requires various and focused techniques (Garcia *et al.,* 2008).

The challenge of systems biology is to integrate proteomic, transcriptomic, and metabolomic information. Advances in these -omics have led to an explosive growth in biological information. To efficiently handle all this information, there is a requirement for bioinformatics, in the form of computerized databases to store, organize, and index the data and the need for algorithms for analysis of biological data.

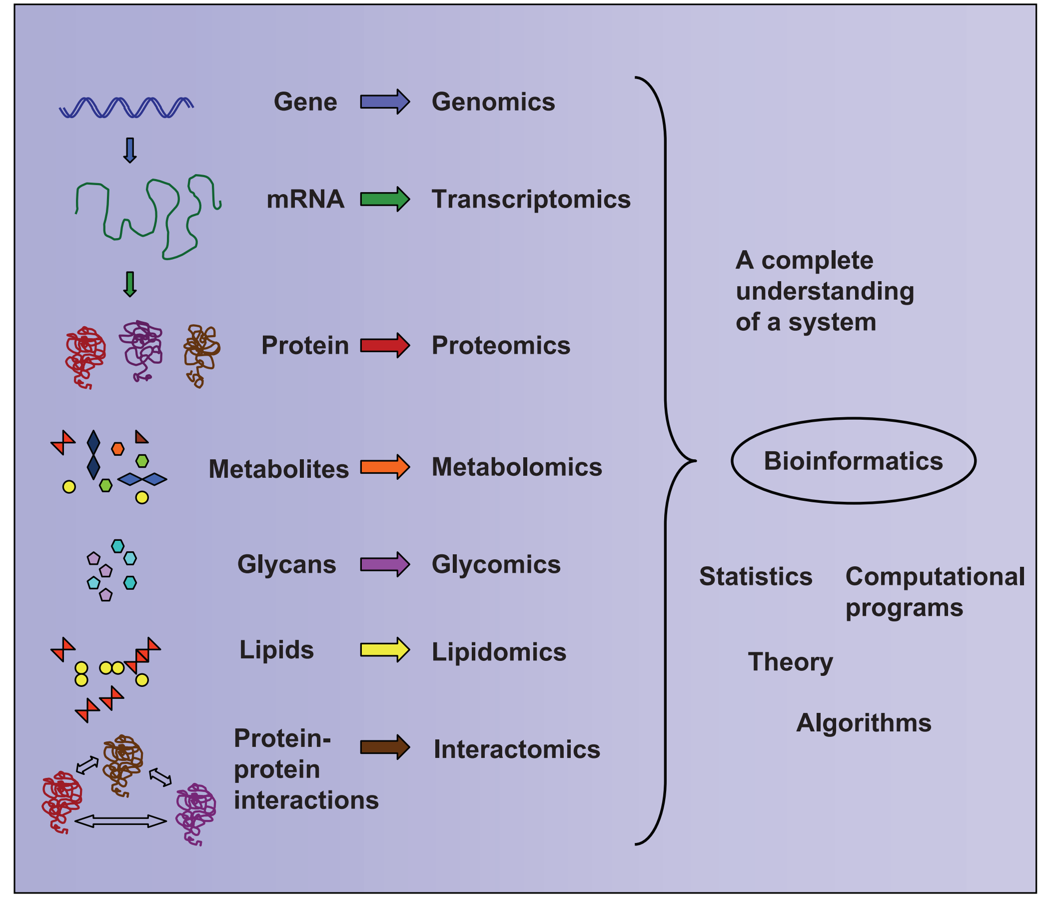

Figure 2. *The overview of the –omics technologies. Genomics is the comprehensive analysis of DNA structure and function. Proteomics involves the systematic study of proteins in order to provide a comprehensive view of the structure, function and regulation of biological systems. Transcriptomics, the genome-wide expression profiling, is the global study of gene expression at the RNA level. Metabolomics is the systematic study of all the metabolites of the biological organism, whereas glycomics is the comprehensive study the entire complement of sugars, weather free or present in more complex molecules. Lipidomics is the large scale study of non-water soluble metabolites. Bioinformatics combines all the data achieved above and provides algorithms and computational and statistical techniques for the management and analysis of this huge amount of data.*

2.2. Separation methods in proteomics

Proteomic experiments using 2-DE aim to visualize a large number of proteins in a proteome, which may consist of tens of thousands of proteins from different types of complex biological samples. Thus, the separation of proteins or their fragments prior to further analysis is one of the key elements in proteomic analysis. The separation of proteins on the whole protein level is most often performed by gel-based electrophoretic or by liquid chromatographic methods. Separations or fractionations on the peptide level can be performed, for instance, by chromatographic methods or by peptide isoelectric focusing (Cargile *et al.,* 2004a, Cargile *et al.,* 2004b, Chick *et al.,* 2008).

2.2.1. Gel-based separation in proteomics

One-dimensional gel electrophoresis (1-DE) is used in resolving relatively simple protein mixtures, usually obtained after purification of the desired protein fraction. In 1-DE the proteins are separated according to their molecular weight (MW). 2-DE has become a standard separation method in gel-based proteomics, enabling the simultaneous separation and visualization of thousands of proteins. In 2-DE, proteins are first separated according to their isoelectric point (pI) by isoelectric focusing (IEF) in a pH gradient, after which they are separated according to their MW. The principle of IEF followed by polyacrylamide gel electrophoresis was first published in 1969 (Macko and Stegeman 1969) and the sample preparation procedures developed by Klose and O'Farrell (Klose 1975, O'Farrell 1975) made 2-DE truly successful.

2-DE is has been successfully used for approximately 25 years to study the expression changes of proteins by comparing 2-DE results from two or more cell populations. With 2-DE, semi-quantitative differences in expression can be revealed and the target proteins readily identified using MS (Gorg *et al.,* 2004). Intrinsic gel-to-gel variability may lead to system variability, which makes it

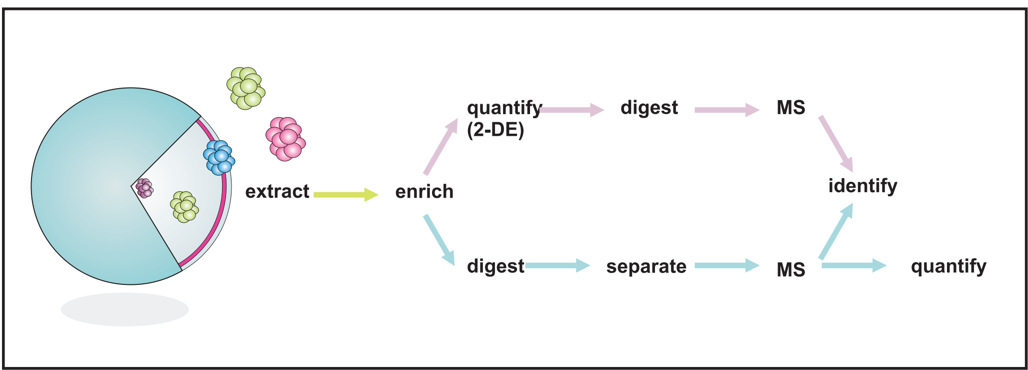

Figure 3. *The two most common processes for quantitative proteome analysis from the cell to the identified protein. At the top, 2-DE is used to separate and quantify proteins, and selected proteins are then identified by MS. At the bottom, LC-MS/MS is used to separate proteins from the mixture and quantitation is achieved by labelling peptides with stable isotopes. The figure is adapted from Patterson & Aebersold (2003).*

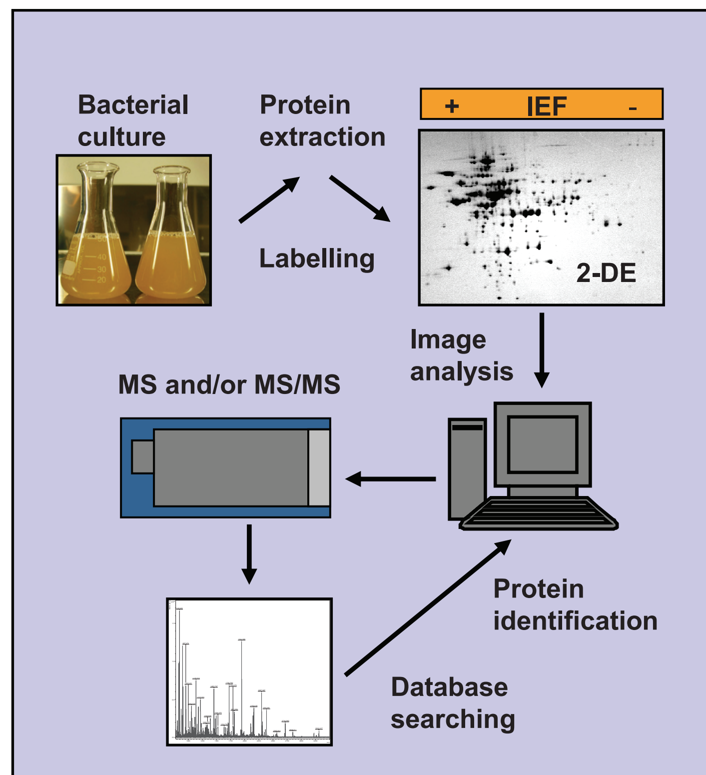

Figure 4. *The principles of proteome analysis by 2-DE gels. Proteins extracted from the cell are first separated according to their pI and subsequently according to their MW. Protein spot patterns from different samples are compared and quantified and the proteins from the spots of interest are identified by MS.*

difficult to distinguish between biological differences and system variation. Moreover, the predominant protein staining methods, silver and CBB, have a limited dynamic range and compromise the quantitative differences between gels. Fluorescent stains provided improvements in staining methods due to their wider dynamic range (Righetti *et al.,* 2004), but did not directly enable quantitation. To make quantitation by 2-DE more accurate, protein reactive cyanine dyes have been developed and used to undertake difference gel electrophoresis (DIGE) (Unlu 1999, Unlu *et al.,* 1997, Marouga *et al.,* 2005). These reactive dyes for proteomics were initially developed at Carnegie Mellon University and then commercialized by Amersham, now GE Healthcare. DIGE builds on 2-DE by adding a highly accurate quantitative dimension, allowing multiplexing of samples and the use of an internal standard. The proteomes to be compared are labelled with different dyes, which react with amino groups of proteins, essentially with side chains of lysine. In minimal labelling, 1-5% of all lysine residues are labelled. The dyes have different fluorophors, so proteins present in one sample can be distinguished from the same proteins present in a second sample.

An internal standard is created by labelling a pooled mixture of all samples with third dye. The differentially labelled proteomes are then combined and subjected to 2-DE in a single gel. Relative quantitation is performed by scanning the gels according the absorption and emission characteristic of each dye, followed by image analysis. Gels are often overstained with appropriate stains, such as silver, and proteins of interest are then excised from the gel and identified by MS. In contrast

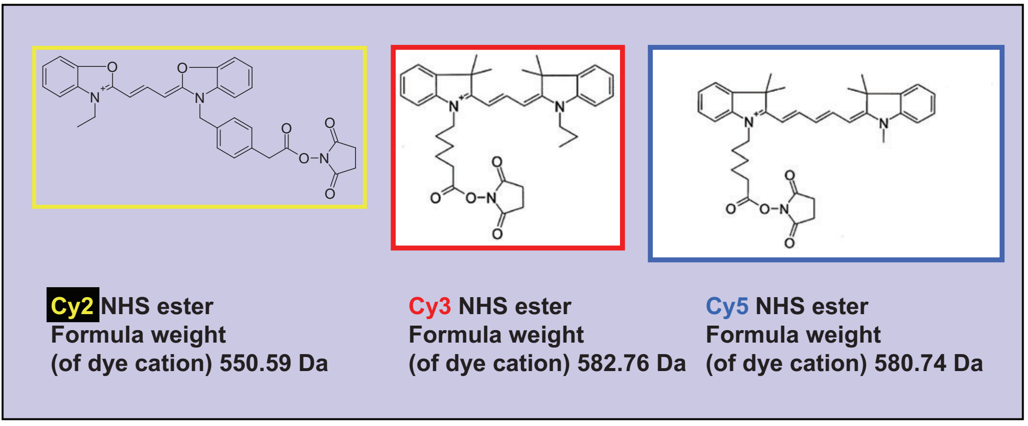

Figure 5. *Chemical structures of CyDye DIGE fluors for minimal labelling. Structures kindly supplied by GE Healthcare.*

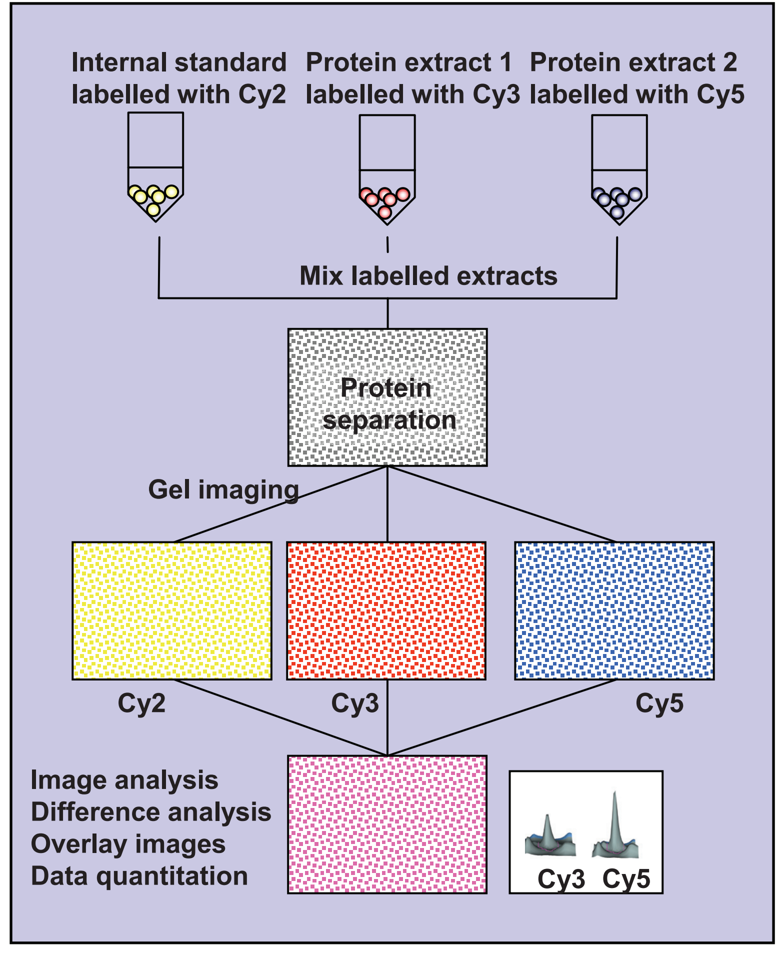

Figure 6. *Schematic diagram of a DIGE experiment.*

with minimal labelling, a set of saturation CyDyes is also available, which react with the thiols of cysteines of reduced proteins. This reaction labels all available cysteines of each protein, and it has been shown that saturation labelling has a 5- to 50-fold lower sample requirement (Greengauz-Roberts *et al.,* 2005, Shaw *et al.,* 2003). DIGE also has the advantage of being applicable to samples from large organisms, which is not possible with metabolic labelling. It is also more applicable to proteins that exist in several isoforms, because these proteins can be separated on a gel, whereas the purely peptide-based LC-MS/MS identifies the proteins based on peptides alone (Wilkins, Appel, Williams and Hochstrasser 2007).

In proteomic studies, 2-DE has commonly been used as a separation method for whole cellular protein lysates. Through the development of proteomic analysis there has been pressure towards high-throughput automated methods.

2.2.2. Non-gel-based separation in proteomics

The need for high throughput methodologies has resulted in the development of non-gel-based strategies for proteomics. The driving force behind the emergence of non-gel-based proteomic methods in recent years has been the application of liquid chromatographic separation, new protein chemistries and enrichment methods and the development of mass spectrometry and software for data analysis. Mass spectrometry based quantitation is an important addition to quantitation by 2-DE. Applications of MS-based technologies have several advantages compared to 2-DE-based ones: they can be automated and they combine high resolution and high sensitivity in the separation of extremely complex peptide mixtures (Kolkman *et al.,* 2005). To use the LC-MS/(MS) combination in proteome analysis, a form of complexity reduction is needed in order to detect and analyze as many components as possible in the sample (Patterson and Aebersold 2003). This is achieved, for instance, by combining two orthogonal peptide separation methods, such as cation exchange and capillary reversed phase chromatography, with MS/MS. This combination of multidimensional chromatography and tandem mass spectrometry became known as MudPit, which has been applied to identify up to tens of thousands of proteins from highly complex protein mixtures (Link *et al.,* 1999, Wolters *et al.,* 2001, Davis *et al.,* 2001, Washburn *et al.,* 2002, Usaite *et al.,* 2008, Lipton *et al.,* 2002, Smith *et al.,* 2002). The use of multidimensional enrichment and separation techniques in proteomic analysis has greatly enhanced protein coverage and dynamics, allowing many previously undetected low-abundance proteins to be identified (Roe and Griffin 2006).

MS-based quantitative methods are essentially an application of stable isotope labelling (Leitner and Lindner 2004, Leitner and Lindner 2006). In general, proteins or peptides from one biological state are tagged with a reagent that has a normal or "light" isotopic distribution, such as ^{1}H, ^{12}C, ^{14}N or ^{16}O. In parallel, proteins or peptides from another biological state are tagged with the "heavy" form of the same reagent ^{2}H, ^{13}C, ^{15}N or ^{18}O (Corthals & Rose in Wilkins, Appel, Williams and Hochstrasser 2007). Labelled peptides may be subsequently enriched to reduce the complexity of the mixture of peptides to be analysed. The mass difference between light and heavy isotopes separates the two forms of the

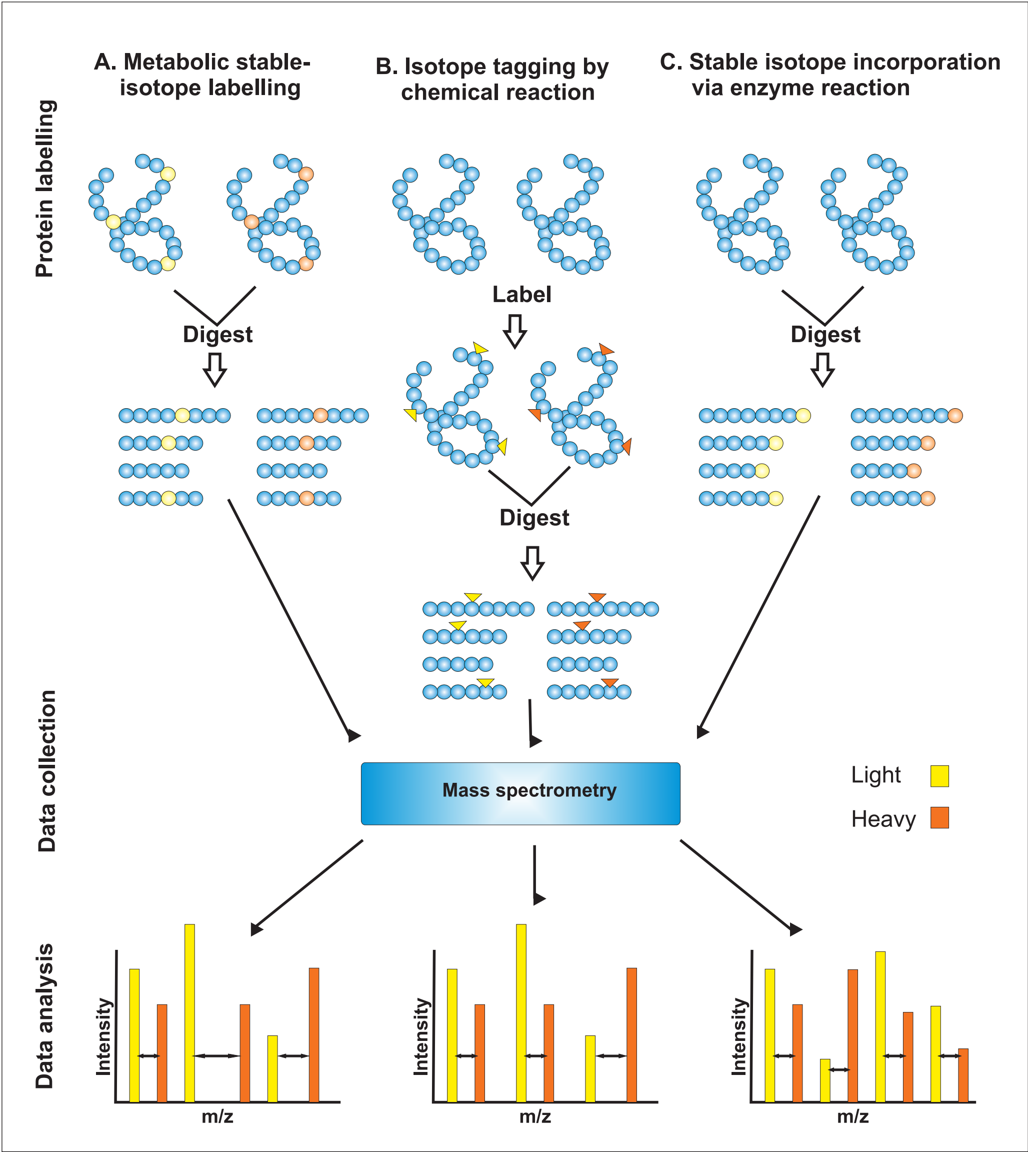

Figure 7. *Incorporation of isotopes into proteins and their use in relative quantitation. A. Proteins are labelled metabolically by culturing cells in media that are isotopically enriched (for instance, containing ^{15}N salts, or ^{13}C-labelled amino acids) or isotopically depleted. B. Proteins are labelled at specific sites with isotopically encoded reagents. C. Proteins are isotopically tagged by means of enzyme-catalysed incorporation of ^{18}O from ^{18}O water during proteolysis. In each case, labelled proteins or peptides are combined, separated and analysed by MS and/or MS/MS for the purpose of identifying the proteins contained in the sample and determining their relative abundance. The patterns of isotopic mass differences generated by each method are indicated schematically. The mass difference of peptide pairs generated by metabolic labelling is dependent on the amino acid composition of the peptide and is therefore variable. The mass difference generated by enzymatic ^{18}O incorporation is either 2 Da or 4 Da, making quantitation difficult. The mass difference generated by chemical tagging is one or multiple times the mass difference encoded in the reagent used. The figure is adapted from Aebersold & Mann, 2003.*

peptide, which are often doubly or triply charged in MS.

An important breakthrough in the relative quantitation of proteins was made in 1999, when Aebersold and colleagues first described isotope coded affinity tagging (ICAT) (Gygi *et al.,* 1999, Aebersold *et al.,* 2000). This technique was made commercially available in the form of an analysis kit by Applied Biosystems (Foster City, CA, USA) in 2000. ICAT enables the enrichment of proteins and peptides prior to quantification by MS analysis. The proteins or peptides to be compared are labelled at cysteine residues with light or heavy, biotin moiety carrying tags. The affinity label can be introduced either at the protein stage or after the digestion step. The labelled samples are then mixed, digested if necessary, and the cysteine-labelled peptides are purified using immobilized biotin-avidin affinity chromatography. Peaks corresponding to the same labelled peptide from the conditions are detected as doublets in MS and can be compared. The peak areas directly correlate with the relative abundances of the proteins in the samples.

ICAT only separates proteins containing cysteines and can be considered to cover most proteins, but some classes on proteins may nevertheless be discarded. Thus, to cover all proteins, the flow-through from the avidin column should be further analysed. On the other hand, the avidin column sometimes suffers from non-specific binding of peptides without cysteines (Moseley 2001). It has been pointed out that liquid chromatographic separation may occur between the light and heavy-forms of ICAT-labelled peptides (Zhang *et al.,* 2001), which can affect the accuracy of quantitation. The original ICAT has been improved with a cleavable ICAT reagent (cICAT), which is considered to enhance the elution characteristics of labelled peptides and to provide better quantitation accuracy (Hansen *et al.,* 2003, Yu *et al.,* 2004, Li *et al.,* 2003). cICAT uses ^{12}C and ^{13}C instead ^{1}H and ^{2}H and therefore does not cause chromatographic separation of the light and heavy forms. It contains a linker group that can be cleaved under acidic conditions, resulting in smaller group attached to the peptide. Several modified versions of ICAT have been developed, including "solid-phase ICAT" (Zhou *et al.,* 2002), which uses isotope-coded tags that are immobilized on glass beads and "visible-isotope-coded affinity tags", e.g. VICATs (Lu *et al.,* 2004), that allow the absolute quantification of protein in a mixture.

iTRAQ, isobaric tag for relative and absolute quantification, was introduced in 2004 (Ross *et al.,* 2004). In this technique, an amine-reactive tagging reagent is currently available in eight isotope-coded variants with identical overall molar mass. After labelling, all peptides with the same sequence but with different labels are indistinguishable during LC separation and MS analysis. Upon fragmentation on the MS/MS level, detached reporter ions create signals in the low mass range (m/z 113-119 and m/z 121) and peptide backbones remain unmodified and generate fragments that are identical in m/z for all samples. Fragmented signature ions provide quantitative information about the peptides from different conditions upon integration of the peak areas. As iTRAQ reagents are available in eight different variants, multiplexing is possible, allowing several samples to be simultaneously compared and quantified.

When working with cultured cells, stable isotopes can be incorporated by using media containing ^{13}C-glucose,

$^{15}NH_3$ or ^{13}C-labelled amino acids (Gu *et al.,* 2004). SILAC, stable isotope labelling by amino acids in cell culture enables the *in vivo* incorporation of isotopic amino acids into proteins as they are synthesized (Ong *et al.,* 2002, Ong and Mann 2006, Blagoev *et al.,* 2003). Protein extracts from samples to be compared are mixed in the early stage of the experiment, before they are subjected to any purification and fragmentation steps, after which they are enriched and subjected to MS analysis. With SILAC, no chemical labelling or affinity purification steps are required, in contrast to the ICAT-procedure. The method is compatible with many cell culture conditions and it has been shown that the incorporation of labelled amino acids is complete and the cells remain normal in the presence of labelled media (Ong *et al.,* 2002).

Relative quantitation of proteins can also be performed computationally without the use of stable isotope labels. This "label-free" approach uses the linearly increasing intensity of the LC-MS ion signal in relation to the molecular concentration by reproducing LC-MS runs. The most important advantage of this technique is that it can be used to track changes in multiple samples, whereas the use of mass tags limits the analysis to at most 8-way comparisons. The technique has been adopted by various groups and recently reviewed (America and Cordewener 2008, Mueller *et al.,* 2008).

2.3. Biological mass spectrometry

Mass spectrometry plays a significant role in biological sciences and is applied in a wide range of applications. In proteomic experiments, MS, including the instrumentation and the methods for data acquisition and analysis, has now reached the level where it is routinely used and applied worldwide to solve a wide range of biological problems (Aebersold and Mann 2003, Yates 2004, Domon and Aebersold 2006).

The beginnings of MS date back to the early part of the last century. The ability to separate molecules based on different size and charge was first described in 1912 by J.J. Thompson, who was a Nobel laureate in 1906, for his investigations of the conduction of electricity by gases. Mass spectrometers consist of three units: the ion source, the mass analyzer, which separates the ionized analytes according to their mass-to-charge (m/z) ratios, and the detector, which registers the number of ions at each m/z value. MS data are recorded as "spectra", which display ion intensity versus the m/z ratio.

Chemical ionization (CI), described by M.S.B. Munson and F.H. Field in 1966 followed by techniques named plasma desorption (PD) by R.D. MacFarlane and D.F. Torgerson in 1976 (Macfarlane and Torgerson 1976) and fast atom bombardment (FAB) by M. Barber and colleagues in 1981 (Barber *et al.,* 1981), were breakthroughs in analysing biomolecules by MS. An initial experiment by the physicist John Zeleny in 1917 preceded the first description of electrospray ionization (ESI) by M. Dole in 1968 (Dole *et al.,* 1968) . The breakthrough of ESI came in 1988 when J. Fenn reported the identification of polypeptides and proteins with a combination of ESI and MS (Fenn *et al.,* 1989).

Biomolecules are large and polar and thus their transfer into the gaseous phase presents challenges. In 1985 M. Karas and F. Hillenkamp showed, that an energy-absorbing matrix could be used to volatilize small analyte molecules

(Karas and Hillenkamp 1988). For large biomolecules a breakthrough was reported in 1987, when K. Tanaka presented the results of a mass spectrometric analysis of an intact protein with soft laser desorption (SLD). He showed that a low-energy nitrogen laser could be used to generate gaseous macromolecules, a technique that was further improved in matrix-assisted laser-desorption ionization (MALDI) by Karas and Hillenkamp (Karas and Hillenkamp 1988). Fenn and Tanaka were the Nobel laureates in 2002 for their work on methods of chemical analysis applied to biological macromolecules.

As mentioned above, ESI and MALDI were the ionization techniques that led to the success of mass spectrometry in life sciences. In proteomic research, four types of mass analysers are mainly used: time-of flight (TOF), linear and three-dimensional ion traps, quadrupole and Fourier transform ion cyclotron resonance (FTICR) (Aebersold and Mann 2003). ESI is often coupled to triple quadrupole, ion trap, orbitrap or hybrid tandem mass spectrometers such as quadrupole time-of-flight (Q-TOF) instruments and is used to generate fragment ion spectra (Morris *et al.,* 1996). MALDI is usually coupled to TOF analysers that measure the mass of intact peptides. As a result of its simplicity, high resolution and sensitivity, MALDI-TOF is still much used to identify proteins at the MS level in proteomic experiments. For tandem mass spectrometry (MS/MS), MALDI ion sources are combined, for instance, with quadrupole ion trap MS (Krutchinsky *et al.,* 2001) and TOF/TOF instruments (Medzihradszky *et al.,* 2000, Loboda *et al.,* 2000).

2.3.1. MALDI MS

In MALDI the sample is co-crystallized with a molar excess of UV-absorbing matrix. Ion formation is accomplished by directing a pulsed laser beam at sample-matrix crystals in a high vacuum. The energy of the laser excites the matrix, causing a proton to be donated to the sample molecules, creating charged ions. The matrix is a solid material that absorbs the laser radiation, resulting in the vaporization of the matrix and sample embedded to it. Matrices consist of fairly

Table 1. *Commonly used MALDI matrices for analysis of peptides and proteins.*

Matrix	Molecular Structure	Molecular Formula	Monoisotopic Mass $[M+H]^+$	Reference
α-Cyano-4-hydroxycinnamic acid, CHCA	O, OH, HO, C, N	$C_{10}H_7NO_3$	190.0502	(Beavis *et al.,* 1992)
3,5-dimethoxy-4-hydroxycinnamic acid, Sinapinic acid	H_3C, O, O, OH, HO, O, CH_3	$C_{11}H_{12}O_5$	225.0763	(Beavis and Chait 1989)
2,5-dihydroxybenzoic acid, DHB	O, HO, OH, OH	$C_7H_6O_4$	155.0344	(Stahl *et al.,* 1991)

low molecular weight organic acids to allow facile vaporisation, but large enough not to evaporate during sample preparation (Table 1). The matrix also serves to minimize sample damage from laser radiation by absorbing most of the incident energy. Once a charged ion is formed, a high voltage is used to eject the analyte from the ion source to the detector. MALDI is most often coupled to a TOF mass analyzer, in which the flight time of the ion from the ion source to the detector is measured. This flight time is converted into a mass-to-charge ratio, determining the molecular weight of the ion. MALDI –TOF/(TOF) MS is a widely used technique in proteomic research. It is considered as easy to use and relatively simple to automate for high-throughput methodologies.

2.3.2. ESI MS

Electrospray ionization is a method used to produce gaseous ionized molecules from a liquid solution. At the end of 1960s, Dole and his collaborators described the idea that spraying a liquid containing analyte molecules under an electric field might liberate these as ions in the gaseous form, and make them suitable for mass spectrometry (Dole *et al.,* 1968). In ESI, the sample solution is sprayed through a conducting capillary and when a voltage is applied a fine spray of highly charged droplets is formed. Upon solvent evaporation the size of the analyte-solvent droplet is reduced and the charge density on the droplet surface is increased until it reaches the point that the surface tension can no longer sustain the charge and the droplet is ripped apart. This is repeated until charged analyte ions are desolvated from the droplet into the gaseous phase (Fenn *et al.,* 1989). The invention of the nanoelectrospray ion source improved the sensitivity of analyses by lowering the flow rate to the level needed for proteomic analysis (Wilm and Mann 1994, Wilm and Mann 1996). Typically, flow rates are around 200 nl/min. The nanoelectrospray tip diameter is 10-50 µm, and it has a smaller spraying orifice and generates smaller droplets than a conventional electrospray.

2.3.3. LC-MS/(MS)

Liquid chromatography coupled to mass spectrometry (LC-MS and LC-MS/MS) is a widely used and powerful technique for the analysis of proteins and peptides. Proteomic samples are usually complex, even after pre-fractionation steps. LC-MS/MS combines efficient separation of proteins and peptides by LC and their sensitive identification with mass spectrometric methods. With LC-MS/MS, mixtures of peptides can be directly analyzed, or the method can be used to simplify the protein digest by fractionating the sample in LC before MS analysis. Certain improvements have transformed LC-MS/MS into a routine laboratory procedure. The development of microscale capillary reversed-phase liquid chromatography (capillary LC, LC-MS) (Karlsson and Novotny 1988) enabled direct coupling of LC into an ESI interface.

2.4. Protein identification

The identification of proteins in proteomics is almost exclusively performed by MS (Aebersold and Mann 2003). The introduction and development of MS methods and computational protein analysis techniques have dramatically enhanced the sensitivity and throughput

of protein identification. The sensitivity has reached a level that allows the identification of virtually any protein that is visible in conventionally stained gels (Shen and Smith 2005). The systematic sequencing of genomes of different organisms has generated massive amounts of data now contained in sequence databases. The development of algorithms and other bioinformatic tools for protein identification has been a great advance in biological MS (Mann *et al.,* 2001).

Proteins can be identified by MS using different techniques. The first, peptide mass fingerprinting (PMF), has been the most common and straightforward way to identify proteins in proteomic experiments. The second, peptide fragmentation analysis, utilizes fragment ion data (partial amino acid sequence) from a peptide together with its molecular mass. PMF is most often performed at the MS level with MALDI-TOF instruments and peptide fragment ion data are derived with tandem mass spectrometry (MS/MS) with MALDI-TOF/TOF or ESI MS/MS.

2.4.1. Peptide mass fingerprinting (PMF)

Mass spectrometry was already used to analyze peptides from proteolytic digests in the 1980s (Gibson and Biemann 1984), but its use for protein identification was published in 1993 when five groups described its use in the identification of gel-separated proteins (Pappin *et al.,* 1993, Henzel *et al.,* 1993, Mann *et al.,* 1993, Yates *et al.,* 1993, James *et al.,* 1993). Peptide mass fingerprinting was rapidly adopted in research. In PMF the protein is first digested with an endoprotease and the molecular masses of these peptides are then measured. The obtained set of peptide masses is unique for each protein. The acquired MS spectra are compared using database search algorithms with theoretical peptide masses calculated from each sequence entry in the database (Pappin *et al.,* 1993, Henzel *et al.,* 1993, Mann *et al.,* 1993, Yates *et al.,* 1993, James *et al.,* 1993). The requirement for a successful identification is that the protein or its very close homology is represented in a sequence database. In the identification procedure the overlapping masses between measured and calculated spectra are compared, leading to similarity scores (Palagi *et al.,* 2006). The accuracy of measured peptide masses has the largest effect on the reliability of results by reducing the number of false positives (Clauser *et al.,* 1999), so the mass accuracy and resolution of the used instrument are of great importance. A variety of scoring algorithms are available, some of which use a simple score based on the number of common masses between the experimental and theoretical spectra. More sophisticated scoring algorithms take into account the nonuniform distribution of protein and peptide masses in the database.

2.4.2. MS/MS analysis

Protein identification with tandem mass spectrometry has been widely explored in bioinformatics research. Before the 1980s, when the MS/MS was first used for protein sequence analysis, Edman degradation was the method of choice for amino acid sequencing (Edman 1949). *De novo* peptide sequencing for MS/MS became possible when computer programs were developed to determine probable amino acid sequences of a peptide by using sequence ion peaks obtained by FAB MS (Morris *et al.,* 1981, Sakurai *et al.,* 1984). In the 1990s, the

number of available protein sequences increased rapidly and their use in protein identification was described: MS/MS spectra were correlated with theoretical peptides (Eng *et al.,* 1994) or fragment ion data together with the peptide molecular weight were used in error tolerant searches (Eng *et al.,* 1994, Mann and Wilm 1994). Following 20 years of constant research and development, MS/MS is nowadays well established as a method for protein identification (Hernandez *et al.,* 2006).

After protein separation the peptides from a protein digest are selected and subjected to fragmentation in the mass spectrometer. Fragmentation of peptides can be achieved by post-source decay (PSD) during MALDI (Spengler *et al.,* 1992b, Spengler *et al.,* 1992a), or collision-induced dissociation (CID) in a collision cell (Shevchenko *et al.,* 1996b). In addition to the peptide mass the tandem mass spectrum contains information on the peptide mass and structural information originating from the peptide sequence. Both PSD and CID result dominantly in the cleavage of the peptide bond along the peptide backbone and generate fragment ion ladders either from the N terminus (b ions) or the C terminus (y ions) (Roepstorff and Fohlman 1984). The mass difference between two consecutive b or y ions reflects the mass of the corresponding amino acid residue. Several approaches exist for the identification of proteins with MS/MS analysis and they are comprehensively reviewed by Hernandez and colleagues (Hernandez *et al.,* 2006).

2.5. Proteomics of baker's yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is one of the simplest eukaryotes. It is classified as a generally recognized as safe (GRAS) microorganism by the USA Food and Drug Administration. *S. cerevisiae* is an effective eukaryotic model system for understanding basic cellular processes due to its ease of manipulation and its genetic tractability (Suter *et al.,* 2006, Botstein and Fink 1988, Cherry *et al.,* 1997). The *S. cerevisiae* genome was the first eukaryotic genome to be sequenced (Goffeau *et al.,* 1996) and open reading frames (ORFs) of *S. cerevisiae* have all been deleted in order to define the functions of all the gene products (Giaever *et al.,* 2002, Shoemaker *et al.,* 1996). Annotated information and the function of the open reading frames and their corresponding protein products are available through several databases, such as the *Saccharomyces* Genome Database (SGD, www.yeastgenome.org), the Yeast Protein Database (YPD; www.proteome.com), the Munich Information Center for Protein Sequences (MIPS) Comprehensive Yeast Genome Database (CYGD;

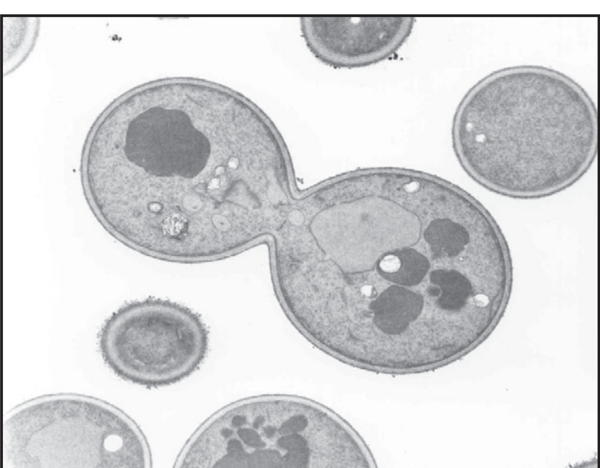

Figure 8. *Baker's yeast, Saccharomyces cerevisiae. The figure is kindly supplied by VTT Biotechnology.*

mips.gsf.de/genre/proj/yeast/index.jsp), and the Yeast Resource Center (depts.washington.edu/~yeastrc).

The availability of the *S. cerevisiae* genome has opened up many possibilities for biotechnological and biomedical research. Several features make this organism a useful research vehicle; it has a relatively short generation time, methods for cultivation under well-controlled conditions are available and it is able to adapt to different, changing environmental conditions such as nutrient limitation, suboptimal temperature, osmolarity, osmotic pressure and acidity (Gasch *et al.,* 2000). *S. cerevisiae* has further established its "super model organism" status as an ideal platform for the development, validation and application of post-genomic techniques, such as those used in large-scale knockout genetics and functional genomics studies, and large-scale analysis of the yeast transcriptome, proteome and metabolome (Barr 2003, Rubin *et al.,* 2000).

The application of 2-DE to yeast proteins has enabled their high resolution separation and visualisation and resulted in the construction of yeast reference maps (Shevchenko *et al.,* 1996a, Maillet *et al.,* 1996, Boucherie *et al.,* 1996, Norbeck and Blomberg 1997, Perrot *et al.,* 2007, Perrot *et al.,* 1999, Wildgruber *et al.,* 2002, Wildgruber *et al.,* 2000). Maps have been presented for sub-proteomes such as the yeast mitochondrial proteome (Ohlmeier 2003) and for other industrially important yeast strains, such as ale fermenting (Kobi *et al.,* 2004), a wine strain (Trabalzini *et al.,* 2003) and a lager brewing strain (Joubert *et al.,* 2000, Joubert *et al.,* 2001).

Due to its wide use in industry and biology, *S. cerevisiae* has to adapt to a large variety of environmental conditions. To survive in the changing environment, yeast cells need to detect the availability of nutrients and rapidly adapt their metabolism. For baker's yeast, comprehensive transcriptome analyses have been performed to study the effects of different nutrient conditions (Boer *et al.,* 2003, Saldanha *et al.,* 2004, Tai *et al.,* 2005). However, the abundance of mRNA and protein expression levels do not necessarily correlate, so it is essential to also examine yeast adaptation and other biological processes at the level of the proteome. Many 2-DE based experiments have been reported with applications to yeast growth under different environmental stimuli. Protein abundance levels were analysed from yeast grown in chemostat cultures limited for glucose or ethanol (Kolkman *et al.,* 2006). This study revealed major changes in the central carbon metabolism pathways upon changing the carbon source. 2-DE has also been applied to obtain a global view of changes in the *S. cerevisiae* proteome as a function of stimuli in the environment, such as cadmium (Vido *et al.,* 2001), lithium (Bro *et al.,* 2003), H_2O_2 (Godon *et al.,* 1998), sorbic acid (de Nobel *et al.,* 2001) and amino acid starvation (Yin *et al.,* 2004).

Quantitative proteomic methodologies have, in many cases, first been developed and validated for *S. cerevisiae*. Washburn et al. applied MudPIT to analyze the proteome of yeast and identified a total of 1484 proteins (Washburn *et al.,* 2001, Washburn *et al.,* 2002). MudPIT methodology was improved by Wei and coworkers (Wei *et al.,* 2005) by adding an additional reversed phase column to the biphasic column, resulting in an on-line 3D LC method and identifying a total of 3109 yeast proteins.

Various groups have applied metabolic stable-isotope labelling in yeast

proteome studies (Washburn *et al.,* 2002, Usaite *et al.,* 2008, Flory *et al.,* 2006, de Godoy *et al.,* 2006, Zybailov *et al.,* 2006, Oda *et al.,* 1999). The efficiency of 2D-DIGE and metabolic stable isotopic labelling was compared in a study where *S. cerevisiae* was grown with ammonium sulphate labelled with either ^{14}N or stable isotope ^{15}N as a nitrogen source (Kolkman *et al.,* 2005). Following lysis and protein extraction, the protein samples were fluorescently labelled using CyDyes. Proteins were separated with 2-DE and further analyzed by MS. Relative ratios of protein abundances between experimental conditions were defined using both 2-D DIGE and metabolic labelling techniques. The correlation between these two methods for relative protein quantification was good: differential average ratios were R(met.lab) = 0.98 R(DIGE) with a correlation coefficient r^2=0.89. Some differences were noted with low intensity spots; they could be detected and quantified with 2-D DIGE but not with the stable isotope labelling approach. In that study, DIGE was stated to be more suitable for the analysis of proteins with low concentrations and with extreme changes in expression. On the other hand, a disadvantage of 2-D DIGE is clearly that separate methods have to be used for quantification (2-D DIGE) and identification (MS).

In proteomics, and in other –omics in systems biology, new technologies are constantly being developed and validated and this is, in many cases, carried out using a model system such as yeast. It is crucial that this is done with as simple an organism as possible, so it is clear that future developments in the proteomics will also largely benefit from *S. cerevisiae* as a model system.

2.6. Proteomics of probiotic lactobacilli

Intimate interactions between bacteria and human or animal cells are increasingly studied by proteomics. Probiotic microorganisms that are capable of communicating with human or animal immune systems are of growing health and economic interest (van Belkum and Nieuwenhuis 2007, Isolauri 1999, Isolauri *et al.,* 2008). Probiotics are microorganisms that help maintain the natural balance of microbiota in the intestine. The premise for a microorganism to be classed as a probiotic includes strain identification, safety confirmations, clearly documented efficacy in clinical studies, and detailed consumer information such as considerations for the route of administration and dose applied (FAO/WHO, 2002). About 10^{14} bacteria live in symbiosis in and with our body (Reid *et al.,* 2003). The most bacteria-rich body part is the large intestine, where bacterial communities can reach densities of 10^{12} per gram of content. Overall, the gut microbiota makes a major contribution to human health and disease (Guarner and Malagelada 2003). Probiotics have a long history of use in the diet to modify the intestinal microbiota and immune system of humans and animals, but the tools to evaluate their effect on the heath and well being of the host have not been available until recently.

Microbes from many different genera are used as probiotics. The main organisms used as probiotics belong to the genera *Lactobacillus* and *Bifidobacterium.* The genomes of probiotic bacteria such as *Lactobacillus johnsonii* (Pridmore *et al.,* 2004)*, Lactobacillus plantarum* (Kleerebezem *et al.,* 2003) and *Lactobacillus acidophilus* (Altermann

et al., 2005) have been sequenced. The probiotic features are strain-specific, but the factors contributing to the health promoting properties are largely unknown. The use of lactobacilli species as probiotics is most probably due to historical reasons. They have been present in the human diet for centuries through fermented dairy products such as cheese and yoghurt. Probiotics are available to consumers mainly in the form of dietary supplements and food and the use of food products containing probiotic microorganisms is of increasing economic importance.

The most intensively studied probiotic effect is the prevention and reduced duration of diarrhoea. *Lactobacillus rhamnosus* and *L. reuteri* are effective against diarrhoea of infants (Rosenfeldt *et al.,* 2002, Szajewska and Mrukowicz 2005). *L. acidophilus* is reported to reduce the incidence of diarrhoea during antibiotic treatment (Beausoleil *et al.,* 2007). Besides Lactobacilli and Bifidobacteria, other genera including *Escherichia*, *Enterococcus*, *Bacillus* and *Saccharomyces* are also used and their efficacy has been clinically proven (Reid *et al.,* 2003).

Proteomic methods are increasingly used to study, for example, the changes in the bacterial proteomes in different growth phases and the bacterial responses to different stress conditions. A proteome of the potentially probiotic *L. plantarum* WCFS1 strain was investigated in different growth phases (Cohen *et al.,* 2006). 2-DE was used to generate proteome maps from mid- and late-log, early and late stationary phases of growth. A total of 200 proteins were identified, and the results indicated that each growth phase has its own metabolic status. For example, in the log-phase, proteins associated with metabolic pathways for energy generation were specifically present, and in the late-log phase the synthesis of macromolecules was increased (Cohen *et al.,* 2006).

Lactobacillus salivarius subsp. *salivarius* UUC118 is a probiotic bacterium isolated from human intestinal tissues and has been shown to alleviate the symptoms of Crohn's disease (Venturi *et al.,* 1999). The bacterial growth phase and the presence of a previously documented cell-wall-associated protein were shown to be correlated using proteomic and enzymatic techniques in *L. salivarius* subsp. *salivarius* strain (Kelly *et al.,* 2005). With 2-DE it was reported that proteins increasingly expressed from the lag to log to stationary phase were analogous to those reported to be associated with the cell-wall proteome of the pathogenic bacterium *Listeria monocytogenes* (Schaumburg *et al.,* 2004). To act as a probiotic in the gastrointestinal tract (GIT), bacteria have to adhere to the tract. In this study it was proposed that some valuable effects of *Lactobacillus salivarius* subsp. *salivarius* UUC118 may be due to the mimicking of pathogenic adherence to the gut epithelium.

The first proteomic study on the probiotic *Lactobacillus crispatus* aimed to reveal the overall changes in metabolic pathways caused by aggregation processes (Siciliano *et al.,* 2008). An aggregation process of *L. crispatus* M247 was studied using a mutant strain that had lost its autoaggregation phenotype and its isogenic control strain. The results demonstrated an overall lower growth rate of *L. crispatus* M247 compared to the mutant strain and higher amounts of enzymes involved in carbohydrate transport and metabolism in the mutant strain. This was probably caused by nutrient limitation due to the aggregation phenomenon in *L. crispatus* M247 (Siciliano *et al.,* 2008).

Passage through the GIT exposes probiotic bacteria to stress due to the lowered pH and bile. The effect of lowered pH on protein synthesis by *Lactobacillus reuteri* was investigated using 2-DE (Lee *et al.,* 2008) and 40 consistently and significantly altered proteins were identified with PMF. General stress responsive proteins and key metabolic components from the glycolytic and pentose-phosphate pathways were identified, indicating the complexity the acid stress response of *L. reuteri*. Survival and gene expression of *L. reuteri* after a sudden shift in environmental acidity to a pH close to the conditions prevailing in the human stomach (Wall *et al.,* 2007) and the physiological concentration of human bile (Whitehead *et al.,* 2008) were investigated in genome-wide analysis using microarrays. In both conditions, several genes were differentially expressed. *clpL*, an ATPase encoding chaperone activity, was selected for an insertion mutation analysis and reported to contribute to the sensitivity to acid stress as well as the ability to survive bile exposure (Whitehead *et al.,* 2008). Thus, *clpL* could contribute to the survival of *L. reuteri* in the gastrointestinal tract. The effect of pre-adaptation to stress on the viability of *Lactobacillus rhamnosus* was examined with proteomics (Prasad *et al.,* 2003). Exposure of *L.rhamnosus* cells to a sub-lethal salt concentration or heat stress considerably improved the storage stability of the bacterium, suggesting that the ability of *L. rhamnosus* to withstand stressful storage conditions can be improved by stress adaptation.

2.7. Proteomics of streptococci

As with probiotic bacteria, proteomics represents an established technology for the study of pathogenic bacteria. The genus *Streptococcus* includes bacteria that are part of the normal microbiota of humans and animals, but some of them are also pathogens. Proteomic studies can provide better understanding of protein production and cell metabolism during different biological situations. *Streptococcus pyogenes* is an important human pathogen causing infections in the upper respiratory tract and on the skin epithelium. Many of the associated virulence factors are expressed in a growth phase-dependent manner (Kreikemeyer *et al.,* 2003). Changes in the transcriptome and proteome of *S. pyogenes* cells in the exponential and stationary phase have been analyzed (Chaussee *et al.,* 2008). At the transcript level, the expression of 689 genes differed between the two growth phases. At the proteome level, 527 and 403 spots on 2-DE gels were detected in the exponential and stationary phases of growth, respectively. Changes in transcriptome and proteome levels were similar, even though transcriptome data seemed to be more comprehensive, which was most probably due to the sensitivity of protein detection. Several growth phase associated proteins were detected on the proteome level that were not detected on the transcriptome level. *Streptococcus pneumoniae* growth phase proteomes have been investigated, focusing on the transition from the log phase to the exponential growth phase (Lee *et al.,* 2006). Growth curves and profiled protein maps based on growth stages were generated and 22 growth-phase-dependent proteins were identified.

Streptococcus mutans is a primary etiological agent of human dental caries. Its growth from planktonic culture to biofilm was studied with traditional 2-DE (Svensater *et al.,* 2001, Rathsam *et al.,*

2005a) and with 2-D DIGE (Rathsam *et al.,* 2005b). The use of 2-D DIGE led to a 3-fold increase in the detection and in the number of significantly altered protein spots on 2-DE gels. The results revealed that in a mature biofilm the general metabolic proteins were down-regulated, indicating a reduction in growth rate. Up-regulated proteins were competence proteins and proteins involved in glucan and cysteine synthesis. This study revealed that 2-D DIGE, as a more accurate and more reproducible technique, leads to a more comprehensive view of the phenotypic changes during the planktonic to biofilm transition in *S. mutans*. In another study, biofilm development in *S. pneumoniae* was followed by 2-DE (Allegrucci *et al.,* 2006). The number of detectable spots markedly increased between time-points of biofilm formation, with most proteins being house-keeping and metabolic proteins. An increased abundance of proteins with virulence and resistance was also noted.

Bacterial surface proteins play a significant role in host-pathogen interactions and pathogenesis and are targets for immune systems (Lindahl *et al.,* 2005, Lin *et al.,* 2002). Thus, knowledge of protein consistency on the bacterial surface is important in drug and vaccine development. One method for proteomic analysis of cell-surface-associated proteins includes degradation of the cell wall by digestion of the peptidoglycan part with an enzyme such as lysozyme. This is followed by the release of cell-wall-associated proteins, their separation with 2-DE, digestion and identification by MS. By this approach, surface proteins have been analyzed from *Streptococcus agalactiae* (Hughes *et al.,* 2002), *S. pyogenes* (Cole *et al.,* 2005) and *S. pneumoniae* (Ling *et al.,* 2004). However, only a few proteins that were predicted to be covalently attached to peptidoglycan with cell wall anchoring motifs (Marraffini *et al.,* 2006) were identified using this technique. This may be due to the high hydrophobicity of these proteins and resulting challenges in the first dimension of 2-DE. Moreover, digestion of the cell wall increases the susceptibility to cell lysis contamination of samples with cytoplasmic proteins. To overcome potential difficulties in gel-based separation, surface proteins can be digested directly from the cell surface and subsequently identified with LC-MS/MS. This “shaving” method has been used in proteomic analysis of surface proteins from *S. pyogenes* (Rodriguez-Ortega *et al.,* 2006, Severin *et al.,* 2007). Together with *in silico* prediction of protein localisation (Nakai 2000), this method provides a relatively broad view of the organization of surface protein identity.

Proteomics has been used in studies on the environmental stress responses of Streptococci. *Streptococcus mutans* is an acidogenic organism generating acidic end products from fermentable carbohydrates available in the oral cavity. Acidogenity and also aciduricity, the ability to survive at a low pH, are considered as important pathogenicity factors for dental caries. The effect of lowered pH on the *S. mutans* proteome has been intensively studied. Altogether, 18 proteins were up-regulated and 12 down-regulated at an acidic pH when 2-DE proteomes grown in pH 7.0 and pH 5.2 batch culture were compared (Wilkins *et al.,* 2002). A total of 27 proteins were identified by MS, most of them involved in metabolism, sugar transport and response processes. The growth rate in the acidic medium was reduced as were the abundancies of most of the identified proteins related to translation. Enzymes involved in glycolysis as well

general stress proteins, such as DnaK, were up-regulated following growth at low pH. A combination of steady-state continuous culture and medium-range IPG strips were applied to compare 2-DE proteomes of *S. mutans* grown at a steady state in continuous culture at pH 7.0 and 5.2 (Len *et al.,* 2004a, Len *et al.,* 2004b). Among the differentially expressed and identified proteins were proteins associated with stress responsive pathways, and also five novel findings of proteins associated with acid tolerance in *S. mutans* (Len *et al.,* 2004a). Changes in the expression of metabolic proteins under the same experimental conditions showed differential expression of glycolytic proteins, alternative acid production and amino acid biosynthesis proteins (Len *et al.,* 2004b). A significant increase was also observed in the abundance of a proton translocating F_1F_0-ATP-ase that extrudes H^+ from the cell. This observation was consistent with previous studies reporting an incerase in H^+-ATPase in response to acidification of the environment of *S. mutans* (Belli and Marquis 1991, Kuhnert *et al.,* 2004). These studies support existing knowledge of the ability of *S. mutans* to survive at a low pH, tolerate acid stress and induce acid tolerance.

Comparisons of the cellular proteome grown of *Streptococcus oralis* at pH 5.2 and 7.0 (Wilkins *et al.,* 2002) and the surface proteome under similar conditions (Wilkins *et al.,* 2003) have also been published. *S. oralis* and *Streptococcus gordinii* are part of the microbial flora of dental plaque (Whiley and Beighton 1998), but when accessing the vascular system they are associated with infective endocarditis (Douglas *et al.,* 1993). When *S. gordonii* gene expression was examined during a pH change from slightly acidic to the pH of the blood stream, genes coding for surface proteins were differentially expressed (Vriesema *et al.,* 2000).

The effect of fluoride exposure on cariogenic streptococci was assessed by 2-DE (Cox *et al.,* 1999). *Streptococcus pyogenes*, a non-cariogenic group A streptococcus, was also exposed to fluoride (Thongboonkerd *et al.,* 2002). Proteome study revealed a total of 60 unique proteins and the down-regulation of several virulence factors, suggesting that fluoride exposure might inhibit *S. pyogenes* virulence factors. During infection, *S. pyogenes* interacts with human plasma (Johansson *et al.,* 2005). In a proteome study it was revealed that exposure to human plasma had a profound influence on the *S. pyogenes* proteome (Johansson *et al.,* 2005). Altogether, 31 protein spots were up-regulated on plasma-exposed bacteria, of which 24 were present only in gels from plasma-exposed cells. Most of the identified proteins were important for cell maintenance, metabolism and glycolysis. Two surface-associated virulence factors were also identified in this study. The effect of blood on *S. pneumoniae* protein expression was assessed using 2-DE by Bae et al. (2006). Differentially expressed proteins were involved in various cellular metabolisms, and the results demonstrated that *S. pyogenes* alters its metabolism when exposed to blood.

Proteomic studies on different strains of streptococci are still in their infancy. Experiments are conducted with one strain to resolve one problem and with another to address a second research question. This is most probably due to the heterogeneity of this group of bacteria, including pathogenic, non-pathogenic and strains used in the dairy industry. Thus, the methods for proteomic experiments need to be validated for each individual strain.

3. Aims of the study

The general goal of this work was to establish, optimize and exploit proteomic methods from protein extraction to protein separation, quantification and identification. Methods were adapted for proteomic studies on the eukaryote baker's yeast *Saccharomyces cerevisiae*, the probiotic bacterium *Lactobacillus gasseri* and the mastitis pathogen *Streptococcus uberis*.

The specific goals of the present study were:

I) To adapt and evaluate methods for analysing and identifying proteins from baker's yeast, *Saccharomyces cerevisiae*. The work included optimization of protein extraction methods and separation by 2-DE and the establishment of a PMF and a nanoLC-MS/MS method for protein identification.

II) To reveal the metabolic responses of recombinant xylose-fermenting *Saccharomyces cerevisiae* with 2-DE, and protein identification with MALDI-TOF PMF and nanoLC-MS/MS.

III) To adapt proteomic methods, including 2-D DIGE and PMF, to bacterial proteins and to use them in the study of the overall heat-shock response and the role of ClpL ATPases in the stress response in probiotic *Lactobacillus gasseri*.

IV) To utilize 2-D DIGE and PMF protein identification to reveal the responses of the mastitis pathogen *Streptococcus uberis* to the mutagenesis-inducing antibiotic ciprofloxacin.

4. Materials and methods

All materials and methods are described in detail in the original publications I-IV.

4.1. Strains and plasmids

Table 2. *Strains and plasmids used in this study*

Strain	Relevant property or genotype(s)	Article	Source or Reference
Yeast strain and plasmids			
Saccharomyces cerevisiae			
H2490	CEN.PK2 derivative (*MAT*α, *leu2*-3/112, *ura3-52*, *trp1-289*, *his3-1*, *MAL2-8*^{c}, *SUC2*)	I, II	(Boles *et al.,* 1996)
YEplac 195	Multicopy plasmid (uracil selection)	I, II	(Gietz and Sugino 1988)
YEplac181	Multicopy plasmid (leucine selection)	I,II	(Gietz and Sugino 1988)
Bacterial strains and plasmids			
Lactobacillus gasseri		III	
ATCC 33323a	neotype, DSM 20243	III	ATCC
AS1	ATCC 33323 derivative with 0.9-kb deletion in *clpL*	III	III
AS2	ATCC 33323 harboring plasmid pKTH2095	III	III
AS3	AS1 harboring plasmid pKTH2095	III	III
AS4	AS1 harboring plasmid pAS2	III	III
E. coli			
M15(pREP4)	Cloning host with inducible expression	III	Qiagen
pTN1	conditionally replicating vector for an allelic replacement	III	(Neu and Henrich 2003)
pAS1	pTN1 with 1.1-kb fragment containing 0.9 kb in-frame deletion in the *clpL*	III	III
pKTH2095	lactobacillar expression vector	III	(Savijoki *et al.,* 1997)
pAS2	pKTH2095 with 2.4-kb fragment containing the *clpL* region	III	III
pQE30	IPTG-controlled overexpression	III	Qiagen
pKTH5202	pQE30 with 1.1 kb fragment encoding HrcA	III	III
Streptococcus uberis			
aATCC BAA-854 (0140J)		IV	(Hill 1988, Leigh *et al.,* 1990, Smith *et al.,* 2003)

[a] ATCC, American Type Culture Collection.

4.2. Sample preparation for 2-DE

4.2.1. Chemostat cultivation

A detailed description of the chemostat cultivation of *Saccharomyces cerevisiae* is presented in original publication II.

H 2490 precultures were grown in yeast nitrogen base/medium (YNB, Sigma, USA) supplemented with 20 g/l glucose. Cells were harvested and resuspended into 50 ml of cultivation media aiming at a starting optical density OD_{600} = 1 in the fermenter cultivation. Both fermentations were started as aerobic batch cultures. Chemostat cultures were performed on either 56 mM (10 g/l) glucose or 17 mM (3 g/l) glucose + 180 mM (27 g/l) xylose (Pitkanen *et al.,* 2003). The cultures were switched to anaerobic chemostat conditions after six residence times. Samples of 10-20 ml were taken from steady-state cultures and at the time points of 5, 30 and 60 min after the switch-off of the oxygen supply.

4.2.2. Bacterial cultivations

In studies III and IV the experimental set-up was performed for four biological replicate samples. In study III, *Lactobacillus gasseri* cells from four independent cultures were heat-stressed by cultivating them at 37 °C in MRS to an OD_{600} = 0.25, at which point aliquots of cells (5 ml) were further incubated at 37 °C (4 x control sample) or shifted to 49 °C for 30 min (4 x stress sample). In study IV, *Streptococcus uberis* cells in four independent overnight cultures were diluted 1:100 in THY broth and allowed to grow at 37 °C until OD_{600} = 0.2. Cultures were then divided into 15 ml aliquots and 0 µg/ml (control sample), 0.5 µg/ml (=0.5 x minimum inhibitory concentration, MIC) or 1.0 µg/ml ciprofloxacin (=1.0 x MIC) (stress samples) was added to the cultures, which were allowed to grow until OD_{600} = 0.5. In both III and IV, cells were harvested by centrifugation and washed with ice-cold Tris-HCl pH 8.0 (+ 30% EtOH in study IV). Cells were stored at -20 °C until they were disrupted.

4.2.3. Protein extraction for 2-DE

In studies I and II, 5-10 µg dry weight of yeast cells were disrupted with glass beads (0.5 mm diameter; Biospec Products, USA) in a MiniBeadBeater (Biospec Products) in 150 µl of a solution of 50 mM Hepes, pH 7, 1 mM $MgCl_2$, 0.1 M EDTA supplemented with phenylmethylsulphonyl fluoride (Sigma, USA) to 1 mM and Pepstatin A (Sigma) to a concentration of 13µM. After cell breakage, 400 µl of lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% Pharmalytes 3-10 and 1% dithiothreitol (DTT) was added and the mixture was gently shaken for 20 min at room temperature and then centrifuged for 5 min at 13 000 rpm. The supernatant was collected and the protein concentration of the extract was determined using a non-interfering protein assay (Geno Technology Inc., USA). Protein extracts were stored at -70 °C before 2-DE.

In studies III and IV, bacterial cells were disrupted with glass beads in 30 mM Trizma base in a FastPrep FP120 homogenizer (Thermo Scientific, USA). Proteins were solubilized in 400 µl of 7 M urea, 2 M thiourea, 4.0% CHAPS and 30 mM Trizma base and incubated at room temperature for 1 h. The cell debris and glass beads were removed by centrifugation. Recovered proteins were concentrated using a 2-D Clean-Up Kit (GE Healthcare, Sweden) and solubilized in 50 µl (III) and 20 µl (IV) of 7 M urea,

2 M thiourea, 4.0% CHAPS and 30 mM Trizma base. Protein extracts were stored at -20 °C before 2-DE.

4.2.4. Protein labelling

In studies III and IV, samples were labelled with CyDye Fluor minimal cyanine dyes (GE Healthcare) according to the manufacturer's instructions. Prior to labelling the pH of the samples was monitored and adjusted to pH 8.5 with 2.0 M Trizma base. In study III, 12.5 µg of protein was labelled with 100 pmol of Cy3 and 12.5 µg was labelled with Cy5 dye. In study IV, two 25 µg protein samples were labelled with 200 pmol of Cy3 and Cy5 dyes, respectively. As an internal standard, aliquots from each sample (a total of 12.5 µg in III and 25 µg in IV) were combined and labelled with Cy2 dye. Labelling reactions were performed for 30 min on ice in the dark, after which 1 µl of 50 mM lysine was added to stop the reaction. Labelled samples were stored at -20 °C.

4.3. Two-dimensional gel electrophoresis (2-DE)

In studies I and II, *S. cerevisiae* proteins (100 µg in study I and 75 µg in study II) were dissolved in 350 µl of IPG strip rehydration buffer containing 9 M urea, 0.5% CHAPS, 0.2% DTT and 0.5% Pharmalytes 3-10. Proteins were applied to IPG strips pH 3-10 (Immobiline™ Dry Strips, Amersham Pharmacia Biotech AB, Sweden) by in-gel rehydration. IEF was carried out using an IPGphor™ (Amersham Pharmacia Biotech AB) device. Prior to the second dimension the IPG strips were first equilibrated in a buffer containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 1% DTT and then in the same buffer where DTT was replaced with 4.5% iodoacetamide (IAA) in order to alkylate the free sulfhydryl groups of the cysteine residues. The second dimension, 12% SDS-PAGE, was carried out using a Protean® II xi apparatus (Bio-Rad) in study I and in a Hoefer DALT electrophoresis tank (Amersham Pharmacia Biotech AB) in study II. After electrophoresis, the gels were fixed in 30% ethanol and 0.5% acetic acid and then silver stained as described in I and II.

In studies III and IV, differentially labelled samples were mixed and combined with sample buffer containing 7 M urea, 2 M thiourea, 4.0% CHAPS and 30 mM Trizma base to a final volume of 20 µl in study III and 25 µl in study IV. IEF in studies III and IV was performed in a Protean IEF Cell (Bio-Rad). ReadyStrip™ IPG Strips (Bio-Rad) were rehydrated overnight in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Trizma base, 1% Biolyte 3-10 buffer for pH 3-6 and 5-8 strips. Biolyte 7-10 buffer was used for pH 7-10 strips. DTT and tributylphosphine (TBP) were added to 50 mM and 4 mM final concentrations, respectively. Samples containing 37.5 ug of *Lactobacillus gasseri* proteins in study III and 75 ug *Streptococcus uberis* protein in study IV were applied to rehydrated IPG strips by anodic cup loading. After IEF the strips were equilibrated first in 50 mM Tris-HCl pH 6.8, 6 M urea, 2% SDS, 20% glycerol and 2% DTT followed by a second equilibration step with 2.5% IAA. In study III the second dimension SDS-PAGE was run in a Criterion Dodeca Cell (Bio-Rad) with Criterion Pre Cast gels (8-16%, Bio-Rad). In study IV the second dimension was run in self-made 12% gels on an Ettan DALTsix Large Vertical System (GE Healthcare). After gel imaging, described in the original publications and in section 4, gels were fixed and stained with silver for visualization and spot cutting.

4.4. 2-DE image analysis

A detailed description of the image analysis parameters is given in the original publications.

In studies I and II, 2-DE gels were stained with silver and scanned with a GS-710 imaging densitometer (Bio-Rad). Raw scans were automatically processed by the PDQuest software (Bio-Rad). In studies III and IV, 2-DE gels with Cy-labelled proteins were for each of the three dyes imaged immediately after the second dimension between low fluorescent glass plates with an FLA 5100 scanner (Fujifilm Europe GmbH, Germany). Gel images were analyzed using Image Master Platinum 6.0 DIGE Software (GE Healthcare) in study III and with DeCyder 5.02 software (GE Healthcare) in study IV.

4.5. Protein identification by MS

Protein spots were manually cut out from the gels, reduced with DTT and alkylated with IAA before digestion with trypsin (Sequencin Grade Modified Trypsin, Promega Corporation, USA) at 37 °C overnight. The generated peptides were extracted, pooled and desalted. Peptides for MALDI-TOF MS analysis were, after desalting, eluted directly onto the MALDI-target. For LC-MS/MS analysis the peptide mixture was desalted, eluted and injected directly into an LC (I) or desalted and concentrated in a pre-column after which it was injected into an LC (II).

MALDI-TOF mass spectra of tryptic digests were acquired using a Bruker Biflex instrument (Bruker Daltonik, Germany) equipped with a nitrogen laser operating at 337 nm in positive ion reflector mode in studies I and II. In studies III and IV, the spectra were acquired using an Ultraflex II mass spectrometer (Bruker Daltonik). For LC-MS/MS analysis in studies I and II, reversed-phase chromatography was performed on an Ultimate nano-chromatography apparatus (LC Packings, the Netherland). ESI-MS/MS spectra were recorded using a Q-TOF hybrid mass spectrometer (Micromass Ltd, UK) equipped with an orthogonal electrospray source (Z-spray) and a modified nano-ES interface (LTQ-ADP, New Objective, Inc., USA).

The programs and servers used in protein identification with PMF data in studies I and II were ProFound (http://prowl.rockefeller.edu), the PepSea peptide map from Protana Inc. and MS-Fit (http://prospector.ucsf.edu/). For identification with sequence tag data, the PepSea sequence tag from Protana Inc, ProteinInfo (http://prowl.rockefeller.edu) and MS-Tag (http://prospector.ucsf.edu/) were used. In studies III and IV the spectra were interpreted using a local Mascot (Mascot 2.2.03, Matrix Science, UK) server. The searches were performed against the *Lactobacillus gasseri* ATCC 33323 (Version CP000413.1 GI:116094265) database produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) and against the *Streptococcus uberis* 0140J database, the sequence data obtained from the *S. uberis* sequencing group at the Sanger Centre (ftp://ftp.sanger.ac.uk/pub/pathogens/su/). The results were blasted against the nonredundant protein sequence database in NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) with a blastp algorithm (BLASTP 2.2.17).

5. Results and discussion

Methods in proteomics have significantly developed over last 15 years. Improvements have emerged in sample preparation techniques, electrophoretic separation methods and protein quantification as well as in new mass spectrometers. The growing amount of genomic data has also facilitated proteomic analyses of a growing number of species. In this thesis, gel-based proteomics have been exclusively used. Studies for original publications I and II were performed in 1999-2002 and studies for III and IV during 2006-2008. For the first two manuscripts, the proteomics technology platform was established to differentially display and subsequently identify the proteins. In the following two studies, a new technology at that time, DIGE, was applied for two different biological systems and proved to be superior in quantitation to previous studies. The development of proteomic methods between these years has been quite apparent and this thesis provides an interesting viewpoint on the development of these methods as well as of microbial proteomics.

5.1. Adaptation and evaluation of a method for identifying of *S. cerevisiae* proteins separated by 2-DE.

To adapt, develop and validate mass spectrometric methods for protein identification from metabolic pathways in a genetically modified yeast strain, MALDI-TOF PMF and LC-MS/MS methods were established (I). Spots of different intensities, pI values and molecular weights were analyzed. The aim was to collect data and validate the methodologies for future studies.

At the time of this work, MALDI-TOF PMF was the most commonly used method for protein identification. PMF is a rapid method and in many cases produces enough information for accurate protein identification. In our study a total of 27 silver stained spots from 2-DE gels were subjected to MS analysis. Of these, 21 were analysed with MALDI-TOF PMF, 19 of which were successfully identified (I, Table 1). Two unidentified spots were further analyzed and identified with sequence tag analysis by LC-MS/MS. As could be expected, the faintest spots with an estimated protein amount in the low femtomole range produced a weaker signal than the intensive ones. LC-MS/MS analysis was carried out on 17 of the 27 spots and all of these were successfully identified from acquired sequence information (I, Table 2 and Table 3).

PMF does not separate peptides with similar sequences from each other, whereas sequence tag analysis does. As an example, one of the analyzed spots was identified as phosphoglycerate kinase by PMF. It was not identified with a sequence tag algorithm, such as PepSea (http://www.unb.br/cbsp/paginiciais/pepseaseqtag.htm), but was successfully identified with the amino acid sequence search algorithm ProteinInfo (http://prowl.rockefeller.edu/prowl/proteininfo.html), in which the peptide molecular weight was not used as search parameter. In the database, the two last amino acids of this tryptic peptide were lysine-lysine [KK], which did not match the measured MW of the peptide. As was observed from the CID spectrum, the amino acid adjacent to the last amino acid was in fact arginine [RK], which indicates a point mutation (I, Figure 5).

The putative biological importance of this mutation remains to be studied.

Some proteins are observed as multiple spots on a 2-DE gel. This is due, for instance, to proteolytic processing of proteins creating protein fragments with different molecular masses and pIs or post-translational modifications, which also change the chemical character of a protein. Pyruvate decarboxylase isozyme (Pdc1) was identified in our study from one isolated and abundant spot and from one spot with a lower MW, potentially representing a protein fragment. The variability of Pdc1 has previously been discussed (Parker *et al.,* 1998), when in-depth analysis was performed for one of the densest regions in the total proteome 2-DE gel of *S. cerevisiae*. With the dimensions pI 5.5-6.5 and MW 35-65 kDa, 420 proteins were expected to be present (Hodges *et al.,* 1998). In that particular part of a 2-DE gel, Pdc1 and other abundant proteins (Adh1, Eno2 and Fba1) were clearly overloaded, so Parker et al. (1998) also expected to find peptides from these four abundant proteins from other protein spots. However, fragments of Pdc1 were found from discrete locations throughout the gel, from a total of 20 spots. The heterogeneity of Pdc1 was explained by potential proteolytic heterogeneity and residue specific modifications. Moreover, the three genes *PDC1*, *PDC5* and *PDC6* are rather homologous and are all expected to be found in the part of the gel studied. However, Parker et al. (1998) found no evidence for Pdc1 and Pdc6 proteins alone, although they found several peptides that are common to all three of these proteins. Pdc1 is reported to appear on gels in multiple spots according to the Yeast Protein Database (Perrot *et al.,* 1999).

As a conclusion from I, PMF was in most cases accurate enough for protein identification. LC-MS/MS, however, proved to be even more sensitive and provided more accurate data such as individual peak characterisation, and was very useful when analysing proteins with low abundances. When comparing the traditional nanospray MS/MS method with a combination of nano flow-rate LC combined with ESI-MS/MS, the latter provides better sensitivity with unseparated peptide mixtures. This makes it possible to identify proteins from amounts not sufficient for identification using the traditional nanospray method.

5.2. Proteome analysis of recombinant xylose-fermenting *S. cerevisiae.*

In study II, the platform technology established for yeast proteome analysis in I was applied in the study of recombinant xylose-fermenting yeast. This was done by comparing the proteomes in conditions in which glucose or xylose was the major carbon source. This was the first proteome-wide study of xylose-fermenting recombinant *S. cerevisiae*, providing a broad insight into the effects of xylose on cellular metabolism.

Because *S. cerevisiae* does not naturally utilize xylose or other pentose sugars, the introduction of an active xylose utilization pathway into *S. cerevisiae* was likely to have a major effect on cellular metabolism. The different cofactor preferences of xylose reductase and xylitol dehydrogenase are believed to disturb the cellular cofactor pool during xylose consumption. One of the objectives of this study was to determine how the redox imbalance affects cellular metabolism. Two different types of chemostat cultivation both with aerobic (20% oxygen) and anaerobic

(0% oxygen) phases, were carried out. The first cultivation was carried out with 10 g/l glucose and the second with 3 g/l glucose and 27 g/l xylose. The small amount of glucose was added to the latter feed in order to enable anaerobic growth on xylose. The residual glucose on both glucose and glucose + xylose cultures was zero under both aerobic and anaerobic conditions, so cells were under both conditions in a glucose derepressed state (Meijer *et al.,* 1998, Sierkstra *et al.,* 1992, ter Linde *et al.,* 1999).

Proteins were identified from spots showing consistent differences in intensity specific to either glucose or xylose as the carbon source (II, Table 2 and Figure 2). Proteins were identified mainly by PMF and when the identity of proteins was not established by PMF analysis alone, the previously described (I) LC-MS/MS method was applied. The proteins responding to the carbon source could be divided into 5 major categories: proteins of central carbon metabolism, TCA cycle and energy generation proteins, proteins of amino acid metabolism, proteins related to nucleotide metabolism, heat shock proteins and proteins having other functions.

The most significant changes in protein abundance between glucose and xylose-grown cells were detected in proteins of central carbon metabolism. These proteins were glycerol-3-phosphate dehydrogenase isoenzyme 1 (Gpd1), DL-glyserol-3-phosphate phosphatase isoenzyme 1 (Gpp1), enolase 2 (Eno2), alcohol dehydrogenase 2 (Adh2) and both mitochondrial and cytoplasmic acetaldehyde dehydrogenases, Ald4 and Ald6, respectively. Changes between xylose and glucose were seen in proteins related to glycerol metabolism, since the NADH-consuming production of glycerol has an important role in balancing the intracellular redox potential. Both Gpd and Gpp are encoded by two isogenes in *S. cerevisiae*. *GPD1* and *GPP2* are osmotically induced, while *GPD2* and *GPP1* are induced under anaerobic conditions. In our proteome study, Gpp1 had strongly elevated levels in anaerobicity and it responded more rapidly to the switch-off of oxygen in xylose than in the glucose culture. Both Gpd1 and Gpp2 had a lower abundance under oxygen depletion.

Five of six proteins of the TCA cycle and energy generation responding to the carbon source behaved in a quantitatively similar fashion in our culture conditions: anaerobic steady-state levels on both carbon sources were equal, but clearly lower than the corresponding steady-state aerobic level. Overall, a lower level was detected in all xylose samples. The abundance of one protein, identified as L-lactate cytochrome *c* oxidoreductase cytochrome b_2 (Cyb2), was shifted on the aerobic xylose, but was otherwise at a relatively low and constant level.

Heat shock proteins Hsp26 and Hsp78 were present at lower levels in the anaerobic glucose cultivation and somewhat higher levels during the entire xylose cultivation, probably suggesting that cells relying solely on xylose were stressed to some degree. Sse1, a heat shock protein belonging to the Hsp70 family, was strongly induced in the aerobic xylose cultivation, but remained at a very low level in the other cultivation steps.

A total of 12 proteins were identified from spots that responded only to the change in aerobicity in the two chemostat cultures (II, Table 3 and Figure 3). Under anaerobic conditions, oxidation of intramitochondrial NADH by the respiratory chain is stopped. Thus, the functioning of the TCA cycle is also

diminished. Mitochondrial glycerol-3-phosphate dehydrogenase (Gut2), mitochondrial manganese superoxide dismutase (Sod2), and heat shock proteins Stp1 and Sgt2 had a lower abundance in the anaerobic cultivations, and the 20S proteasome subunit (Pre3) could not be detected on anaerobic samples at all, indicating very strict control of their expression during anaerobic conditions. The changes detected between aerobic and anaerobic conditions are in accordance with the transcriptional analysis of aerobic and anaerobic chemostat cultures of *S. cerevisiae* (ter Linde *et al.,* 1999).

Altogether, 26 proteins were identified with constant abundance in these conditions (II, Table 4). The analysis showed that many proteins appeared in the gels as more than one spot with essentially the same molecular weight but different pI and abundances. This may be due to post-translational modifications. For all these proteins, the relative amounts of the different pI forms did not change with the culture modifications (Gygi *et al.,* 2000, Corthals *et al.,* 2000) (II, Tables 2, 3 and 4, Figure 1). Thus, these modifications may be considered as insensitive to the changes in conditions we applied (glucose/xylose, aerobic/anaerobic). We also identified five proteins that were novel identifications in the proteome of *S. cerevisiae*.

This study (II) was the first proteome-wide study of xylose-fermenting *S. cerevisiae*. The research project continued with a transcriptomic study (Salusjarvi *et al.,* 2006) and with another proteomic study, which revealed phosphorylation of several proteins with different abundance in cells grown on xylose and glucose. In the transcriptomic study (Salusjarvi *et al.,* 2006) DNA microarrays were used to analyze the samples derived from the aerobic phase from the same chemostat cultures that were studied with 2-DE in (II). Both downregulation of the citric-acid cycle and upregulation of reactions balancing the cellular redox state were also observed in the transcriptomic study and in a metabolic flux study carried out earlier (Pitkanen *et al.,* 2003). As a result of all these studies, it can be stated that xylose seemed also neither a repressive nor a derepressive carbon source in *S. cerevisiae*. As xylose fermenting point of view, the more efficient utilization of xylose will most probably require more complex and global changes in cellular metabolism.

5.3. 2-D DIGE analysis of *Lactobacillus gasseri* heat shock proteome.

In study III 2D-DIGE was used to obtain an overall picture of the cellular heat-shock response of *Lactobacillus gasseri*. Our analysis revealed ClpL as one of the most highly induced proteins in response to heat stress. To study the physiological role of ClpL, a *clpL* deletion mutant was created. The *clpL* deletion mutant derivative of *L. gasseri* showed substantially reduced survival at a lethal (60 °C) temperature. Moreover, the *clpL* deletion mutant was not able to induce the thermotolerance at the same temperature. These results demonstrate that *clpL* is essential for both constitutive and induced thermotolerance in this potentially probiotic strain. DNA-protein binding assays (III, Figure 2) revealed that *clpL* is regulated by a different protein from the *clp* gene regulator protein in other lactic acid bacteria (Varmanen *et al.,* 2000). However, the putative biological implication of the heterogeneity in *clpL* regulation among LAB remains to be determined.

The 2-D DIGE approach exposed a total of 20 spots that were up-regulated after 30 min heat shock at 49 °C. The most strongly induced proteins were identified as general heat shock proteins DnaK (4.4-fold induction) and GroEL (3.8-fold), HflX GTPase (4.4-fold), a pyrimide operon attenuation protein (2.5-fold), an ATPase of the ABC-type polar amino acid transport system (2.1-fold) and ClpL ATPase (4.4-fold) (Table 4 in III). The relative amounts of Clp proteins, ClpL, ClpE, ClpC and ClpE, were increased by at least 1.5 fold after heat stress. This most likely indicates the importance of Clp family proteins under stress conditions.

HflX and PyrR have not previously been identified as heat-inducible before. While the HflX family is almost universally conserved in all three superkingdoms of life (Caldon and March 2003), its role in the regulation of cellular functions is largely unknown. Heat-shock-related induction in our study might indicate that HflX is an essential regulator during stress in ATCC 3323. The pyrimidine syntesis regulator, PyrR, was one of the proteins found to be up-regulated after heat shock. The 2.5-fold increase in its expression could be part of the mechanism by which cells slow down their pyrimidine synthesis in response to the reduced replication rate following heat shock.

In this thesis, study III was the first in which DIGE was used to accurately detect abundance changes in 2-DE. Compared to traditional 2-DE used in studies I and II, 2D-DIGE provided an efficient overview of the total proteome and a simultaneous view of differentially expressed proteins. In 2D-DIGE, changes in protein abundance can be instantly monitored with different colours after a gel scan. The software for 2D-DIGE analysis reveals information about every spot that is differentially regulated. In recent years, DIGE has been applied in bacterial proteome studies on several organisms (Rathsam *et al.,* 2005b, Rathsam *et al.,* 2005a, Hu *et al.,* 2003, Hongsthong *et al.,* 2007, Lopez-Campistrous *et al.,* 2005, Yan *et al.,* 2002, Jeamton *et al.,* 2008, Park *et al.,* 2007).

For study III, the pH range 3-10 was divided into three overlapping regions; 3-6, 5-8 and 7-10, with medium-range IPG strips. Such overlapping strips widen the area used to display the proteins in the pI range, which improves the separation of adjacent spots in gels. The narrow-range IPGs (nrIPGs), e.g. with one pH unit / 18 cm, allow the loading of a higher protein concentration per pH unit (Corthals *et al.,* 2000). Thus, proteins are displayed over a greater distance with increased resolving power. Narrow and overlapping pH gradients (4-5, 4.5-5.5, 5-6, 5.5-6.7 and 6-9) were compared with traditional pH 3-10 and pH 4-7 strips in a study where the aim was to determine the maximum number resolvable *S. cerevisiae* proteins (Wildgruber *et al.,* 2000). As expected, the number of detected spots was considerably higher with narrow pH range strips; a total of 2286 protein spots were detected compared to 775 spots with traditional pH range IPG strips. For our research question, division of the pH range 3-10 into three ranges, instead of using narrow-range strips, proved to be sufficient and we were able to overcome the difficulty of resolving and detecting proteins in the basic region.

5.4. Proteomic profiling of the response of mastitis pathogen *Streptococcus uberis* to antibiotic ciprofloxacin using 2-D DIGE and MALDI-TOF PMF.

In study IV, previously adapted 2-D DIGE and MALDI-TOF PMF protein identification were used to analyse global changes in the proteomes of *Streptococcus uberis* ATCC BAA-854 strain in response to ciprofloxacin (CF) challenges and demonstrated that *S. uberis* responds to CF by controlling the synthesis of enzymes with a potential role in the oxidative damage response and in nucleotide biosynthesis.

S. uberis is an important environmental mastitis pathogen. Mastitis is the most common and costly disease in dairy cows and remains a major problem for the dairy industry, causing severe economic losses resulting partly from decreased milk production and partly from increased management costs. In the past the biggest economic losses were due to contagious pathogens such as *Streptococcus agalactiae* and *Staphylococcus aureus* (Erskine *et al.,* 1987, Myllys *et al.,* 1998, Pyorala 2002). During recent decades the proportion of environmental pathogens as mastitis-causing agents has increased in many countries (Bradley *et al.,* 2007, Dingwell *et al.,* 2004, Kossaibati *et al.,* 1998, Peeler *et al.,* 2003). Mastitis is the most common reason for antimicrobial treatment of dairy cows (Grave *et al.,* 1999, Valde *et al.,* 2004). Altogether, the use of antimicrobials against human and veterinary diseases has been extensive during the last decades. Thus, pathogenic bacteria are repeatedly exposed to antimicrobials, which is likely to induce a variety of survival strategies to enhance viability and successful virulence.

It is suggested that *S. uberis* species are able to adapt to antibiotic treatments (Milne *et al.,* 2005). However, the mechanisms underlying this adaptive mutagenesis in *S. uberis* and in other *Streptococcus* species are largely unknown. The use of fluoroquinolones, such as ciprofloxacin, is coming into greater focus, since exposure to these antibiotics has been shown to increase genetic variation, and promote the evolution of resistance by altering metabolism and transiently increasing the mutation rate in a growing number of Gram-negative and Gram-positive bacteria (Cirz *et al.,* 2006, Cirz and Romesberg 2006, Power and Phillips 1992, Ysern *et al.,* 1990, Blazquez *et al.,* 2002, Beaber *et al.,* 2004, Cirz *et al.,* 2005, Cirz *et al.,* 2007, Henderson-Begg *et al.,* 2006, Ubeda *et al.,* 2005, Prudhomme *et al.,* 2006).

In study IV, the proteomes of untreated and CF-treated (0.5 or 1.0 μg/ml) *S. uberis* cells were compared. At a CF concentration of 1.0 μg/ml (1.0 x minimum inhibitory concentration, MIC), 24 spots showed a significant change in the level of expression between control and CF-treated cells (IV, Table 2). The spots were identified by MS and corresponded to 20 separate proteins. The comparison of *S. uberis* proteomes treated with CF at 0.5 MIC to those of untreated proteomes revealed seven protein spots exhibiting statistically different changes. These changes were parallel to but somewhat lower than the changes detected in the proteomes of *S. uberis* treated with CF at 1.0 MIC. In this study, protein identifications revealed two classes of proteins that might be involved in *S. uberis* mutation mechanisms and stress responses: proteins that are potentially involved in the balance of deoxyribonucleotide triphosphate (dNTP) pools and proteins

that function in maintaining the peroxide balance of the cell.

5.4.1. Ciprofloxacin exposure induces an oxidative damage in *S. uberis*.

CF exposure is reported to cause oxidative stress in pathogens such as *S. aureus* (Becerra and Albesa 2002). In study IV, the F subunit of the alkyl hydroperoxide reductase (AhpF) was the most up-regulated protein (IV, Table 2). In *S. pyogenes* it is reported to function in maintaining the peroxide balance in cell by controlling endogenously produced peroxide (Brenot *et al.,* 2005). It was recently shown that cellular death induced by all classes of bactericidal antibiotics occurs by promoting the formation of harmful hydroxyl radicals (Kohanski *et al.,* 2007). Bacteria adapt to the presence of hydroxyl radicals and other reactive oxygen species (ROS) by increasing the expression of detoxification enzymes such as superoxide dismutase, catalase, and peroxidase in order to reduce the level of ROS (Storz and Imlay 1999). Streptococci, including *S. uberis*, do not express catalase and therefore they must rely on other strategies to adapt to peroxide stress. In *S. uberis* the up-regulation of potential detoxification enzymes, such as AhpF, may play a role in controlling the peroxide level in the cell when the quantity of reactive oxygen species is increased by ciprofloxacin exposure.

Nicotinamine adenine dinucleotide (NADH) is a reducing agent that carries electrons from one cellular reaction to another. A reduction in the available pool of NADH has been shown to increase the survival of *E. coli* cells after fluoroquinolone exposure by decreasing the formation of ROS (Jonsson *et al.,* 2007). Thus, the enzymes involved in the generation of NADH could be potential mediators of the response to oxidative damage in *S. uberis* cells. The enzymes sorbitol-6-phosphate 2-dehydrogenase, fructose-6-phosphate amidotransferase, glyseraldehyde-3-phosphate dehydrogenase and phosphoglyserate kinase were all down-regulated during CF treatment (IV, Table 2). In oral streptococci, sorbitol metabolism has also been shown to be reduced upon oxidative stress (Iwami *et al.,* 2000). Thus, it can be predicted that metabolic pathways leading to the reduced availability of NAHD and to an increase in the synthesis of detoxifying enzymes will provide *S. uberis* with increased resistance to oxidative damage.

5.4.2 Enzymes controlling the deoxynucleotide pool in *S. uberis* were affected by ciprofloxacin

Aerotolerant anaerobic bacteria, such as streptococci, are able to synthesize DNA both in the presence and the absence of oxygen. This can be achieved by different ribonucleotide reductases (RNRs), enzymes that provide the building blocks for DNA synthesis and repair (Nordlund and Reichard 2006). The regulation of RNR synthesis has a direct effect on dNTP pools (Nordlund and Reichard 2006) and the sizes of intracellular dNTP pools are highly regulated (Herbig and Helmann 2001, Reichard 1988). Both balanced accumulation and unbalanced dNTP pools are known to be mutagenic (Kunz *et al.,* 1994). In *E. coli*, overexpression of two aerobic RNRs resulted in roughly proportional increase in dNTP pools and the rate of spontaneous mutation was increased 40-fold (Iwami *et al.,* 2000).

In study IV, CF exposure significantly increased the abundance of some proteins involved in the dNTP pool. Eight protein

spots corresponding to 5 different proteins potentially involved in the synthesis of dNTPs were up-regulated in response to CF challenge; ribonucleoside diphosphate reductase (NrdA), deoxyribose aldolase (DeoC), ribose-phosphate pyrophosphokinase (Prs), adenylosuccinate synthetase (AdsS) and inositol-5-monophosphate dehydrogenase (ImpDH) abundances were positively regulated by 2.2, 1.6, 1.7, 1.5 and 2.0 fold, respectively.

The proteomic data from study IV suggest that *S. uberis* copes with oxidative damage caused by CF exposure by reducing the available pool of NADH. This might have forced *S. uberis* to consume dNTPs as an alternative energy source, supported by the finding that the abundance of deoxyribosephosphate aldolase (DeoC) was increased as a result of CF challenge (IV, Table 2). This enzyme is known to play a role in the catabolism of dNTPs arising from dead cells (Sgarrella *et al.,* 1997). Thus it gives a selective advantage to microorganisms with a capability to consume DNA as an alternative energy source and may confer

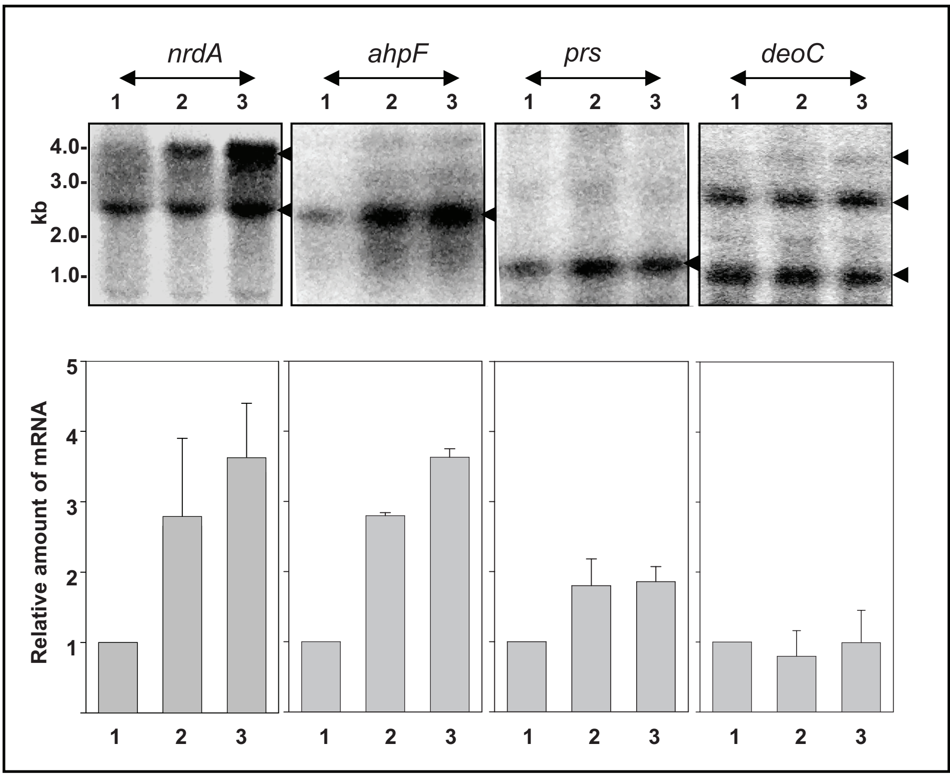

Figure 9. *Northern blot analyses of* nrdA *(SUB1225), ahpF (SUB1753), prs (SUB0020) and deoC (SUB0952) expression in S. uberis ATCC BAA-854 before (lane 1) and 60 min after the addition of CF at 0.5 µg/ml (lane 2) and 1.0 µg/ml (lane 3). Bar diagrams show the relative mRNA induction ratios calculated by dividing the signal from the RNA sample by the signal from the RNA sample at time zero. RNA amounts were corrected after rRNA hybridization (data not shown), and results represent the mean values of two independent experiments with standard errors. The sizes of mRNAs were estimated according to an RNA molecular weight marker (Promega). Detected transcripts are marked by black triangles.*

a growth advantage over other bacteria, as evidenced by previous studies on *S. mutans* (Han *et al.,* 2004).

The transcriptome studies on CF-challenged *P. aeruginosa, S. aureus*, and *S. pneumoniae*, as well as its FQ-resistant derivative strain, demonstrated the induction of pathways contributing to increased mutagenesis after CF exposure (Cirz *et al.,* 2006, Cirz *et al.,* 2007, Marrer *et al.,* 2006). Other metabolic changes associated with CF challenge were suggested to provide *S. aureus* and *P. aeruginosa* time to persist and evolve resistance (Cirz *et al.,* 2006, Cirz *et al.,* 2007). In the case of *S. pneumoniae*, the acquisition of CF resistance was shown to lead to a metabolic state in which the error-prone DNA repair pathways are altered to correct the potential effects of CF (Marrer *et al.,* 2006). On the other hand, previous studies on *S. uberis* (Varhimo *et al.,* 2008, Varhimo *et al.,* 2007) and *S. pneumoniae* (Henderson-Begg *et al.,* 2006) suggest a minor role for error-prone polymerases in CF-induced mutagenesis in *Streptococcus*. It has been shown, that *S. uberis* employs distinct mechanisms for UV- and ciprofloxacin-induced mutagenesis (Varhimo *et al.,* 2008). Although ciprofloxacin induces the expression of *umuC,* a gene coding for an error-prone polymerase V, *umuC* is not necessary for ciprofloxacin-induced mutagenesis in *S. uberis*. Ciprofloxacin does not induce the expression of dnaE or dinP, the polymerase subunits of other known error prone polymerases, implying that alternative, as yet unknown mechanisms that contribute to induced mutations exist in *S. uberis*.

The proteomic analysis and results of study IV demonstrate that *S. uberis* responds to CF -exposure by changing its metabolism so that it can survive the changes in conditions. The results obtained here support previous findings reported by Varhimo et al. 2008, and show that other, as yet unknown mechanisms exist for the induction of mutagenesis. RNA-level studies (Figure 9. unpublished) based on the findings from IV support the results obtained here by also showing a clear induction of *ahpF* and *nrdA* at the RNA level.

6. Concluding remarks

Proteomic studies involve diverse analytical techniques, data analyses and the use of databases for the systematic study of the proteome. Proteomics was initially considered as the identification of proteins from 2-DE gels, but is now associated with a great variety of analysis techniques covering protein identification, characterisation and quantification.

In this thesis, proteomic methods were established and applied to study the proteomes of eukaryotic baker's yeast *Saccharomyces cerevisiae*, the probiotic bacterium *Lactobacillus gasseri* and the mastitis pathogen *Streptococcus uberis* under different biological conditions. Methods in proteomics, from sample preparation to protein identification, have developed during the years these studies were performed. In the 1990s, protein identification methods rapidly developed and many sample preparation methods and instrumental enhancements took place. For example, MALDI-TOF MS in studies I and II was an older generation instrument than that applied in studies III and IV, which were carried out using MALDI-TOF/TOF.

In the studies forming the first part of this thesis (I and II), proteomic investigations were carried out on baker's yeast *S. cerevisiae*. In study I, protein separation by 2-DE as well as methods for protein identification were established. Proteins were identified by MALDI-TOF PMF and LC-MS/MS sequence tag analysis, for which LC with a low flow rate was coupled with an ESI-Q-TOF instrument. These methods were applied and further developed in study II, where proteome analysis of recombinant xylose-fermenting baker's yeast, *S. cerevisiae*, was performed. In study II the LC was further equipped with pre-column for sample desalting and concentration, which enabled the direct injection of peptide digest into the LC without manual desalting. Study II was the first proteome-wide study of recombinant xylose-fermenting *S. cerevisiae*, revealing a broad insight into the effects of xylose on cellular metabolism.

In the background to studies I and II lies increasing concern over global climate change and the search for alternatives to petroleum-derived fuels. Nowadays, bioethanol is the most common replacement product (Gray *et al.,* 2006, Zaldivar *et al.,* 2001, Nevoigt 2008). Bioethanol production from cellulosic biomass by fermentation with *S. cerevisiae* is one of the most studied areas in this field (Cardona and Sanchez 2007, Chu and Lee 2007, Hahn-Hagerdal *et al.,* 2007, Zaldivar *et al.,* 2005). Xylose is abundant in the hemicellulose fraction of the biomass, and the utilization of pentose sugars such as xylose has been widely studied in *S. cerevisiae* (Chu and Lee 2007, Hahn-Hagerdal *et al.,* 2007). The research project on recombinant xylose-metabolising *S. cerevisiae* continued with a transcriptomic (Salusjarvi *et al.,* 2006) and another proteomic study (Salusjarvi *et al.,* 2008). Together, these revealed numerous changes in gene expression, protein abundance and post-translational modifications of proteins in cells metabolising xylose compared to those growing on glucose. This, combined with all the other metabolic engineering studies on xylose-utilising *S. cerevisiae*, remains a challenging task that will require further progress in bioinformatics.

In the second part of this work, 2-D DIGE, an advanced quantitative electrophoretic protein separation method based on traditional 2-DE, was applied to

bacterial proteomics. In study III, the heat shock response of the probiotic bacterium *L. gasseri* was analysed. Probiotics are of growing economic interest as components of the consumables available to provide health benefits for humans and animals. Probiotics and the mechanisms by which they exert positive effects on the composition of the gut microbiota are subjects of intensive research. Proteomic methods, among others, have succeeded in revealing the stress responses of probiotic bacteria, and in the future they will most probably also provide new insights into the probiotic nature of bacteria.

In study IV, the mastitis pathogen *S. uberis* was challenged with mutagenesis-inducing antibiotic ciprofloxacin, and the changes in proteome were monitored with 2-D DIGE. An inevitable side effect of the use of antibiotics is the emergence and spread of resistant bacteria and resistance genes. The use of antimicrobials in pet animals and animal husbandry is extensive, and imposes selective pressure on certain bacteria of animal origin. Fluoroquinolones are broad-spectrum antibiotics that inhibit the replication of DNA and are widely used in veterinary medicine. Bacterial resistance to fluoroquinolones has unfortunately, been reported in various studies (Marrer *et al.,* 2006, Varhimo *et al.,* 2008, Angelakis *et al.,* 2008, Descloux *et al.,* 2008, Gerboc *et al.,* 2008, Kim *et al.,* 2008, Maki *et al.,* 2008). Fluoroquinolone antibiotics are sometimes released into the environment due to incomplete waste-water treatment (Batt *et al.,* 2006) and they appear to be quite stable in the environment (Turiel *et al.,* 2005). Bacteria and fungi are among the first components in ecosystems affected by pollutants from waste water and other sources. The relatively small size and simplicity of the bacterial genome makes them particularly attractive models for the detection of changes on the proteome level. In environmental proteomics (Nesatyy and Suter 2007) two of the subjects discussed in this thesis, antibiotic resistance and stress response of bacteria, meet in a fascinating way. Environmental proteomics allows the analysis of the interactions between environmental and biological factors, such as how exposure to antibiotics or other chemical agents and physical stress can alter the proteome expression patterns leading to health effects and disease in humans and in animals.

In proteomic studies, MS is considered as one the core technologies. Developments and improvements in MS-based techniques have revolutionized the identification of proteins. Instrumentation, software and data management capabilities enable proteomics to be widely applied in biological research as well as in the medical and food industry. Ongoing developments in automation and sophisticated data acquisition and interpretation will make it possible to obtain a greater amount and quality of data. Eventually, this could enable the study of all protein complexes and organelles that can be purified from a cell. However, the identification of the proteins present in a sample is only the first step in the process of understanding their functions. Thus, a successful model of protein function and regulation pathways in the cell requires a broad understanding of protein interaction with other proteins and a comprehensive understanding of cellular metabolism.

The proteomic methods established, developed and applied in this thesis proved their strength in visualizing, detecting and identifying the proteins of interest. Proteomics is a rapidly developing area of research and new technologies are being developed and validated. The combination of proteomics and other -omics data,

such as genomics, transcriptomics, metabolomics and bioinformatics, will lead to a more complete understanding of the biology of systems at the molecular level.

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