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GEL-BASED PROTEOMIC COMPARISON OF TWO STRAINS OF *PROPIONIBACTERIUM FREUDENREICHII* SSP. *SHERMANII*

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Tiivistelmä — Referat — Abstract

The literature review describes the characteristics, uses and probiotic potential of propionibacteria and gives an overview of proteomic methods focusing on 2-DE studies related to probiotic bacteria and *P. freudenreichii.* The aim of the experimental part was to use 2-DE to obtain proteome maps of two strains of *P. freudenreichii* and detect inter-strain differential protein expression. The influence of a number of parameters on the quality of 2-D gels was also investigated.

The pH 3-7 and pH 3-11 NL proteome maps obtained displayed 305 and 356 spots, respectively, and detected the differential expression of 60 and 72 spots, respectively. The 3-11 NL proteome map showed spot over-crowding in the pH 4-6 region. Addition of 50 mM dithiothreitol (DTT) to the samples prior to isoelectric focusing (IEF) and an increase of IPG buffer concentration greatly improved spot resolution. Silver staining achieved higher spot counts than Coomassie staining. The influence of the number of lysis cycles on protein extraction was marginal, while lower protein sample loads produced superior 2-D gels.

Results show that data from 2-DE proteome maps can provide an overview of inter-strain differential protein expression in *P. freudenreichii*. The optimal 2-DE experimental setup would include pH 4-7 IPG strips, a low protein load and inclusion of DTT in the IEF sample, as well as silver staining for spot visualisation.

Avainsanat — Nyckelord — Keywords
2-DE, proteomic, probiotic, Swiss cheese, vitamin B_{12}, *Propionibacterium freudenreichii*

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PREFACE

This study was carried out at the Department of Food and Environmental Sciences, Diary Technology division, Faculty of Agriculture and Forestry of the University of Helsinki.

Firstly, I would like to thank my supervisor, Docent Pekka Varmanen, to whom I am very grateful for the guidance and the support provided during this study and for the enjoyable working environment he created. My warm thanks to Kirsi Savijoki, PhD, and Kerttu Koskenniemi, PhD, for their invaluable help during the experimental work.

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Finally, I am eternally grateful to my parents for their constant encouragement and affection.

Helsinki, June 2012

Andrei Prodan

LIST OF ABBREVIATIONS

2-DE	2-dimensional gel electrophoresis
ACNQ	2-amino-3-carboxi-1,4-naphtoquinone
BCAA	branched-chain amino acids
BGF	bifidogenic growth factor
CFU	colony-forming units
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DHNA	1,4-dihydroxy-2-naphtoic acid
DTT	dithiothreitol
DIGE	difference gel electrophoresis
ESI	electrospray ionization
GIT	gastrointestinal tract
GRAS	Generally Recognized As Safe
IPG	immobilized pH gradient
LAB	lactic acid bacteria
MALDI	matrix-assisted laser desorption/ ionization
M_r	molecular mass
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
ORF	open reading frame
pI	isoelectric point
PCA	principal component analysis
PMF	peptide mass fingerprinting
PTM	post-translational modification
QPS	Qualified Presumption of Safety
SCFA	short chain fatty acids
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis

TBP	tributhylphosphine
TCA	tricarboxylic acid
TOF	time-of-flight mass analyzer

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1 INTRODUCTION

Propionibacterium freudenreichii is the representative species for dairy propionibacteria, best known for its long tradition of use in the production of Swiss-type cheeses where it has a critical role in flavor development and the formation of the distinctive holes (“eyes) (Thierry et al. 2011). Propionibacteria are relatively slow-growing, resilient bacteria which display a distinctive metabolism using lactate as a main substrate and producing propionate, acetate and CO_2 as the principal end-products (Piveteau 1999). They show excellent resistance to starvation and cold stress due to their array of carbon and energy storage molecules (Falentin et al. 2010). The importance of propionibacteria extends beyond cheese-making. The constitutively high production of cobalamin is the reason why *P. freudenreichii* is one of the main bacteria used in vitamin B_{12} industrial biosynthesis.

Probiotics have been defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2002). Health effects include improvement of gastrointestinal tract (GIT) integrity, protection from GIT colonization by pathogens as well as immunomodulatory and anti-inflammatory action (Oelschlaeger 2010). Resistance to digestive and technological stresses is an important criterion for probiotic strain selection (Saarela et al. 2000). Evidence extracted from *in vitro* or animal model studies pointing to pathogen antagonism, anti-inflammatory or anti-mutagenic effects is also commonly employed in the screening for probiotic candidates (Sanders 2008).

During the last two decades data has accumulated pointing to *P. freudenreichii* strains as potential probiotics (Cousin et al. 2010). Arguments supporting this view include thorough safety certifications as well as good resistance and cross-protection effects to acid and bile stresses. The main health benefit associated with *P. freudenreichii* is the increase in the counts of beneficial *Bifidobacterium* ssp. in the colon due to the production of bifidogenic growth factors (BGFs) (Warminska-Radyko et al. 2002). *P. freudenreichii* has also been shown to inhibit the gastric pathogen *Helicobacter pylori* and has putative anti-mutagenic and anti-carcinogenic effects (Jan et al. 2002a; Vorobjeva et al. 2008; Nagata et al. 2010).

Proteomics – the study of the entire protein complement of a cell - has emerged as a powerful tool in bacterial research. It relies on either a gel-based or gel-free separation procedure, with mass spectrometry analysis used for the identification of the individual proteins. Two-dimensional gel electrophoresis (2-DE) has been used extensively to advance the understanding of bacterial physiology. 2-DE can provide proteome reference maps for inter-strain comparisons or detect differential expression of specific proteins caused by different culturing or environmental conditions. Proteomic studies have obtained valuable information on probiotic bacteria stress responses, GIT adhesion mechanisms and host interaction. Furthermore, proteomics promises to offer solutions for improved probiotic strain selection (Siciliano and Mazzeo 2012). However, the extent of proteomic research into *P. freudenreichii* is still very limited.

This small-scale study aimed to optimize a few preliminary steps and 2-DE protocols for application to propionibacteria and visualize the proteomes of two strains of *P. freudenreichii* ssp. *shermanii* showing different phenotypic traits. The proteomes of the two strains were then compared in order to detect differential protein expression which might provide future clues to the cause of phenotypic dissimilarities.

The first part of the theoretical review looks at scientific literature on propionibacteria focusing on *P. freudenreichii* and describes its characteristics, metabolism and technological importance. The second part gives a brief introduction into probiotics and reviews the features of *P. freudenreichii* which support its status as a candidate probiotic. The third part offers an overview of proteomic research applied to microbiology, discusses the advantages and limitations of 2-DE techniques and reviews previous proteomic studies on probiotic bacteria as well as the yet limited number of proteomic studies centered on *P. freudenreichii.* The experimental section describes the protein extraction, purification and 2-DE protocols and the attempts made to improve them for application to *P. freudenreichii.* The proteome maps obtained from the two strains are subsequently shown and some factors influencing their quality are discussed. Finally, the observed protein differential expression is examined.

2 LITERATURE REVIEW

2.1 Propionibacteria – characterization and importance

2.1.1 General description of propionibacteria

Propionibacteria were discovered in Swiss cheese more than a hundred years ago by von Freudenreich and Orla-Jensen, who were the first to propose the genus *Propionibacterium* and to link the presence of *Propionibacterium freudenreichii* to the formation of the characteristic round holes ("eyes") in Emmental cheese (Von Freudenreich and Orla-Jensen 1906). Propionibacteria belong to the Actinobacteria class containing Gram-positive, high G+C (64-68%) bacteria and have been described as being non-sporing, non-motile pleomorphic rods. All members of *Propionibacterium* are mesophilic, aerotolerant, generally catalase positive, with optimal growth at 30 °C and in neutral pH; they produce propionate as the main final product of metabolism (Cousin et al. 2010; Thierry et al. 2011). The 12 species composing the genus are divided into two functional groups, based on their habitat of origin: dairy propionibacteria and cutaneous propionibacteria (Cousin et al. 2010; Euzeby 2012). The following review will deal only with dairy propionibacteria.

P. freudenreichii, the most important species of the genus, has been split into two subspecies based on the ability to ferment lactose and reduce nitrate: *P. freudenreichii* ssp. *freudenreichii* which cannot use lactose but is nitrate reductase-positive, and *P. freudenreichii* ssp. *shermanii* which shows the opposite characteristics. According to this classification, the two strains examined in the experimental part of this thesis belong to *P. freudenreichii* ssp. *shermanii*. However, it should be noted that this subspecies classification has recently come under attack as *P. freudenreichii* strains displaying the other two possible phenotypes have also been discovered (Dalmasso et al. 2011). The annotation of the first dairy propionibacteria genome sequenced (that of the type-strain *P. freudenreichii* CIRM-BIA1^T) revealed that the *lacZ* gene coding for galatosidase is hosted on a mobile element which has probably been acquired through horizontal gene transfer through phage infection, further indicating that this taxonomic division might be unsatisfactory (Falentin et al. 2010).

2.1.2 Metabolism of propionibacteria

Dairy propionibacteria display a relatively slow growth rate - with a generation time of around 5 h in optimal conditions – partially explained by the fact that their genome contains only 2 rRNA operons (Falentin et al. 2010). A low copy number of rRNA operons is indicative of bacteria that have evolved to use nutrients in a very efficient manner, sacrificing growth speed under optimal conditions in order to adapt to low-nutrient substrates (Klappenbach et al. 2000; Lee et al. 2009).

Propionibacteria are able to metabolize a wide range of energy substrates, including galactose, fructose, glycerol, lactate and pyruvate. Lactate is the main substrate used by dairy propionibacteria in their typical ecological niche, Swiss cheese (Piveteau 1999). Propionibacteria are prototrophic for all amino acids and nucleotides, as well as for most vitamins. All *P. freudenreichii* strains are however auxotrophic for panthotenate (vitamin B5) and biotin (vitamin B7), while some strains also need thiamine (vitamin B1) and p-aminobenzoic acid in order to grow (Falentin et al. 2010). The metabolic pathway distinctive for propionibacteria and the source of its characteristic production of propionate is the Wood-Werkman cycle - also known as the transcarboxylase cycle (Wood 1981; Piveteau 1999; Thierry et al. 2011).

Propionibacteria metabolism is complex and consists of several parallel and interlinked metabolic pathways. Substrates are converted into pyruvate through glycolysis or by the pentose phosphate pathway. Pyruvate may then follow two alternative routes: oxidative decarboxylation to acetate and CO_2, or reduction to propionate via the Wood-Werkman cycle linked to a series of tricarboxylic acid (TCA) reactions, as shown in Figure 1 (Falentin et al. 2010). As a result, the main end-products of propionibacteria fermentation besides propionate are succinate, acetate and CO_2.

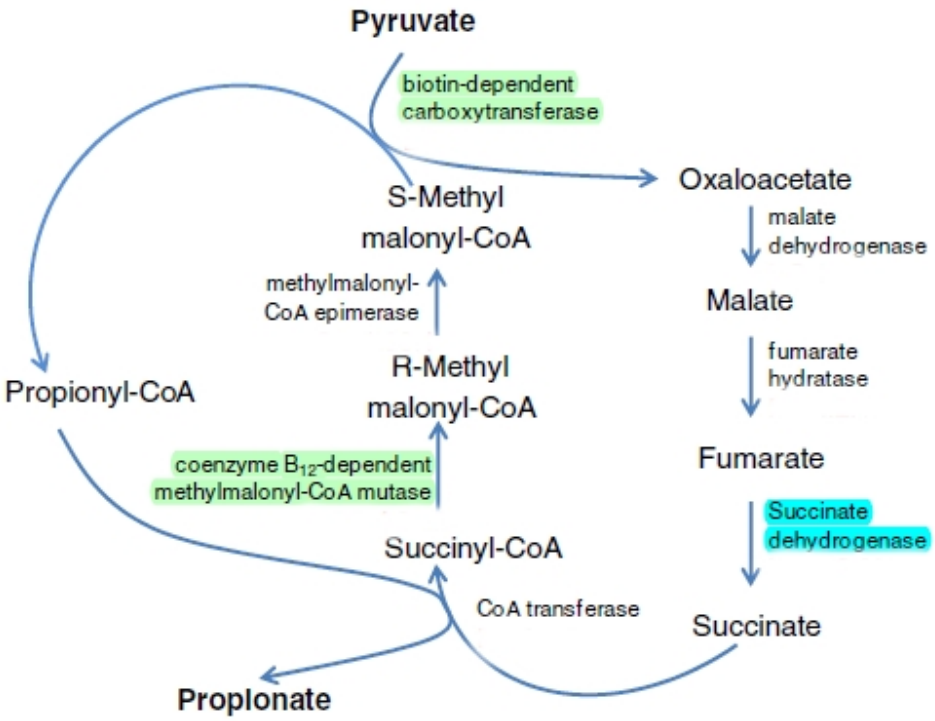

Figure 1. Simplified schematic of the Wood-Werkman cycle in propionibacteria (adapted from Thierry et al. 2011).

Propionibacteria are capable of co-metabolizing aspartate / asparagine together with lactate through the deamination of aspartate to fumarate and its subsequent reduction to succinate. The use of aspartate as a substrate causes a shift in pyruvate conversion, decreasing the amount undergoing reduction through the Wood-Werkman cycle and increasing the proportion of pyruvate oxidized to acetate and CO_2. The molar ratio of end-products may vary in propionibacteria fermentation depending on the range of substrates used, as the cell adjusts the ratio of pyruvate being oxidized / reduced in order to maintain the redox balance (Piveteau 1999; Thierry et al. 2011).

Three enzymes which are particular for propionibacteria are emphasized in Figure 1. Succinate dehydrogenase (highlighted in blue) is a membrane-bound protein which catalyzes the reduction of fumarate to succinate, a respiratory process associated with the production of

ATP and reduced coenzymes through cytochrome b-mediated electron transport (Falentin et al. 2010). Two cofactor-dependent enzymes (highlighted in green, left side of the schematic) catalyze the transfer of carboxyl groups. The first one is a biotin-dependent carboxytransferase directing the reaction between methylmalonyl-CoA and pyruvate in order to form oxaloacetate and propionyl-CoA. Oxaloacetate then enters a succession of TCA reactions and is converted into succinate, which subsequently receives a CoA group from propionyl-CoA in a step linking the TCA and Wood-Werkman cycles and resulting in succinyl-CoA and propionate. The other distinctive enzyme involved in carboxyl transfer in propionibacteria is the coenzyme B_{12}-dependent methylmalonyl-CoA mutase catalyzing the isomerization of succinyl-CoA to methylmalonyl-CoA (Thierry et al. 2011). As there is practically no free CO_2 fixation involved in the Wood-Werkman cycle and CoA is recycled, the production of propionate is a energetically efficient process, adding to the energy yield obtained from glycolysis and pyruvate oxidation (Piveteau 1999).

Propionibacteria are capable of prolonged survival in conditions of starvation and stationary phase and display a range of characteristics that allow for an efficient multi-stress response. The key to their resilient nature is the ability to use a varied array of molecules to store carbon and energy (Falentin et al. 2010; Thierry et al. 2011). Firstly, in a manner often observed in bacteria adapted to harsh ecological environments, propionibacteria predominantly rely on inorganic polyphosphate (polyP) reserves as a means of energy storage, instead of the more usual ATP accumulation (Seufferheld et al. 2008). PolyP are linear molecules composed of tens to hundreds of orthophosphate groups linked by energy-rich phosphoanhydride bonds whose formation in propionibacteria is catalyzed by a polyphosphate kinase (Thierry et al. 2011). Enzymes using polyP as an energy supply allow for an increased number of reactions to run reversibly. This is true for all the reactions of the Wood-Werkman cycle, thus providing a significant deal of metabolic flexibility (Deborde et al. 1999; Falentin et al. 2010). Secondly, propionibacteria can store carbon either in the form of glycogen (α-1,4 linked, α-1,6 branched glucose chains) or as trehalose (a non-reducing disaccharide with a α,α-1,1-glucoside bond between two glucose monomers) giving them enhanced long-term survival capabilities under

conditions of carbon starvation (Deborde et al. 1996; Falentin et al. 2010). Trehalose also acts as a compatible solute and a molecular chaperone, although the level of trehalose accumulation in *P. freudenreichii* is strain-dependent. It is synthesized at the beginning of the stationary phase or under stress conditions, while the build-up of polyP and glycogen occurs during the exponential phase (Deborde et al. 1996; Cardoso et al. 2007; Falentin et al. 2010). Finally, propionibacteria have been found to produce glycine betaine (N,N,N-trimethylglycine), another compound with chemical chaperone activity that aids in long-term, stationary stage survival (Falentin et al. 2010).

2.1.3 Technological importance of propionibacteria

The main historical use of propionibacteria - particularly *P. freudenreichii* - was as a ripening culture in Swiss-type cheeses, where they are responsible for the development of flavor compounds as well as for the formation of the characteristic “eyes” through the intense production of CO_2 (Paulsen et al. 1980; Noel et al. 1999; Steffen et al. 1999). They may occasionally be present in a range of other cheeses, although at low counts (Thierry et al. 2011). Propionibacteria are not milk-adapted and grow poorly in milk; they cannot hydrolyze casein, but their growth in cheese is aided by the proteolytic action of the lactic acid starters which provide a reservoir of readily-available peptides (Dupuis et al. 1995; Falentin et al. 2010). Partially due to their high resistance to thermal stress compared to starter cultures, propionibacteria survive the usual heat treatment of 50 - 55 °C for 30 min applied to Swiss-type cheese curd and may reach densities of 10^8 - 10^9 CFU / mL during the ripening stage (Beuvier et al. 1997; Thierry et al. 2011). The critical characteristic allowing propionibacteria to thrive during the ripening stage is their ability to anaerobically ferment lactate produced by lactic acid bacteria (LAB). As previously mentioned, propionibacteria can co-metabolize aspartate / asparagine in tandem with lactate, a trait that is strain-dependent and significant for cheese-making. High aspartase activity in dairy *P. freudenreichii* strains leads to increased CO_2 and acetate production per mol of fermented lactate in the detriment of propionate formation and is an important favoring factor in the occurrence of secondary fermentation and split defects in Swiss-type cheese (Wyder et al. 2001; Daly et al. 2009).

The crucial contribution of propionibacteria to the flavor of Swiss-type cheese is not only due to main fermentation end-products - propionate and acetate - but also to a number of other flavor compounds formed as a result of lipolysis and amino acid catabolism. As the thermal treatment used in the initial stages of the cheese-making process inactivates the casein-linked lipoprotein lipase present in milk and the lipolytic activity of the LAB starters in Swiss-cheese is low, propionibacteria are the principal agents involved in the production of free fatty acids – mainly palmitic, oleic, myristic and stearic (Fitz-Gerald 1995; Chamba and Perreard 2002; Dherbecourt et al. 2010a). Flavor compounds continue to be produced by propionibacteria throughout the latter stage of ripening, when the cheese is stored at 4 to 6 °C, and *P. freudenreichii* metabolism has been shown to remain active under these conditions, with the expression of the genes involved in flavor formation almost unchanged (Dalmasso et al. 2012). The lipolytic activity is believed to be exercised mainly through the action of a secreted esterase (Dherbecourt et al. 2010b). Most strains of propionibacteria also show a high capacity to convert amino acids into flavor compounds - mainly organic acids and esters - during cheese ripening. This applies particularly to branched amino acids such as leucine and isoleucine, but also to methionine and phenylalanine (Thierry and Maillard 2002; Thierry et al. 2004). The most important flavor compounds in this category according to their influence on cheese flavor are 3-methylbutanoic acid, 2-methylbutanoic acid, phenyllactic acid and methanethiol (Chamba and Perreard 2002; Thierry and Maillard 2002; Thierry et al. 2005b). Indeed, the flavor-generating qualities of propionibacteria are such that they have occasionally been used in non-Swiss-type cheeses that do not require “eye” formation and even in yoghurt-type products (Thierry et al. 2005a; Ekinci and Gurel 2008).

Another major industrial application of *P. freudenreichii* ssp. *shermanii* is in the production of vitamin B_{12} (cobalamin). Vitamin B_{12} is the generic name given to all corrinoid compounds displaying cyanocobalamin biological activity. Cobalamins are required by human metabolism in quite small amounts (1 - 2.5 µg / day) and serve as cofactors for only two enzymes (methionine synthase and R-methylmalonyl-CoA mutase). Chronic deficiency can cause severe problems in cell division (pernicious anemia) and in the functioning of the peripheral

nervous system (peripheral neuritis). A current topic still being debated by nutritionists and emphasizing the importance of vitamin B_{12} is the dangerous masking effect that folate has on vitamin B_{12} deficiency (Shane 2003; Ulrich and Potter 2006; Johnson 2007). Cobalamin is the only vitamin that is synthesized *de novo* exclusively by some species of bacteria and archaea (Burgess et al. 2009). Vitamin B_{12} has the most complex chemical structure of all vitamins and its 70 step-long chemical synthesis is prohibitively expensive to utilize on an industrial level. Instead, it has been obtained for decades through fermentation technologies and propionibacteria are among the main agents used (Yongsmith et al. 1982; Martens et al. 2002).

Cobalamin biosynthesis in propionibacteria is biologically justified by its role as a cofactor for one of the crucial enzymes in the transcarboxylase cycle: methylmalonyl-CoA mutase. The complete pathway of vitamin B_{12} synthesis has been revealed by biochemical and genomic studies and was found to contain more than 20 distinct steps involving more than 30 genes (Murooka et al. 2005; Roessner and Scott 2006; Falentin et al. 2010). Advances in the use of genetic manipulation tools in *P. freudenreichii*, such as solving the issue of its natively low efficiency of transformation, have led the way to the development of strains that overproduce vitamin B_{12} (Van Luijk et al. 2002). Random mutagenesis by UV light and chemical reagents, as well as genome shuffling techniques have been successfully applied to obtain *P. freudenreichii* strains with enhanced cobalamin production (Martens et al. 2002; Zhang et al. 2010). Moreover, the development of insertion vectors containing multi-gene expression systems has led to a more targeted, metabolic engineering approach (Kiatpapan and Murooka 2002; Piao et al. 2004; Murooka et al. 2005).

2.2 *P. freudenreichii* as a potential probiotic

The earliest studies investigating the probiotic properties of *P. freudenreichii* date back to the ‘80s, when it was administered in mixed culture with *Lactobacillus acidophilus* to infants with gastrointestinal disorders (Mantere-Alhonen 1995). True hints to its probiotic potential came with the discoveries that it stimulated the growth of beneficial *Bifidobacterium* ssp. and that it appeared to decrease the levels of some carcinogen-producing enzymes in the colon of model

mice (Kaneko et al. 1994; Pérez Chaia et al. 1999). Although probiotic dietary supplements containing *P. freudenreichii* have recently become commercially available (Sécuril®, from NutriCology®), their probiotic claim has not been evaluated by the FDA (http://www.nutricology.com). Probiotic dairy products have yet to be marketed. However, evidence has been accumulating pointing towards the probiotic potential of *P. freudenreichii*.

2.2.1 Probiotics: definition, health benefits and selection criteria

Beneficial gut bacteria ferment non-digestible fiber and mucus and produce trophic factors (short-chain fatty acids, particularly butyrate) and vitamin K, while others, such as *Clostridia*, metabolize proteins and generate potentially toxic and carcinogenic compounds (e.g. amines, phenol, indol) (Wong et al. 2006; Blachier et al. 2007). Increasing evidence indicates that the complex ecosystem that makes up the gut microbiota and its interactions with host enterocytes and gut-associated immune cells play an important role in maintaining homeostasis and preventing disease (Saarela et al. 2000; Dethlefsen et al. 2007; Isolauri et al. 2008).

Probiotics, as defined by the United Nations Food and Agriculture Organization (FAO) and World Health Organization (WHO), are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2002). Probiotic bacteria are claimed to protect the gastrointestinal tract (GIT) from pathogen colonization by competing for ecological niches, maintaining the integrity of the epithelial barrier lining the GIT, reducing local pH, stimulating mucus production and secreting antimicrobial molecules (H_2O_2, bacteriocins) (Sherman et al. 2009; Oelschlaeger 2010; Aires and Butel 2011). Gut microbiota has a major immune function, and can interact with immune cell surface receptors to trigger signaling cascades. Probiotics have been shown to modulate both the innate and the adaptive immune systems, and the cross-talk between bacteria and immune cells may act to decrease chronic inflammation of the GIT as well as the incidence of allergies and atopic dermatitis (Delcenserie et al. 2008; Oelschlaeger 2010). Finally, there is evidence of an important detoxifying and anti-carcinogenic action owed to the anti-inflammatory effect as well as to the inhibition of putrefactive bacteria (and the associated reduction in the level of

mutagenic compounds present in the lumen), binding of other genotoxic molecules (e.g. mycotoxins) and the modulation of the immune response against neoplastic cells (Hirayama and Rafter 2000; Oelschlaeger 2010).

On a cautionary note, it should be mentioned that most studies supporting probiotic health were based on *in vitro* rather than *ex vivo* or *in vivo* methods and not all the claims are backed up by randomized double-blind human clinical trials. Probiotic effects are highly strain-dependent, the mechanisms behind them are not yet thoroughly understood and the large diversity of strains and methods used makes comparisons difficult (Marco et al. 2006; Weichselbaum 2009). However, evidence is continuously building up in support of their great potential for human health.

A set of criteria used for the selection of probiotic microbial strains is illustrated in Figure 2 below. However, there is still no consensus on a systematic selection scheme for probiotics and some criteria are being questioned. For example, the “human origin” of a strain may not be a valid argument, as proven by counter-examples of strains with beneficial health effects which are not commonly isolated from humans - such as *Bifidobacterium animalis* ssp. *lactis* (Sanders 2006). Another debatable trait which is still widely used as predictor of probiotic activity is the ability of the bacterial cells to adhere to the GIT epithelium. This attribute is challenging to prove *in vivo* and does not negate the fact that probiotics do not persist in the host GIT long after intake and that regular doses are needed to achieve health effects (Sanders 2008).

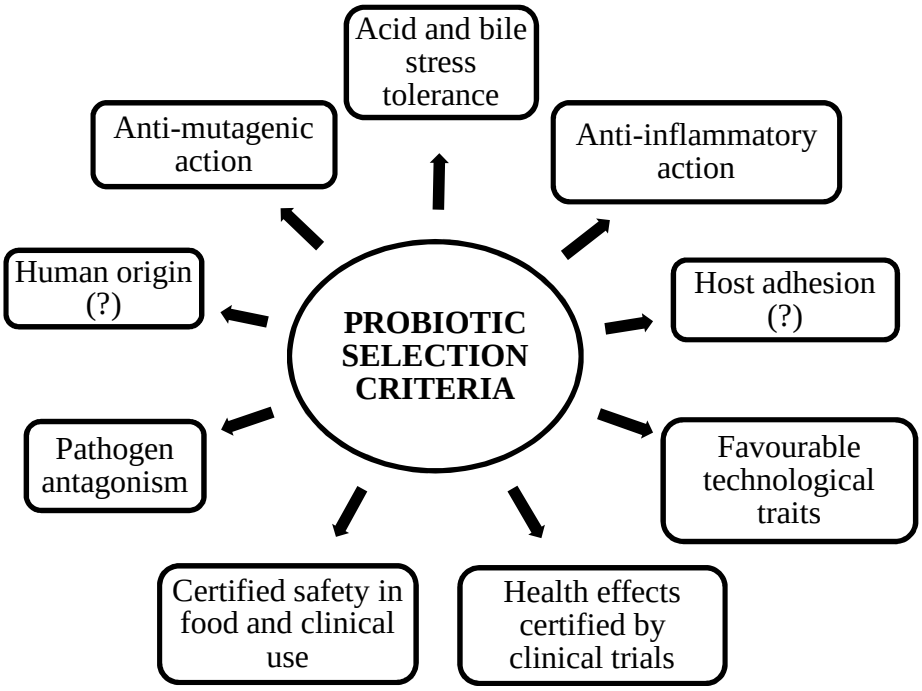

Figure 2. Overview of selection criteria for probiotic strains (adapted from Saarela et al. 2000).

In view of the current issue of the spread of antibiotic resistance genes among pathogenic and opportunistic pathogenic bacteria, a trait that should also be required is the lack of mobile genetic elements containing antibiotic resistance genes (Arias and Murray 2009). Indeed, FAO/WHO recommendations also refer to this aspect (FAO/WHO 2002).

Due to the difficulties inherent to rigorously certifying the health effects of particular strains, in industrial practice most strains used in probiotic products were initially chosen on the basis of attributes that are simpler to measure: stress resistance and ability to tolerate acid, gastric juice and bile, antagonism against common GIT pathogens, anti-inflammatory and anti-mutagenic activities action in *in vitro* and animal models. Certain technological criteria also need to be fulfilled in order for a strain to be used successfully in a commercial product: good sensory properties, resistance to phages and the ability to remain viable during processing and storage (Saarela et al. 2000). In the following subchapters we examine the characteristics of *P. freudenreichii* that support its inclusion on the candidate probiotic list.

2.2.2 Safety characteristics of *P. freudenreichii*

Due to its long use in cheese-making *P. freudenreichii* has been granted both the "Generally Classified as Safe" (GRAS) status by the United States Food and Drug Administration (FDA) and the "Qualified Presumption of Safety" qualification (QPS) by the European Food Safety Agency (EFSA) (Mogensen et al. 2002; Andreoletti et al. 2008). Numerous studies – virulence and cytotoxicity tests *in vitro* and on animal models, as well as human trials - have shown that consumption of *P. freudenreichii* does not have any detrimental effects on health (Meile et al. 2008; Cousin et al. 2010). Moreover, genomic and phenotypic comparisons with the related opportunistic pathogen *P. acnes* revealed that there are no equivalent pathogenicity factors in *P. freudenreichii*, which was therefore considered to be a true commensal (Falentin et al. 2010; Delgado et al. 2011). *P. freudenreichii* was found to contain no mobile genetic elements carrying antibiotic resistance genes, and showed no resistance to relevant antibiotics (Suomalainen et al. 2008; Falentin et al. 2010; Delgado et al. 2011). Summing up, *P. freudenreichii* strains thoroughly satisfy the safety criteria required for use in probiotic commercial products.

2.2.2 Survival and stress responses of *P. freudenreichii*

A number of metabolic characteristics contributing to the long-term survival capabilities of *P. freudenreichii* under starvation conditions and while subjected to technological stresses during cheese-making have already been discussed in chapter 2.1.2 (i.e. the ability to accumulate and use polyP, trehalose, glycogen and glycine betaine). Here, we examine aspects relating specifically to acid and bile stress, the types of stress most relevant to probiotic applications and which probiotic bacteria must survive in their passage through the GIT. Homologous stress tolerance and cross-protection effects - including that of heat treatment - are also scrutinized.

Acid stress is the first challenge encountered by bacteria as they enter the GIT and reach the stomach. Probiotic strains must survive up to a few hours in human gastric juice (with pH usually between 2.0 and 3.5) before being exposed to pancreatic enzymes and bile salts in the

small intestine (Huang and Adams 2004). As with most probiotic traits, acid resistance of *P. freudenreichii* is highly strain-dependent (Jan et al. 2001). Several studies have found that all *P. freudenreichii* strains maintain viability at pH = 4.0, with most strains also able to withstand pH = 3.0 for a few hours. However, exposure to pH = 2.0 for the same duration resulted in a severe reduction in colony-forming unit (CFU) counts of 5 log or more (Zarate et al. 2000; Jan et al. 2002b; Huang and Adams 2004; Leverrier et al. 2004; Suomalainen et al. 2008). A highly effective acid tolerance response was documented, with a 60 min of pre-treatment at pH = 5.0 preventing any significant loss of viability at subsequent exposure to pH = 2.0 (Jan et al. 2001). When bacterial cells were incorporated into food matrixes prior to exposure, even non-adapted cells of some strains were able to successfully survive pH = 2.0, depending on the respective food matrix (Huang and Adams 2004). Acid stress caused the overexpression of chaperonins GroEL and GroES (macromolecule damage repair proteins) (Jan et al. 2001).

After surviving the low pH in the stomach, probiotic bacteria must resist the membrane-disrupting action of detergent-like bile salts present in the intestinal juice. Exposure to a concentration of 0.15 – 0.30 g / L bile salts for a 1 to 4 h period has been proposed for the screening of probiotic candidates for bile stress resistance (Huang and Adams 2004). *In vitro* studies have shown *P. freudenreichii* strains to exhibit high survival rates in bile stress conditions, as no significant loss of viability was detected after exposure to bile concentrations ranging from 0.2 to 0.5 g / L (Jan et al. 2002b; Leverrier et al. 2003; Huang and Adams 2004; Leverrier et al. 2004; Suomalainen et al. 2008). A concentration of 1.0 g / L (higher than normally found in the duodenum of healthy humans) was found to be strongly inhibitory, causing a 4 to 5 log decrease in CFU counts. However, just as in the previous case of acid stress, a non-lethal pre-exposure of 2 h at 0.2 g / L bile salts led to a dramatic increase in survival, with only a 0.1 to 0.7 log decrease in viability as determined from CFU counts (Jan et al. 2002b; Leverrier et al. 2003). This indicated that besides good constitutive resistance, *P. freudenreichii* strains also show a marked adaptive response to bile salt stress. The physiological state of the cells was also deemed to be relevant, as non-adapted cells in the stationary-phase were found to display the same resistance as adapted cells (Leverrier et al.

2003). Incorporation in a food matrix had the same protective effect as observed as for the acid stress.

Thermotolerance was found to vary among different *P. freudenreichii* strains, and they were classified by their survival at 30 min at 55 °C as being either thermotolerant (less than a 1 log reduction in CFU counts) or thermosensitive (more than a 2 log reduction) (Anastasiou et al. 2006). Homologous heat stress tolerance was observed for multiple strains of *P. freudenreichii*, as pre-treatment for 60 min at 42 °C resulted in increased resistance. This effect was substantial for thermosensitive strains (showing a 3 to 4 log increase in survival), but less so for thermoresistant strains – only a 4-fold increase. Thermotolerance was linked with constitutive over-expression of certain heat-shock proteins involved in dealing with protein denaturation: chaperonin Cpn60, Hsp70 and an ATP-dependent chaperonin homologous to ClpB. Non-lethal heat pre-treatment of the thermosensitive strains resulted in the up-regulation of molecular chaperones including members of the Clp family, GroEL and DnaK (Anastasiou et al. 2006). Heat pre-treatment of *P. freudenreichii* at 42 °C was shown to induce significant cross-protection to bile stress, improving survival by a factor of 5000 (Leverrier et al. 2003).

General stress adaptation genes of major importance for the ability to survive digestive stress were found to be present in multiple, redundant copies in the *P. freudenreichii* genome (Falentin et al. 2010). While the activation of the general stress response by thermal stress gave good cross-protection against bile stress, acid pre-treatment had a sensitizing action, increasing the viability loss due to bile stress by a additional 2 log decrease in CFU counts (Leverrier et al. 2003; Leverrier et al. 2004). This effect could pose a problem for digestive stress survival of *P. freudenreichii*, as in the GIT acid stress if followed by bile stress. *In vivo* studies have proven that *P. freudenreichii* strains can indeed retain viability in the passage through the human GIT and reach counts of 6 to 7 log CFU / g in the faeces of human volunteers (Jan et al. 2002b; Suomalainen et al. 2008). However, as observed with most probiotic strains, *P. freudenreichii* counts did drop to initial levels a few days after intake ceased. Another study detected methylmalonyl-CoA mutase mRNA in volunteer faeces (Herve

et al. 2007). Methylmalonyl-CoA mutase is distinctive for the propionibacteria Wood-Werkman cycle, and the finding suggests that some strains of *P. freudenreichii* not only survive, but are also able to maintain active metabolism in the GIT.

2.2.3 Bifidogenic growth factor (BGF) production by *P. freudenreichii*

Bifidobacteria are a normal component of intestinal microflora in healthy humans and are believed to play an important and beneficial role in maintaining GIT homeostasis. As such, *Bifidobacterium* strains are among the best studies and most commonly used probiotics (Picard et al. 2005; Minocha 2009). One of the main attributes recommending *P. freudenreichii* as probiotics is their ability to support and enhance the growth of bifidobacteria in the colon (Warminska-Radyko et al. 2002). Studies have linked the intake *of P. freudenreichii* with an increase in bifidobacteria counts as well as an increase of the bifidobacteria / total gut microflora ratio in human volunteers (Bougle et al. 1999; Hojo et al. 2002). This positive effect was caused by the production of two bifidogenic growth factors (BGFs), precursors of menaquinone (vitamin K_2), which were identified as 1,4-dihydroxy-2-naphtoic acid (DHNA) and 2-amino-3-carboxi-1,4-naphtoquinone (ACNQ) - shown in Figure 3 (Mori et al. 1997; Isawa et al. 2002).

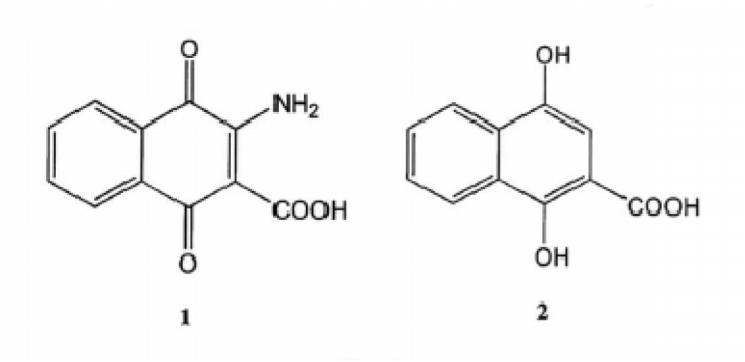

Figure 3. Chemical structure of BGFs: ACNQ (1) and DHNA (2) (from Isawa et. al 2002).

These BGFs are believed to act as electron transfer mediators aiding the regeneration of $NAPD^+$ in bifidobacteria (Yamazaki et al. 1999). They are heat-stable and resistant to enzymatic degradation (Kaneko et al. 1994). BGF production was a trait common to all *P. freudenreichii* strains out of 27 examined in a study, including some strains that were found to

be able to survive acid and bile stresses (Warminska-Radyko et al. 2002). The ability to produce BGFs that sustain bifidobacteria growth in the human colon is therefore one of the most important characteristics supporting the status of *P. freudenreichii* as a candidate probiotic.

2.2.4 *Helicobacter pylori* inhibition *by P. freudenreichii*

Helicobacter pylori infection is considered to be the most important risk factor for gastritis and gastric and duodenal ulcers, as well as a significant risk factor for stomach cancer. The problem is exacerbated by the fact that *H. pylori* is beginning to develop resistance to common antibiotics (Fox and Wang 2001). *In vitro* studies found that *P. freudenreichii* reduced the ability of *H. pylori* to adhere to intestinal cells and to induce membrane leakage and decreased *H. pylori*-induced inflammation (Myllyluoma et al. 2007; Myllyluoma et al. 2008). Interestingly, another *in vitro* study showed that *P. freudenreichii*-produced DHNA had a strong and specific inhibitory effect on *H. pylori*, with a minimum inhibitory concentration of 1.6-3.2 µg / mL. It also decreased *H. pylori* CFU counts in the stomach of infected mice from 3.4 log CFU / stomach down to non-detectable levels (Nagata et al. 2010). The effect was thought to be caused by the inhibition of cellular respiration by DHNA. Finally, an *in vivo* randomized double-blind human trial showed that *P. freudenreichii* helped to improve patient tolerance to a *H. pylori* eradication treatment (Myllyluoma et al. 2005). While the frequency of the symptoms was unchanged, the overall symptom severity was decreased. The data points to the possibility that *P. freudenreichii*-containing probiotic products may be used in the future to combat the effects of *H. pylori* infection and improve patient tolerance to the required antibiotic treatment, although further research needs to be done in this direction.

2.2.5 Anti-mutagenic and anti-carcinogenic effects of *P. freudenreichii*

Mycotoxins are known mutagenic and carcinogenic compounds which may be present in some foodstuffs (Chu 1991). Several studies have looked at the proposed ability of *P. freudenreichii* to bind mycotoxins (El-Nezami et al. 2000; El-Nezami et al. 2006; Niderkorn et al. 2006). However, the mycotoxin-binding capacity of propionibacteria is low compared to LAB and

studies documenting significant binding were undertaken using a mix of *P. freudenreichii* and probiotic LAB strains, so the exact contribution of *P. freudenreichii* to the effect is uncertain.

P. freudenreichii was shown to decrease the *in vitro* effect of chemical mutagens such as 4-nitro-quinoline, 9-aminoacridine and N-nitro-N-nitrosoguanidine, as observed by using the Ames test (looking at the action of DNA mutagens on *Salmonella enterica*) (Vorobjeva and Abilev 2002). Vorobjeva et al. attributed this protective effect to the action of *P. freudenreichii* cysteine synthase, a constitutively expressed protein that escapes during bacterial lysis and was thought to inhibit the action of mutagens by a yet unknown mechanism, possibly through electrophilic mutagen scavenging and free radical inhibition (Vorobjeva et al. 2004; Vorobjeva et al. 2008). As this effect has yet to be confirmed in animal models or *in vivo* studies and its mode of action is unresolved, the claim needs to be examined further.

Short chain fatty acids have been known to beneficially affect the functioning of the colon. Butyrate is an important energy source for enterocytes while propionate and acetate may exert other physiological effects, including inhibition of neoplastic cells (Scheppach et al. 1995) An *in vitro* study found that *P. freudenreichii* co-incubated with colorectal cancer cell lines kills them by inducing apoptosis through mitochondrial permeabilization (Jan et al. 2002a). The active components were identified as the SCFAs acetate and propionate, main fermentation products of propionic bacteria, with a strong, concentration-dependent killing effect in the range 10 to 40 mM. Another study using human microbiota-associated mice showed that *P. freudenreichii* lowered cell proliferation and increased cell apoptosis in the colon of mutagenized mice, with no similar effect on normal mice (Lan et al. 2008). Finally, *P. freudenreichii* growth supernatant appeared to induce NKG2D ligand expression on the surface of cancer cells, an event which targets them for destruction by the immune system (Andresen et al. 2009). These results hint at the possibility of *P. freudenreichii* being used to lower the risk of colorectal cancer.

To conclude, *P. freudenreichii* appears to be a viable candidate for use in probiotic dairy products. It has excellent safety characteristics, good digestive stress resistance as well as

exploitable adaptive stress responses and exhibits stress cross-tolerance behavior. Its ability to induce beneficial health effects by supporting the growth of bifidobacteria in the human gut is well documented. The observed antagonistic effect on the gastric pathogen *H. pylori*, as well as the proposed anti-mutagenic and anti-carcinogenic properties appear promising, though requiring further investigation. Still, it must be emphasized that some of these traits are strongly strain-dependent. Most importantly, commercial probiotic claims should be supported by proof of sufficient viable cell counts in the finished product, by precise strain characterization as well as by double-blind randomized human trials certifying the advertised health effects.

2.3 Proteomics as a tool for the study of propionic and probiotic bacteria

Emerging in the early ‘90s, the term “proteome” referred to the entire protein complement of a organelle, cell, tissue or organism (Wilkins et al. 1996). It has gradually acquired a wider meaning and is now considered to include the global pattern of protein activities, structural modifications, localizations and protein-complex interactions attributable to a specific biological sample (Tyers and Mann 2003). The field of proteomics (the study of *prote*ins encoded by the gen*ome*) has benefited extensively from major advances in genome sequencing, mass spectrometry (MS) and bioinformatics, as well as from increasingly sensitive, reproducible and affordable analytic technologies (Brewis and Brennan 2010). Although the development of DNA microarrays now makes it possible to rapidly obtain a global gene expression profile of a cell with a single analysis, transcriptomics has significant limitations. Crucially, the amount of a certain species of mRNA measured at a point in time often does not correlate with the amount of the corresponding protein present in the cell. Among the factors responsible are the different stability of the various mRNAs as well as differences in mRNA translation efficiency and in individual protein turn-over rate (determining the average amount of time a protein persists before being degraded) (Gygi et al. 1999). Proteins also display a wealth of information beyond their sequence and concentration: post-translational modifications (PTMs), phosphorylations, glycosylations and other types of isoforms all provide biological clues (Brewis and Brennan 2010). Proteomics therefore offers

a unique insight into the inner workings of a biological system. It is used synergistically together with genomics, transcriptomics and metabolomics in the new “systems biology” approach to biological sciences (Patterson and Aebersold 2003).

2.3.1 An overview of proteomics methods

There are two main technical approaches to proteomics, both of which rely on mass spectrometry (MS) for the identification of proteins / peptides after they are separated. In both approaches, sample pre-treatment is highly important and must ensure complete disaggregation, solubilization, denaturation and reduction of the proteins (Lopez 2007).

The first to evolve was the gel-based workflow involving the separation of the proteins on a gel matrix. A single dimension is insufficient for the visualisation of complex protein mixtures. Therefore, a 2^{nd} dimension of separation is performed after the 1^{st} in a perpendicular direction, hence the name of the method: 2-dimensional gel electrophoresis (2-DE). The proteins are separated in the 1^{st} dimension according to their overall charge in a step called isoelectric focusing (IEF), on a gel strip containing an immobilized pH gradient (IPG). An electric voltage is applied to the IPG strip, the sample is loaded and each of the proteins migrates towards the electrode of opposite sign to its net charge until it reaches a specific pH zone on the IPG strip corresponding to its own isoelectric pH (pI). Here the positive and negative charges on the protein’s surface balance each other out. As a result the protein’s overall electrical charge is null, the electrical field can no longer exert its effect and the protein stops moving. The 2^{nd} dimension involves the separation of the proteins as a function of their apparent molecular mass (M_r). The IPG strip containing the “focused” sample is embedded on top of a polyacrylamide gel and again a voltage is applied, in a similar fashion to the classic 1-D SDS-PAGE analysis. The proteins migrate through the gel at different speeds, with smaller proteins travelling faster than larger ones. The gel is subsequently stained (either with colloidal silver, Coomassie Blue stain or fluorescent dyes) allowing the proteins to be visualized at a glance as spots on a two-dimensional map. Alternatively, immuno- or

radiolabeling can also be used. In the end, each protein from the sample is characterized by the specific pI / M_r orthogonal coordinates of its spot on the 2D gel map (Lopez 2007).

Dedicated software can process and compare gel images, identify spots corresponding to proteins that show relevant variation and automatically calculate the statistical significance of the findings (e.g. p-value). Interesting spots can then be excised from the gel, subjected to trypsin digestion and the peptides obtained analyzed with MS (for 2-DE samples, generally with MALDI-TOF). The reasoning behind the proteolysis step is that resulting peptides are easier to solubilize and have a very convenient size for MS analysis (around 1100 Da) thus greatly improving sensitivity and mass precision. Trypsin is most often the enzyme of choice due to its excellent stability, specificity and the peptide size range it produces (Steen and Mann 2004). The usual approach for the identification of proteins from 2-D gel spots is "peptide mass fingerprinting" (PMF). PMF does not attempt to directly obtain the amino acid sequence of the peptides. Rather, a software uses the sequenced genome of the respective organism to translate the nucleotide sequences of all predicted open reading frames (ORFs) into amino acid sequences and performs a simulated (*in silico*) trypsin proteolysis in order to predict the masses of all the possible resulting peptides. The set of theoretical peptide masses is then matched against the set of MS-measured masses of the sample-derived peptides and the protein is identified. A statistical score is calculated expressing the probability that the resulting protein identification is correct (Steen and Mann 2004). Although convenient and generally effective, PMF has the drawback of relying on the protein in question being present in sequence databases (Brewis and Brennan 2010). It is not absolutely necessary that the sequenced genome of the organism in question be available. Particularly in the case of closely related bacteria, proteins can sometimes be identified on the basis of sequence homology with proteins from other species/strains whose genomes have been sequenced (Santos et al. 2007). When PMF fails, tandem mass spectrometry (MS/MS) can be used to obtain the *de novo* amino acid sequence of the peptides.

The other main approach to proteomics employs liquid chromatography (LC) as the method of separation and is sometimes referred to as "gel-free" or "shotgun" proteomics. The reason is

that in the LC-based workflow the protein sample is enzymatically digested prior to separation, and it is this resulting peptide mixture (analogous to “shotgun fragments”) that is actually injected into the HPLC column. Usually a reverse phase capillary column is used and the peptides elute at different times depending on their hydrophobicity – more hydrophobic peptides adhere better to the non-polar stationary phase and elute later than hydrophilic peptides. ESI-MS/MS is often directly integrated with nano-scale HPLC in an automated, “on-line” setup, which can continuously analyze the peptides as they are eluted (Brewis and Brennan 2010). Some setups may also include multiple dimensions of LC-based separation prior to MS besides reverse phase HPLC, most common of which is cation-exchange chromatography. This is referred to as “multidimensional protein identification technology” (MudPIT) (Washburn et al. 2001).

2.3.2 Gel-based proteomics: advantages and limitations

Gel-based techniques have been around for a long time, as they were the first to be employed in early proteomic studies starting with the ‘70s, even before the term “proteomics” was coined. As such, both their advantages and their intrinsic drawbacks are well defined (Patterson and Aebersold 2003).

One of the crucial technical advances leading to the spread of gel-based proteomics was the development of IPG strips, greatly easing and improving reproducibility of the IEF step. However, for several reasons, IEF probably remains the most challenging stage of a 2-DE protocol. First of all, very hydrophobic proteins are notoriously troublesome, mainly because they are so difficult to solubilize in the conditions necessary for the IEF process, which requires low ionic strength and is incompatible with strong ionic detergents such as SDS (Brewis and Brennan 2010; Rabilloud et al. 2010). This is unfortunate, as many hydrophobic proteins (such as multi-spanning integral membrane proteins and various multi-protein complexes) play crucial roles in the functioning of the cell (chemical sensing, signal transduction, pathogenic interactions) and are therefore key research targets (Cordwell 2006). Poor focusing of these proteins in the IEF stage leads to the appearance of horizontal smears

on the 2D gels. Although optimizing chaotropes and IEF-compatible detergents, or including differential solubility or enrichment stages in the sample preparation can somewhat alleviate this issue, it is a significant hindrance for 2-DE studies and one that is difficult to get around (Cordwell 2006; Rabilloud et al. 2010). Proteins with a very basic pI (such as ribosomal proteins and histones) or with very high (>150 kDa) or very low (<10 kDa) M_r are also problematic for 2-DE (Timms and Cramer 2008; Brewis and Brennan 2010). IPG strips do however provide the option of selecting a convenient IPG interval, with narrower intervals providing improved resolution at the expense of a reduced selection of proteins. Micro-range IPG strips are currently available that cover ranges as narrow as 1 pH unit, and "ultra-zoom" gels can be obtained from IPG strips covering an array of narrow, overlapping pI ranges (Hoving et al. 2002; Lopez 2007).

Another problem encountered in all proteomic studies - and even more so in gel-based setups - is the inability to discern low-abundance proteins (Rabilloud et al. 2010). The problem lies in the huge variation in the concentrations of specific proteins contained in a biological sample, often stretching over 12 orders of magnitude or more (Brewis and Brennan 2010). A relatively low number of proteins make up a large proportion of the total protein amount and mask low-abundance proteins. 2-DE is more vulnerable to this dynamic range issue partly because it includes a visualisation stage in the selection of proteins for the MS/MS stage: a spot must be distinguishable on the gel in order for the researcher to make the decision of excising it and trying to identify the protein (Rabilloud et al. 2010). In contrast, in the case of on-line LC-ESI-MS/MS systems, all peptides eluted are automatically mass-analyzed. The excellent dynamic range of even the most modern fluorescent dyes used to visualize the gels (e. g. Cye dyes maintain linearity of quantification over a range of 4 orders of magnitude) is still far from sufficient.

Though LC-based setups are now able to discern more proteins in large-scale, global studies and are easier to automate, 2-DE has some notable advantages (Lopez 2007; Brewis and Brennan 2010). Firstly, it allows for parallelism of experiments which increases statistical confidence. Indeed, it is common practice to run 4 to 8 gels simultaneously, in the same setup.

Variability is further decreased in multiplexed experiments such as those performed with difference gel electrophoresis (DIGE). DIGE represents the state-of-the-art in 2-DE analysis and allows for up to 3 samples labeled with different fluorescent cyanine (Cye) dyes to be run and visualized on the same gel, thus eliminating inter-gel variation (Timms and Cramer 2008). Also, the dynamic range hindrance is less pronounced when samples have relatively lower complexity, making 2-DE better suited for studies on bacteria rather than on mammalian cells (Rabilloud et al. 2010).

Perhaps the main advantage of 2-DE over LC-based setups comes from the fact that it involves the separation and visualisation of the intact proteins. This allows for easier distinction between protein species compared to “shotgun” approaches, where the protein-of-origin of a particular MS-analyzed peptide may be harder to determine. 2-DE can also extract biological information that is lost in gel-free proteomics and impossible to obtain from genomic and transcriptomics data, the best example being PTMs. 2-DE is highly useful in the study of PTMs (e.g. phosphorylations, glycosylations or redox modifications) which generally induce changes in the pI of the protein that can be discerned in the IEF stage (Brewis and Brennan 2010). The spot corresponding to the respective protein appears to shift horizontally on the gel, or horizontal “spot trains” are observed corresponding to multiple pI isoforms of the same protein. 2-DE can also be used to detect proteolytic processing of specific proteins (Lopez 2007). Another advantage is that excised gel spots can be stored for a long time before further analysis. Gel-based proteomics is particularly suitable for microbiological studies due to its versatility, lower costs and lower requirements for highly specialized staff compared to LC-based setups. Furthermore, it allows the MS analysis to be outsourced to external, specialized laboratories (Sá-Correia and Teixeira 2010). In conclusion, 2-DE is a robust and versatile method of great value for the study of bacteria.

2.3.3 Role of gel-based proteomics in improving the understanding of bacteria

Generally, proteomics studies of bacteria can be classified into two main categories. The first are systematic studies aimed at obtaining an overall image of protein expression for a specific

strain under a set of fixed baseline conditions. These experiments attempt to visualize and identify the highest possible number of expressed proteins (i.e. build a reference map), as well as assign and investigate the functions of as many of these proteins as achievable (Champomier-Vergès et al. 2002). Though a truly complete proteome map cannot be obtained due to technical limitations, DIGE allows for up to 2000 proteins to be resolved one a single gel (Lopez 2007). Proteome reference maps serve to compare and classify different bacterial strains. They also ease the identification of spots on subsequent gels obtained with identical protocols (Sá-Correia and Teixeira 2010). Lastly, they provide a starting point for the other main category: differential studies.

Differential studies are the most common and powerful approach in bacterial proteomic research and involve comparing the protein expression patterns of a given strain under different culture conditions – various growth media, stresses or stimuli. Observing which proteins are switched on/off (in qualitative studies), or over- / underexpressed (in quantitative studies) as a result of different environmental conditions (varying temperature, pH, redox potential, antibiotics, nutrient composition, etc) can provide clues towards biological questions. The structure and regulation of metabolic pathways, stress and starvation response systems, signal cascades, specific biosynthetic and degradation pathways can be visualized and understood (Hecker et al. 2008). Other studies look for the presence of certain protein biomarkers known to be indicative of properties that do not correlate properly with the presence/absence of a particular gene (Williams et al. 2004). Observing how sets of proteins show linked changes in expression can help map protein interaction networks and group genes/operons into stimulons and regulons. Interaction networks can also be mapped by proteomic studies using mutants that lack certain regulatory proteins (Hecker and Völker 2004).

The diversity of studies undertaken using 2-DE is considerable. All this information can lead to a deeper understanding of bacterial physiology which can then be used to make predictions as to the pathogenicity, technological or probiotic properties of specific strains (Wu et al. 2008; Aires and Butel 2011).

2.3.4 Proteomic studies on probiotic bacteria

During the last decade, an ever increasing collection of sequenced probiotic strain genomes has paved the way for proteomic studies centered on probiotic bacteria. Gel-based reference maps have been constructed for well known probiotic strains belonging to *Bifidobacterium* and *Lactobacillus* as well as for some new candidate strains, while other studies employed LC-based methods to construct detailed proteome catalogues enabling inter-strain comparisons (Yuan et al. 2006; Wu et al. 2009; Majumder et al. 2011; Savijoki et al. 2011). The main focus has been on the acid and bile stress responses that allow probiotics to maintain viability during their passage through the GIT as well as on the mechanisms of probiotic adhesion to the GIT epithelium (Siciliano and Mazzeo 2012). An overview of the molecular effectors identified through proteomic studies is shown in Figure 4, each linked to its respective function.

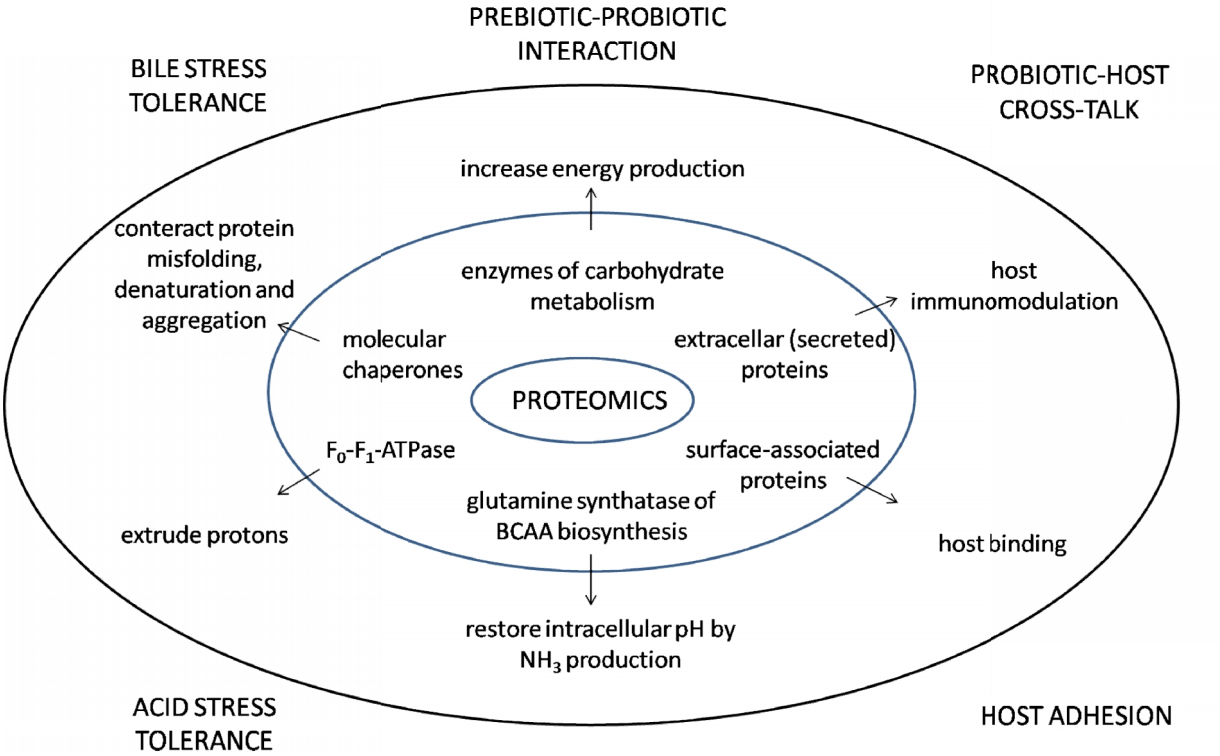

Figure 4. Molecular mechanisms involved in digestive stress adaptation, GIT adhesion and other probiotic interactions with prebiotics and the host (adapted from Siciliano and Mazzeo 2012).

Proteomics studies revealed that some stress response components are common to a range of different probiotic strains. This digestive stress “core response” seems to include overexpression of chaperones (GroES, GroEL, DnaK, DnaJ, Clp family) to deal with protein misfolding, adapting metabolism to boost ATP production (often by glycolysis) and using the proton pump F_0-F_1-ATPase to maintain an acceptable intracelular pH (Siciliano and Mazzeo 2012). Other response components may differ between species or strains. For example, glutamine synthetase - an enzyme that produces ammonia as a by-product of branched chain amino acid (BCAA) synthesis - was overexpressed in *B. longum* under acid stress, while being underexpressed in *L. rhamnosus* GG under the same conditions (Sánchez et al. 2007; Koponen et al. 2012).

Many studies have centered on the surface-associated and extracellular proteins secreted by probiotic strains. These proteins are thought to mediate bacterial interactions with intestinal epithelial cells (Beck et al. 2009; Izquierdo et al. 2009). They seem to play a crucial role in bacterial adhesion, in exercising immunomodulatory effects as well as in effecting molecular cross-talk between bacteria and mucosal cells (Sanchez et al. 2008). One example is the identification in *L. rhamnosus* GG of pilus structure components which may act as effectors of an adhesion mechanism to the intestinal mucus (Kankainen et al. 2009; Savijoki et al. 2011).

There have also been some attempts to decipher the mechanism of prebiotic-probiotic interactions, such as a study by Majumder et al. looking at changes in specific protein abundance triggered when lactitol (a synthetic sugar alcohol prebiotic) was used as a substrate by the probiotic strain *Lactobacillus acidophilus* NCFM (Majumder et al. 2011).

An improved understanding of the above topics could lead to the discovery of reliable protein biomarkers that correlate with gastric and bile stress resistance, adhesion and immunomodulatory capabilities or the predisposition of a strain to be selectively favored by the concomitant administration of a particular prebiotic (Aires and Butel 2011). Such biomarkers would ease the selection of probiotic bacterial strains, pre-treatments and probiotic vectors, while ensuring that only the best candidates reach the expensive and time-consuming

clinical trials that are needed to solidly certificate probiotic action (Leverrier et al. 2003) (Siciliano and Mazzeo 2012).

2.3.5 Proteomic studies on *P. freudenreichii*

To date, the number of research articles containing proteomic investigations of dairy *Propionibacterium* strains is very limited. Detailed searches of online journal databases yielded only 9 original articles (as of May 2012) involving proteomic research on dairy propionic bacteria. All of them involved strains of *P. freudenreichii*, either ssp. *freudenreichii* or ssp. *shermanii*. A 10th study aiming to construct a proteome reference map for Emmental cheese also identified some bacteria-secreted cheese proteins as belonging to *P. freudenreichii* (Gagnaire et al. 2004).

Jan et al. authored the earliest proteomic study of *P. freudenreichii* (using strain SI41), which looked at the mechanism of the acid tolerance response previously documented by the same group (Jan et al. 2000; Jan et al. 2001). It revealed acid-induced changes in protein synthesis, with more than 50 proteins being induced at different stages of the acid tolerance response. These included general-stress proteins such as chaperonins GroEL and GroES, SOS-response enzymes involved in DNA synthesis and repair (RecR, RepB), but also the biotin-dependent carboxyl transferase specific to propionibacteria metabolism. The study suggested that *P. freudenreichii* acid tolerance response had two distinct components providing additive protective effects. The first was a readily-inducible system relying on constitutively-expressed proteins, while the other required *de novo* synthesis of specific general stress and acid stress proteins, as indicated by the fact that chloramphenicol (a protein synthesis inhibitor) only partially decreased the acid tolerance response (Jan et al. 2001). Soon thereafter another 2-DE study examined *P. freudenreichii* (strain SI41) susceptibility and tolerance to bile stress. It found that 24 proteins were bile stress-induced while overall proteins synthesis was decreased and documented a range of cross-protective effects described in chapter 2.2.2 (Leverrier et al. 2003). The differentially-expressed proteins were assigned functions related mainly to signal sensing and transduction and to the general stress response (Hsp20, RecR, ClpB, DnaK,

SodA), but also included an alternative sigma factor and the biotin-dependent carboxyl transferase. More than 10 proteins were shown to be common to both acid stress and bile stress responses, including RecR and the biotin-dependent carboxyl transferase (Jan et al. 2002b). In these early studies, ^{35}S radiolabeling was used to allow for protein visualisation, while partial peptide sequences were obtained through N-terminal amino acid sequencing. As there was no sequenced *P. freudenreichii* genome available and annotated peptide sequences were scarce, these studies had significant limitations in the protein identification step and PMF was not a viable option.

The first study to use mass spectrometry (nanoscale-LC-MS/MS) and perform *de novo* peptide sequencing on proteins isolated from 2-DE gel spots gathered further information on heat, acid and stress responses and analyzed the various potential cross-protective effects (Leverrier et al. 2004). Vorobjeva et al. also employed LC-MS/MS *de novo* sequencing to identify the putative anti-mutagenic action protein cysteine synthase from *P. freudenreichii* cell-free extracts after performing 2-DE (Vorobjeva et al. 2004). The visualisation method used involved a Coomassie Blue dye. As genome sequence information availability and database coverage increased, PMF began to gain importance as a mainstay method for protein identification (Anastasiou et al. 2006). A breakthrough was achieved in 2010 with the publishing of the first complete genome of the type-strain of *P. freudenreichii*, sequenced with an 11-fold coverage (Falentin et al. 2010). The plasmid-free genome of *P. freudenreichii* subsp. *shermanii* CIRM-BIA1^{T} was predicted to hold 2439 protein-coding genes. The proteomic section of the study was successful in identifying 490 proteins (16% of the total predicted number) using a combination of gel-based and gel-free methods: 2-DE coupled with either nano-LC-ESI-MS/MS or MALDI-MS/MS, and 2D-LC-ESI-MS/MS (1^{st} dimension – cation exchange chromatography, 2^{nd} dimension - reverse phase LC).

2-DE was applied in a recent genome-shuffling experiment attempting to obtain *P. freudenreichii* subsp. *shermanii* strains with improved vitamin B_{12} production. Protein spots were identified by gel matching using the SWISS 2DPAGE on-line database. The experiment obtained a strain showing 60 % higher B_{12} synthesis compared to the parent strain (Zhang et

al. 2010). Comparative analysis of protein expression patterns revealed 38 proteins with significant expression variation, 22 of which were overexpressed and 16 underexpressed. Overexpressed proteins included 6 members of the B_{12} synthesis pathway: glutaminyl-tRNA synthase (GlnS), delta-aminolevulinic acid dehydratase (HemB), methionine synthase (MetH), riboflavin synthase (RibE), phosphofructokinase (PfkA) and isocitrate dehydrogenase (Icd). The study points to these proteins as being potential targets of manipulation for the future creation of B_{12} overproducing strains by metabolic engineering.

A recent study employed a combination of transcriptomics and proteomics in order to investigate how *P. freudenreichii* CIRM-BIA1^T adapts and is able to maintain active metabolism in the cold conditions (4 °C) characteristic of the storage stage of the Swiss cheese ripening process. 2-DE was used in combination with nano-LC-ESI-MS/MS to identify 50 proteins by PMF. The experiment showed that during cold storage most constitutively-expressed proteins are underexpressed, while some involved in long-term survival carbon and energy storage (lactate conversion to pyruvate, gluconeogenesis and glycogen synthesis) are in fact overexpressed (Dalmasso et al. 2012). General stress proteins like GroES, GroEL, DnaK, ClpB and Hsp20 which are overexpressed in other types of stress were underexpressed in cold stress conditions. Notably, besides two known cold shock proteins (CspA and CspB), two RNA helicases (thought to act against cold-induced unfavorable changes in RNA secondary structure) were also overexpressed. This type of research could provide clues as to how to obtain probiotic strains that maintain viability during long storage at refrigeration temperatures.

Proteomic research into *P. freudenreichii* is still in its infancy compared to the studies performed on other dairy or probiotic bacteria. With the prospect of more and more *P. freudenreichii* strains genomes being sequenced in the near future and the availability of ever more extensive peptide sequence and functional databases, conditions are favorable for further proteomic research. The improved understanding these study can provide may hold the key to obtaining superior *P. freudenreichii* strains for probiotic or industrial application.

3 EXPERIMENTAL RESEARCH

3.1 Aims

There have been very few proteomic studies dealing with dairy propionibacteria. The first experimental objective of this study was to examine the effect of the bacterial cell lysis step on protein extraction from samples of *P. freudenreichii* and the effect of sample composition on the efficiency of the IEF stage of the 2-DE. The second objective was to obtain proteome maps for the two strains under scrutiny. In the context of this study, we define the "proteome map" as a gel-based visualisation of a bacterial proteome showing the highest achievable number of protein spots at the best possible resolution. The last objective was to detect differential expression of proteins by comparing the proteome maps of the two strains. These protein expression differences may provide a starting point for future proteomic studies uncovering the reasons behind the observed phenotypic differences between the strains.

3.2 Materials and methods

3.2.1 Bacterial strains and culture conditions

The strains used in this study belonged to *P. freudenreichii* ssp. *shermanii* and were cataloged as strain PV256 and strain PV266. Strain PV256 is a dairy strain that has been employed in cheese-making; PV266 is originally isolated from a commercial starter preparation. On-going studies are looking at the potential use of these strains in vitamin B_{12} fortification of cereal foods.

Bacteria were grown and propagated using propionic agar medium and propionic liquid medium. De-ionized water was used for all agar and liquid media. Propionic agar medium composition (per liter) was as follows: 5.0 g tryptone (Sigma-Aldrich, St. Luis, MO, USA), 10.0 g yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA), 15.0 g agar (Becton, Dickinson), 14.0 mL DL-sodium lactate 60 % w/w (Sigma-Aldrich). The pH was adjusted to 7.3 with NaOH (Merck KGaA, Darmstadt, Germany) prior to agar addition. Propionic liquid medium composition was the same as for propionic agar medium, but without the addition of

agar and with the pH adjusted to 6.7. All agar and liquid media were autoclaved at 121 °C for 15 min. Propionic agar plates were streaked with inoculums from bacterial glycerol stocks containing 15 % glycerol (Merck KGaA, Darmstadt, Germany) as a cryoprotectant, stored at -80 °C. The plates were incubated in anaerobic jars (using Anaerocult A, Merck KGaA) at 30 °C for 4 days. Colonies from the streak plates were inoculated in quadruplicate into 15 mL sterile Cellstar® tubes (Greiner Bio-One, Kremsmünster, Austria) containing 10.0 mL propionic liquid media which were incubated microaerobically at 30 °C for 24 h. Aliquots of 2 mL of the cultures were then inoculated into 100 mL propionic liquid media bottles which were incubated microaerobically at 30 °C for 24 h. Optical density was measured at 600 nm using a Novespec® II spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

3.2.2 Protein extraction, purification and quantification

10 mL aliquots of the 100 mL bacterial cultures (with $O.D._{600}$ of 1.15 for strain PV256, and 0.85 for strain PV266, respectively - corresponding to late exponential growth phase for both strains) were centrifuged at 4 °C for 5 min at 4000 g using a model 5810 C centrifuge (Eppendorf, Hamburg, Germany). Water used in the protein sample preparation and for all 2-DE buffers and solutions was purified with the Mili-Q installation (Millipore Corp., Bedford, MA, USA). The supernatant was removed and the cell pellet was washed with ice-cold 50 mM Tris-HCl pH 8.0 (Merck KGaA) followed by centrifugation and removal of the supernatant. The cell pellet was either stored at -20 °C or the analysis immediately continued to the lysis step in order to minimize changes in the state of the proteins. For the lysis step the pellet was transferred to 1.5 mL micro centrifuge tubes with skirt (Greiner Bio-One) with 30 mM Tris (Merck KGaA) containing sterile glass beads Ø = 0.1 mm (Sigma-Aldrich). Bacterial cells were lyzed using a Fast-Prep™-24 (MP Biomedicals, Solon, OH, USA) with 3 lysis cycles of 30 s at 6.5 m / s. The samples were placed on ice for 1 min between cycles to cool the sample and avoid protein denaturation or acceleration of enzymatic proteolysis. Next, 300 µL urea buffer pH 8.0 containing 7 M urea (GE Healthcare, Buckinghamshire, U.K.), 2 M thiourea (Merck KGaA), 4 % 3-[(3-cholamidopropyl) dimethylammonio] - 1-propanesulfonate

(CHAPS) (GE Healthcare), and 30 mM Tris (GE Healthcare) were added to each tube, followed by incubation for 30 min at room temperature with frequent vortexing. Urea was used as a denaturing agent (to disrupt protein secondary and tertiary structures and disassemble multi-protein complexes), CHAPS as a detergent (to solubilize proteins and separate them from lipids), while thiourea and DTT were employed for their reducing action (to convert inter- and intra-molecular disulfide bonds to –SH and to prevent protein oxidation). The samples were then transferred into 1.5 mL Eppendorf tubes and centrifuged for 30 min at 20 °C and 21000 g using a Himac CT 15RE centrifuge equipped with a thermostate. Sample processing is subsequently described for 1-D SDS-PAGE samples, and for 2-DE sample, respectively.

For 1-D SDS-PAGE samples, 70 µL 100 mM Tris-HCL pH 8.0 were added to the each sample following lysis. The samples were subsequently incubated for 30 min at 20 °C. Next, the samples were centrifuged and the supernatant transferred into new tubes together with 30 µL Laemmli 5X buffer (Merck KGaA) followed by another incubation step consisting of 10 min at 95 °C. The same treatment was applied to 5 µL of protein ladder (New England Biolabs, Ipswich, MA, USA) after the addition of 1 µL Laemmli 5X buffer. The protein ladder contained molecular weight references for the interval 10 to 250 kDa. The final composition of the samples was 60 mM Tris-Cl pH 6.8, 2 % v/v SDS, 10 % v/v glycerol, 5 % v/v β-mercaptoethanol and 0.01 % w/v bromophenol blue (Laemmli 1970). β-mercaptoethanol was used as a reducing agent. SDS binds to proteins at a rate of 1.4 g SDS / 1 g protein and therefore forms SDS-protein complexes that exhibit a constant charge-to-mass ratio. Together, β-mercaptoethanol and SDS enable proteins to be separated purely by their molecular mass, regardless of their shape or charge in the native form (Schägger and Von Jagow 1987). Bromphenol blue was used as an indicator dye in order to ease the loading of the protein sample in the wells and allow for the visualisation of protein migration on the gel. The protein ladder and 20 µL aliquots of each sample were loaded on a 12.5 % polyacrylamide gel which was run for 15 min at 100 V followed by 40 min at 200 V. The gel was subsequently stained with Coomassie Brilliant Blue R dye (Sigma-Aldrich) as described in subchapter 3.2.4.

For 2-DE samples, a 2-D Clean-Up Kit (GE Healthcare) was used to process 220 µL of each sample according to the manufacturer's specification. Care was taken during the protein precipitation and centrifugation steps as not to disturb the protein pellet while removing the supernatant. The resulting protein pellet was allowed to air dry for 2 min to remove traces of the 2-D Clean-Up Kit wash buffer and then resolved in 40 – 80 µL of urea buffer (see above). Repeated vortexing was used together with brief consecutive room temperature incubation steps in order to ensure complete resolving of the protein pellet. Protein concentration was determined using an Ettan™ 2-D Quant Kit (GE Healthcare) according to the manufacturer's specification.

3.2.3 2-DE

IEF was performed using 11 cm Immobiline™ IPG strips (GE Healthcare) (110 x 3 x 0.5 mm after rehydration). IPG strips with pH ranges of either 4-7 or 3-11 NL were used for different experimental runs. The IPG strips were rehydrated over night (14-16 h) in 220 µL DeStreak™ rehydration solution (GE Healthcare) containing 1.0 % IPG buffer (GE Healthcare). The amount of protein loaded in samples for IEF was 40 µg for silver-stained gels, and 200 µg for Coomassie Blue-stained gels. For 3-11 NL strips, two sample compositions were tested, hereby referred to as composition A and composition B. Composition A samples contained 10 mM tributhylphosphine (TBP) (Sigma-Aldrich) and 0.5 % IPG buffer (GE Healthcare). Composition B samples contained 4 mM TBP, 1.0 % IPG buffer and 50 mM dithiothreitol (DTT) (Bio-Rad, Hercules, CA, USA). Samples were applied to the IPG strips by cup loading at the acidic (anodic, +) end, with sample volumes of 20 - 25 µL for silver-stained gels, and 75 µL for Coomassie-stained gels, respectively. Sample volume was adjusted to the desired level using urea buffer. IEF was performed on an Ettan™ IPGphor™ 3 (GE Healthcare) at 20 °C, using a current limit of 50 µA. Care was taken to ensure that the loading cups were securely positioned so as to avoid sample leakage. Also, the entire surface of the focusing tray - including the sample loading cups - was covered with Biotechnology Grade Mineral Oil (Bio-Rad) in order to prevent drying of the strips, urea crystallization and the uptake of O_2 or CO_2. For 4-7 IPG strips, IEF protocol was set as follows: 500 V for 500 Vh, linear ramping to 1000

V for 800 Vh, linear ramping to 6000 V for 8800 Vh, hold at 6000 V for 2900 Vh, and step down to 500 V. For 3-11 NL IPG strips, the protocol sequence was: 500 V for 500 Vh, linear ramping to 1000 V for 800 Vh, linear ramping to 6000 V for 7000 Vh, hold at 6000 V for 2200 Vh, and step down to 500 V. After IEF the IPG strips were equilibrated in 2 mL equilibration buffer (50 mM Tris-HCl, 6 M urea, 2 % SDS, 20% glycerol, pH 6.8) with 2 % w/v DTT for 15 min, followed by 15 min in 2 mL equilibration buffer with 2.5 % w/v iodoacetamide (Bio-Rad). Saturating the strips with SDS allowed for the sample proteins to transfer efficiently from the IPG strips to the SDS-PAGE gel, without the use of a dedicated stacking gel. The strips where carefully set on top of a Criterion™ Precast Gel (Bio-Rad), insuring optimal contact between the trips and the upper side of the gel. The running buffer contained 5 mM Tris, 192 mM glycine and 0.1 % SDS, pH 8.6, and was prepared through dilution of 10X TGS buffer (Sigma-Aldrich). The gels were run in 2-gel Criterion™ Cell chambers (Bio-Rad) connected to an EPS 601 power supply (GE Healthcare). Run time was 55 min at 200 V.

3.2.4 Gel staining, image acquisition and data analysis

Gels prepared for silver staining first underwent protein spot fixation by holding for 1 h in a solution containing 30 % v/v ethanol (Merck KGaA) and 0.5 % v/v glacial acetic acid (Merck KGaA). Gel fixation causes proteins to be completely denatured, precipitated and trapped in the gel matrix in the form of large aggregates, ensuring that protein spots could not diffuse during the staining procedure or subsequent storage. The high concentration of organic solvent in the fixation buffer was also aimed at removing the SDS from the gels so that it would not interfere with protein staining. After fixation the gels where rinsed with 20 % v/v ethanol for 10 min and then with Milli-Q water for 10 min. Silver staining procedure was as follows: 1 min sensitization in 0.02 % w/v sodium thiosulfate (Merck KGaA), rinsing twice with Milli-Q water for 20 s, staining with 0.2 % w/v silver nitrate (Merck KGaA) for 30 min, rinsing twice with Milli-Q water for 10 s, followed by 2.5 min development with solution containing 3 % w/v potassium carbonate (Merck KGaA), 0.001 % w/v sodium thiosulfate and 0.0259 % v/v formaldehyde (Merck KGaA). Gel development was subsequently stopped with solution

containing 5 % w/v Tris base (GE Healthcare) and 2.5 % v/v glacial acetic acid. Gels were stored in Milli-Q water.

High-protein load gels were stained with Coomassie Brilliant Blue R-250 dye (Sigma-Aldrich) for 1 h. De-staining was achieved in 5 cycles of 20 min. The de-staining solution (also serving as a protein spot fixation buffer) contained 40 % v/v methanol (Merk KGaA) and 10 % v/v glacial acetic acid.

Gel images were captured with a MultiImage™ Light Cabinet (Alpha Innotech, San Leandro, CA, USA) using a 16-bit depth grayscale TIFF format. Image analysis was performed using Progenesis SameSpots software (Nonlinear Dynamics, Newcastle, U.K.). Images were examined for quality and damaged or saturated areas were masked from analysis. Gel images were automatically normalized to minimize inter-gel background spot abundance variation. Gel alignment was obtained through the manual input of 20 – 30 alignment vectors per gel image followed by automatic software alignment. The gel alignment was subsequently visually verified for all gels using the gel image "transition" and "checkboard" modes provided by the software, while systematically zooming in on the various gel regions. Spot filtering was not used in this study, in order to prevent the accidental discarding of small size spots. As all the cell samples were harvested in the same conditions, in the Experimental Design Setup stage of the SameSpots workflow the images were grouped in a "between-subject design", according to the strain of origin. Spot boundaries and volumes were automatically detected and the results were filtered for spots showing a minimum average spot volume ration between the strains of 1.2 (average ratio < 1.2 or > 1.2) and a Student's test p-value of less than 0.05. Principal Component Analysis (PCA) was performed initially on all spots in order to visualize the data clustering (i.e. to determine whether multivariate analysis can reliably discriminate between the two strains without taking into account the grouping set by the operator). PCA was then repeated using only the spots with a p-value < 0.05. The change in data clustering was observed with the aim of evaluating how well the data differentiates between the two strains after the exclusion of random or insignificant variations.

3.2.5 Experimental design

All experiments were performed with 4 biological replicates for each strain (a total of 8 gels per experimental run). IEF was performed on all 8 IPG strips simultaneously. For 2-D SDS-PAGE and subsequent staining, the experimental runs were divided into 2 batches of 4 gels each, with 2 biological replicates from each strain per batch. Each of the two electrophoresis cells ran one sample from strain PV256 and one from PV266 at the same time. This experimental blocking was aimed at minimizing the bias introduced by experimental variation that could be mistaken as originating from biological variation. Statistical power can be defined as the probability of finding a real difference if it exists. Figure 5 shows that 60 % of data from the pH 4-7 run had power > 0.80. This higher than the 47.9 % of data with power > 0.80 from pH 3-11 NL runs (Figure 6).

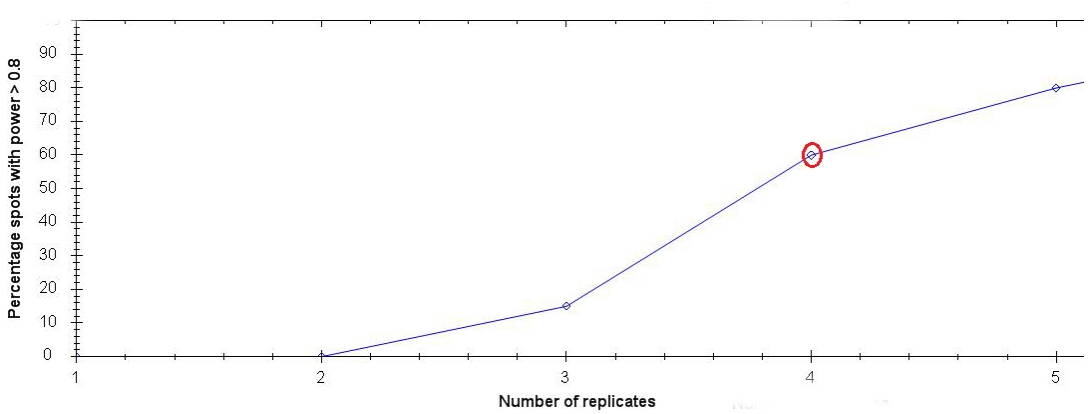

Figure 5. Power analysis for experimental runs using IPG strips pH range 4-7. Graph shows that 60 % of the data in this experimental run had power > 0.8. Using 5 biological would increase that proportion to 80 % of the data.

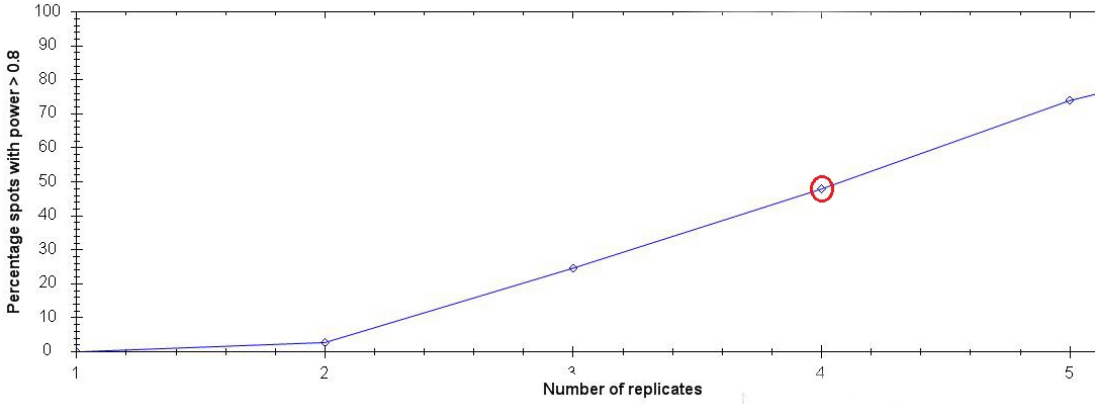

Figure 6. Power analysis for IPG strips pH 3-11 NL. Graph shows that 47.9 % of the data in these experimental runs had power > 0.8. Using 5 biological replicates would increase that proportion to 77 % of the data.

3.3 Results

3.3.1 Growth rate of the strains

The two strains exhibited different growth rates, with strain PV256 growing faster than PV266, as shown in Figure 7 below. The average generation time (calculated for the exponential growth phase region between 8 h and 24 h) was 4.9 h for strain PV256, and 5.4 h for strain PV266, respectively. Furthermore, final $O.D._{600}$ values were higher for strain PV256 (final $O.D._{600}$ around 2.2) compared to strain PV266 (final $O.D._{600}$ around 1.8). Generation time values of both strains fitted well with the 5 h generation time expected of *P. freudenreichii* in optimal growth conditions (Falentin et al. 2010).

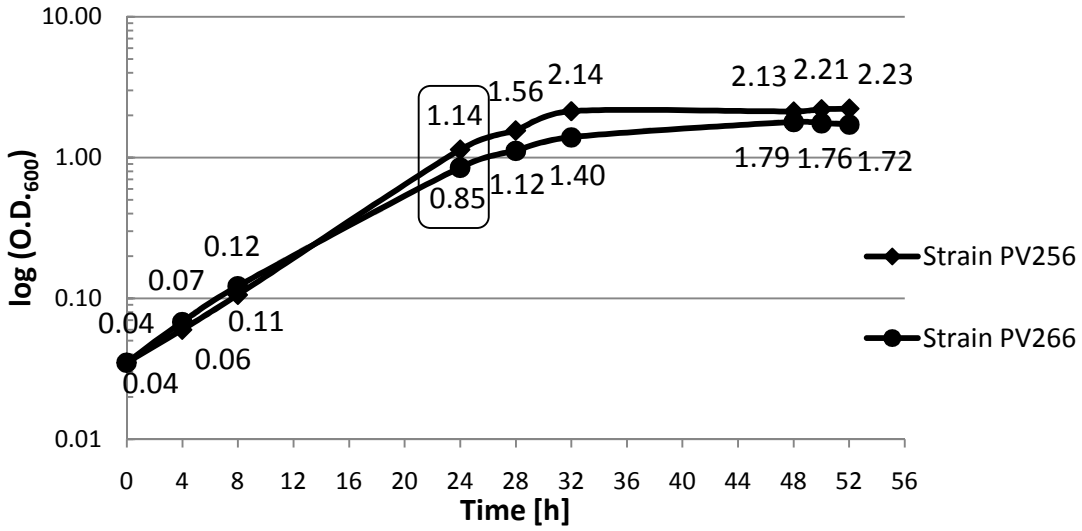

Figure 7. Growth curve of *P. freudenreichii* strains PV256 and PV266. The growth phase at which the cells were harvested (after 24 h) is highlighted together with the corresponding O.D values.

3.3.2 Effect of cell lysis treatment and strain on protein extraction

The influence of the number of cell lysis cycles applied to the cell sample on the amount of protein extracted was examined. For each of the two strains, cell pellets were processed with 1 through 6 cell lysis cycles, and the resulting samples were subsequently run on 1-D SDS-PAGE gels, shown in Figure 8 below.

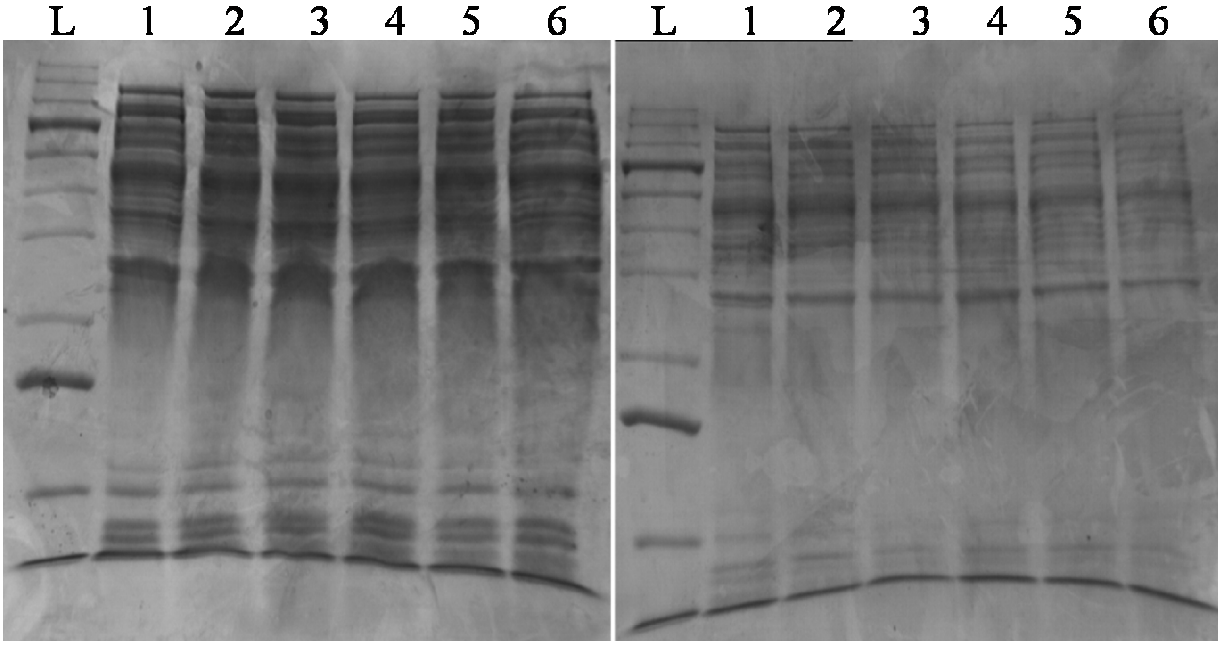

Figure 8. 1-D SDS-PAGE gel images of samples from strain PV256 (gel on the left) and PV266 (gel on the right). Lane L shows the protein molecular mass reference ladder (10 to 250 kDa). Lanes 1 through 6 correspond to samples prepared with 1 through 6 cell lysis cycles (left to right on each gel).

The 1-D SDS-PAGE gels showed that the number of cell lysis cycles had little influence on the amount of protein extracted from the cell samples, although for strain PV256 it appeared that the amount of protein was highest for samples prepared with 2 to 3 lysis cycles. In accordance with this finding, the protein extraction protocol subsequently used throughout this study employed 3 cell lysis cycles.

Proteins were extracted from cell pellets obtained through the centrifugation of 10 mL of bacterial culture. This volume of cell culture produced an amount of protein sufficient for one preparative run for Coomassie Blue staining, or for multiple analytical runs to be stained with colloidal silver, respectively. Samples from strain PV256 had on average higher protein content and higher protein concentration than those from PV266, as shown in Figure 9. This was to be expected as strain PV256 had higher cell density in the late exponential growth phase compared to strain PV266, as inferred from optical density measurements ($O.D._{600}$ 1.15 for PV256 compared to $O.D._{600}$ 0.85 for PV266).

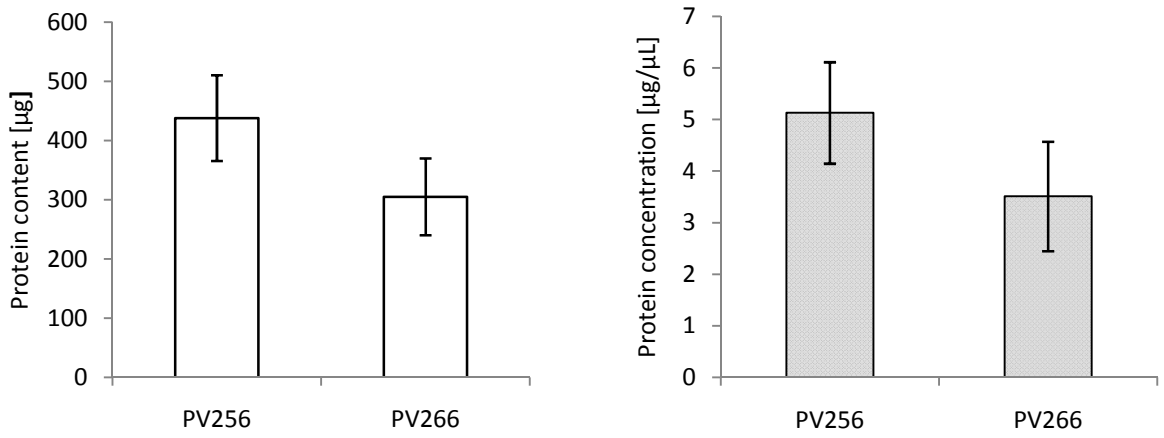

Figure 9. Average amount of protein extracted per sample (left) and average sample protein concentration (right) for strains PV256 and PV266.

3.3.3 Effect of IEF sample composition on proteome map quality

Two different sample compositions were tested for the IEF stage of 2-DE. The two experimental runs used the same IPG strips (pH range 3-11 NL) and the protein component included in the IEF samples was identical (from the same biological replicates). Gels A and B were silver-stained using identical protocols. The only parameter modified was the buffer composition of the sample with regard to reducing agents and IPG buffer (carrier ampholyte) concentration. The samples in the first experimental run (sample composition A) contained 10 mM TBP and 0.5 % IPG buffer, with no DTT added. Silver-stained gels obtained from that run are shown in Figure 10. The second experimental run used sample composition B: 4 mM TBP, 1.0 % IPG buffer and 50 mM DTT.

Gels with without DTT (composition A) showed poor protein spot resolution and severe streaking, particularly in the alkaline pI region. Gels obtained from samples with DTT (composition B) showed visible improvement compared to composition A. Composition B gels presented with overall superior spot resolution, reduced spot streaking, reduced spot overlap and improved protein detection (particularly in the alkaline region of the gel) (Figure 11). Some streaking in the alkaline region remained.

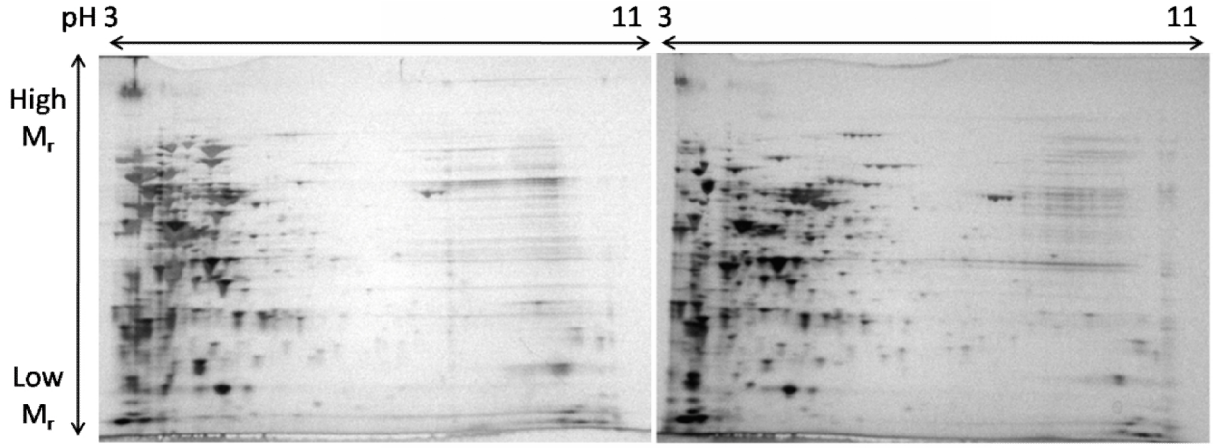

Figure 10. Proteome maps pH 3-11 NL of PV256 (left) and PV (266) right obtained with sample composition A (10 mM TBP and 0.5 % IPG buffer, no DTT added).

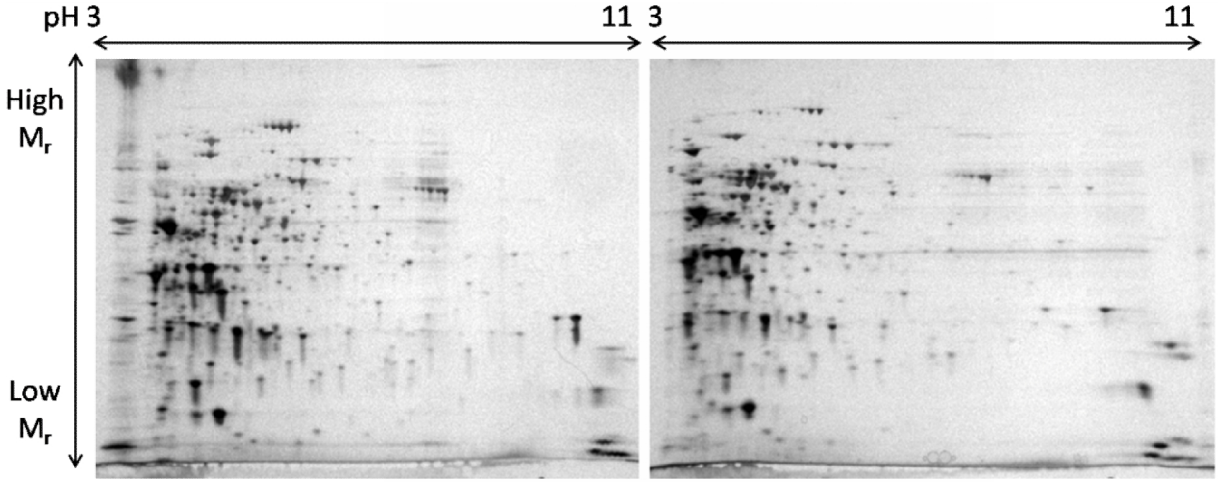

Figure 11. Proteome maps pH 3-11 NL of PV256 (left) and PV (266) right obtained with sample composition B (4 mM TBP, 1.0 % IPG buffer and 50 mM DTT).

The total number of protein spots detected by the software was higher in gels B (356 spots) compared to gels A (279 spots). The number of detected proteins showing significant inter-strain differential expression was more than 3 times higher in gels B (72 spots with $p < 0.05$ and volume ratio > 1.2) compared to gels A (23 spots). Improved spot resolution for gels B meant that pI isoforms of proteins (horizontal “spot trains”) were more easily visible than on gels A.

3.3.4 Effect of protein content and staining method on proteome map quality

Gels obtained with IEF sample composition A on IPG strips with pH 4-7 with a protein load of 200 µg / sample were stained with Coomassie Blue dye. These gels showed a comparatively low total number of spots, extensive horizontal streaking and high inter-gel variation (Figure 12). Gels obtained with the same sample composition (A) on the same type of IPG strips but with 40 µg / sample and stained with colloidal silver are showed in Figure 13. They displayed greatly improved spot resolution, separation and visibly less horizontal streaking compared to the 200 µg / sample, Coomassie-stained gels.

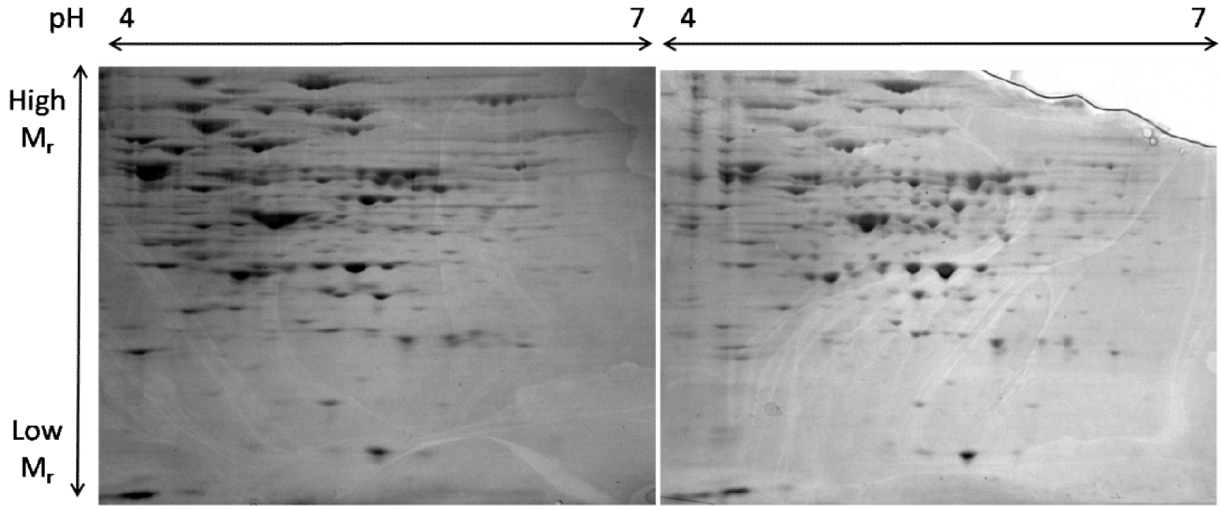

Figure 12. Proteome maps of PV256 (left) and PV266 (right) obtained with 200 µg protein samples on pH 4-7 IPG strips and stained with Coomassie Blue dye.

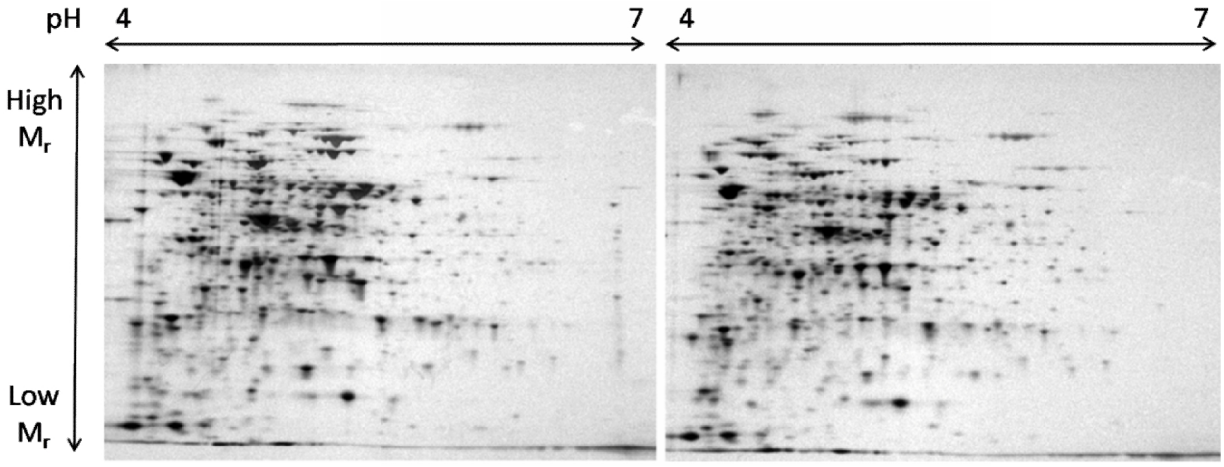

Figure 13. Proteome maps of PV256 (left) and PV266 (right) obtained with 40 µg protein samples on pH 4-7 IPG strips and stained with colloidal silver.

The total number of protein spots detected was 38.6 % higher on the silver-stained gels (305 spots) compared to the Coomassie-stained gels (220 spots). There were 60 detected proteins showing significant inter-strain differential expression on the silver-stained gels compared to just 18 on the Coomassie-stained gels.

3.3.5 Proteome maps and differential expression

In this study, the best quality proteome maps were produced with pH 4-7 strips (composition A) and with pH 3-11 NL strips (composition B). All the above gels were obtained using loads of 40 µg protein / sample and stained with colloidal silver. Gels produced with 3-11 NL strips and composition A samples, as well as the higher-protein load Coomassie-stained gels were of lower quality (not shown). The first half of this subchapter will deal with the pH 4-7 proteome map (Figure 14), with the pH 3-11 NL proteome map presented in the second half.

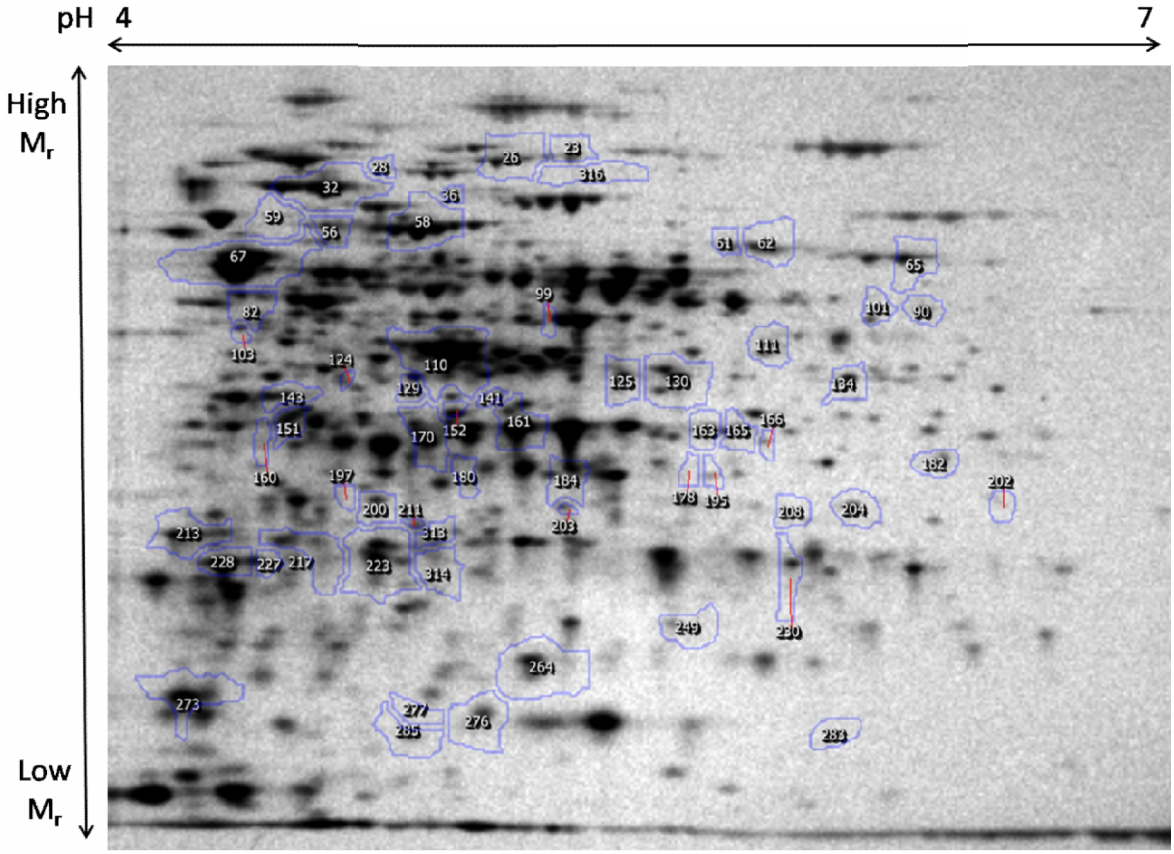

Figure 14. pH 4-7 proteome map of *P. freudenreichii* strain PV266 highlighting spots with significant differential expression (p-value < 0.05) compared to strain PV256.

There were 305 spots detected on the pH 4-7 proteome map. The 60 spots which showed inter-strain differential protein expression are listed in Table 1 below.

Table 1. Spots on the pH 4-7 proteome map showing differential protein expression between strain PV256 and PV266 (in increasing order of p-value).

Spot number	Anova p-value	Fold-change	Power	Spot number	Anova p-value	Fold-change	Power
59	3.39E-05	4.6	1.00	141	0.012	1.7	0.88
316	1.85E-04	5.5	1.00	61	0.013	2.5	0.84
200	1.94E-04	2.0	1.00	134	0.015	1.2	0.83
182	4.58E-04	1.4	1.00	228	0.016	1.3	0.80
160	6.54E-04	2.0	1.00	151	0.017	1.4	0.79
56	6.67E-04	1.7	1.00	230	0.022	1.7	0.77
273	7.57E-04	2.0	1.00	283	0.022	1.5	0.73
197	9.84E-04	2.7	1.00	129	0.024	1.3	0.73
211	0.001	3.0	1.00	67	0.026	1.2	0.70
203	0.001	3.1	1.00	99	0.026	1.9	0.69
101	0.002	2.0	0.99	23	0.026	2.0	0.69
213	0.002	1.7	0.99	180	0.027	1.6	0.69
204	0.002	1.6	0.99	249	0.028	1.3	0.68
58	0.002	1.2	0.98	124	0.029	1.9	0.67
152	0.003	2.6	0.98	130	0.029	1.6	0.67
264	0.003	1.4	0.98	276	0.030	1.4	0.67
28	0.003	5.4	0.98	217	0.033	1.2	0.65
165	0.003	1.9	0.98	32	0.035	1.4	0.63
163	0.003	1.8	0.98	161	0.035	1.5	0.62
178	0.003	2.4	0.97	26	0.036	2.0	0.62
36	0.003	2.2	0.97	208	0.036	1.4	0.62
314	0.004	1.7	0.97	103	0.040	1.5	0.61
202	0.004	1.6	0.97	227	0.041	1.6	0.59
166	0.004	2.0	0.96	65	0.042	1.3	0.59
143	0.004	2.1	0.95	170	0.043	1.3	0.58
82	0.005	2.3	0.95	195	0.043	1.5	0.57
62	0.005	2.4	0.95	285	0.043	1.4	0.57
111	0.007	1.4	0.92	125	0.045	1.7	0.57
90	0.007	1.8	0.92	184	0.047	1.2	0.56
277	0.009	3.1	0.89	223	0.047	1.5	0.55

The lowest power encountered in spots showing significant expression changes ($p > 0.05$) was 0.55. Of the 60 spots, 34 spots displayed power > 0.80.

The highest fold-change for the pH 4-7 proteome map was detected for spot 316, which had a 5.5-fold increase in average standardized spot volume in gels from strain PV256 compared to gels from PV266. The spot also had a low p-value, 1.85 E-04. The gel area containing spot 316 is shown below in Figure 15 for all 8 gels of the respective experimental run. An intense

spot showing multiple pI isoforms is visible on the gels from strain PV256 (left), while completely absent in the gels from strain PV266 (right).

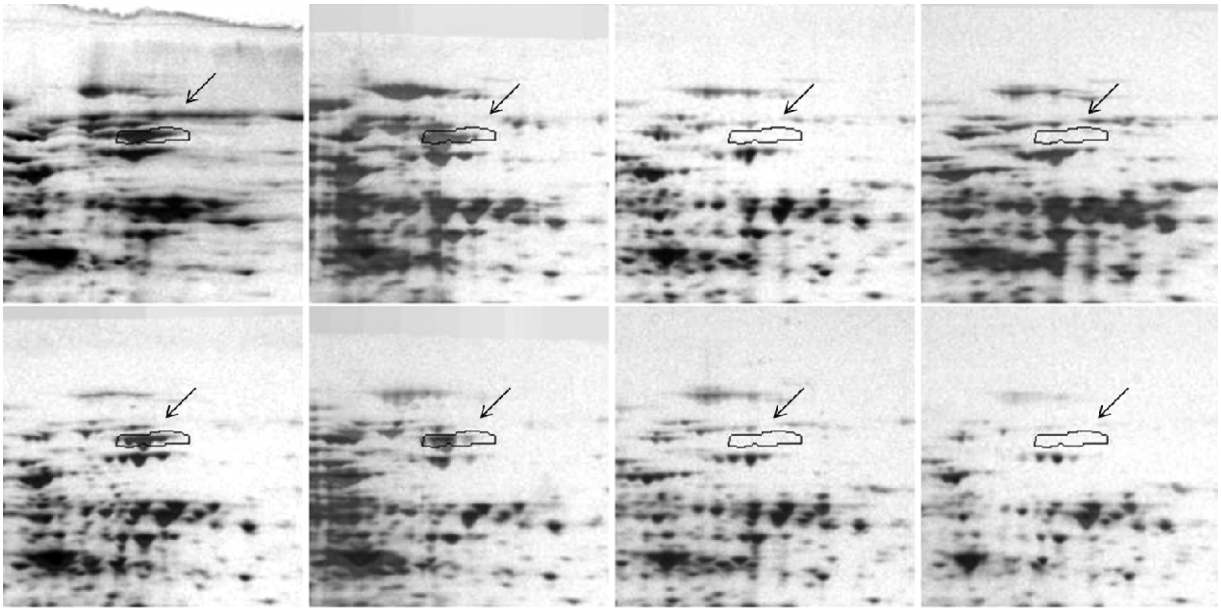

Figure 15. Example of differential protein expression in the pH 4-7 proteome map (spot 316). The 4 gels on the left are strain PV 256, the 4 gels on the right, PV266.

The Progenesis SameSpots software also allows for the 3-dimensional visualisation of protein spots. An example of differential protein expression in this display mode is shown below in Figure 16. Spot 276 of the pH 4-7 proteome map (p-value 0.03, 1.4-fold change) is clearly distinguishable in strain PV266 (right) while absent in strain PV256 (left).

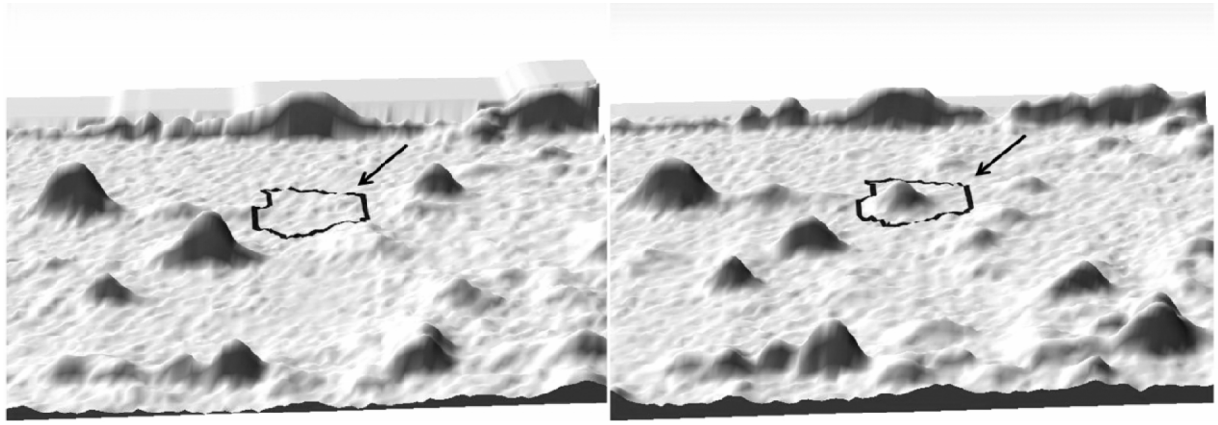

Figure 16. 3-D visualisation of differential protein expression, spot 276 of the pH 4-7 proteome map (p-value 0.03, 1.4-fold change). The protein spot is present in PV266 (right) while absent in PV256 (left).

A statistical overview of the pH 4-7 proteome spot data was obtained through a Principal Component Analysis (PCA) performed through the SameSpots software. PCA was initially performed on all spots regardless of p-value (Figure 17). This showed that the data clustering was clearly grouping the gels according to the strain of origin. The gels from strain PV266 clustered better than those from stain PV256. However, the separation between the two categories was clear.

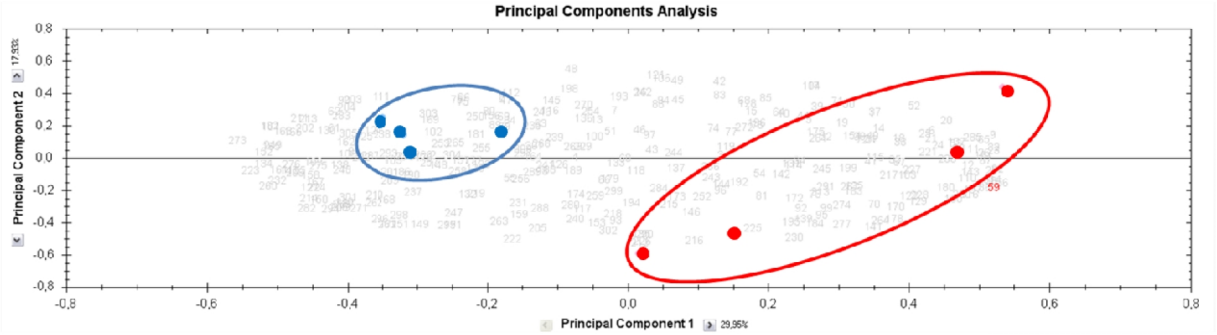

Figure 17. PCA analysis on all spots from pH 4-7 proteome map. Gels from strain PV256 are indicated by the red ellipsoid (right) while gels from PV266 are indicated in blue (left).

An additional PCA analysis included only the spots showing statistically significant variation between the two strains (Figure 18). This increased the amount of variation accounted for by principal component (PC) 1 to 70.05 % compared to 29.95 % in the first PCA (Figure 17), where all the spots were included. The exclusion of non-significant or random spot variations improved the clustering of PV256 gels, although they maintained a slightly larger spread than those of stain PV266.

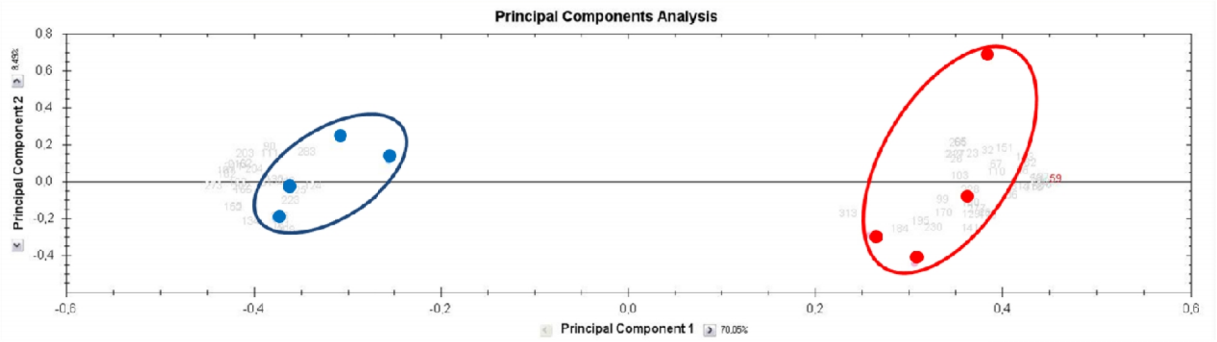

Figure 18. PCA analysis on spots from the pH 4-7 proteome map with significant inter-strain expression variation (p-value < 0.05). Gels from strain PV256 are indicated by the red ellipsoid (right) while gels from PV 256 are indicated in blue (left).

The proteome map produced using pH 3-11 NL trips and sample composition B also is shown below in Figure 18. It contained a total of 356 protein spots of which 72 showed significant differential expression (highlighted in Figure 18 and listed in Table 2).

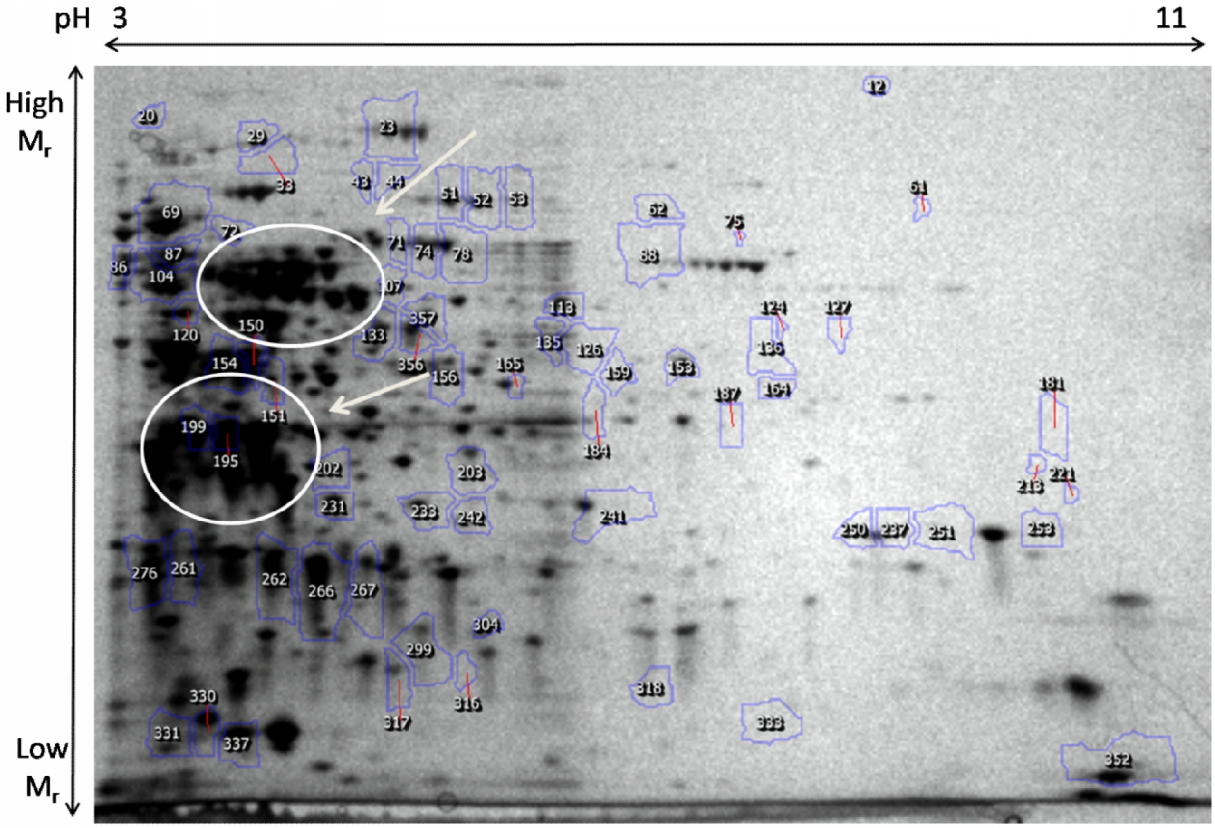

Figure 18. pH 3-11 NL proteome map of *P. freudenreichii* strain PV266 showing spots with differential expression compared to strain PV256. Saturated gel area in the approximately 4-6 pH region are highlighted in circles.

While the pH 3-11 NL proteome map showed several proteins that could not be visualized on the pH 4-7 gels, it displayed spot crowding in the area roughly corresponding to pH range 3-5 (left side of the map) which lead to saturation of those areas and hampered spot detection and image analysis.

Table 2. Spots on the pH 3-11 NL proteome map showing differential protein expression between strain PV256 and PV266 (in increasing order of p-value).

Spot number	Anova p-value	Fold-change	Power	Spot number	Anova p-value	Fold-change	Power
267	8.45E-05	2.0	1.00	333	0.016	1.4	0.79
356	1.03E-04	1.9	1.00	242	0.016	1.5	0.79
159	2.72E-04	2.2	1.00	12	0.016	1.6	0.79
33	3.52E-04	2.6	1.00	331	0.017	1.5	0.78
330	3.54E-04	2.7	1.00	203	0.017	1.2	0.78
304	3.78E-04	2.3	1.00	195	0.019	1.3	0.76
261	0.001	2.1	1.00	133	0.020	1.5	0.75
124	0.001	1.9	1.00	75	0.022	1.9	0.73
150	0.001	1.7	1.00	250	0.022	1.5	0.73
78	0.001	2.1	1.00	352	0.022	1.3	0.73
44	0.002	2.2	0.99	86	0.022	2.6	0.72
71	0.003	2.6	0.98	221	0.023	1.6	0.71
153	0.003	2.1	0.97	69	0.024	1.4	0.71
126	0.004	1.5	0.97	43	0.025	1.4	0.70
317	0.004	1.9	0.96	104	0.025	1.6	0.69
29	0.004	1.4	0.96	299	0.026	1.2	0.69
74	0.005	1.7	0.95	253	0.026	2.5	0.69
233	0.005	1.8	0.95	266	0.029	1.2	0.69
53	0.006	1.8	0.93	337	0.030	1.3	0.66
199	0.006	1.7	0.93	52	0.031	1.6	0.66
276	0.006	2.1	0.93	23	0.031	1.5	0.65
20	0.008	1.4	0.90	51	0.032	1.6	0.65
231	0.008	1.4	0.89	120	0.035	1.5	0.64
251	0.009	1.4	0.89	72	0.037	1.3	0.62
165	0.009	1.6	0.88	187	0.037	1.4	0.61
113	0.009	1.9	0.88	184	0.040	1.3	0.61
136	0.010	1.3	0.87	213	0.041	1.4	0.59
62	0.010	1.5	0.86	87	0.041	1.6	0.58
61	0.012	1.5	0.84	151	0.042	1.7	0.58
357	0.012	1.3	0.83	135	0.044	1.4	0.58
316	0.013	1.4	0.83	262	0.044	1.3	0.57
154	0.013	1.4	0.83	181	0.045	1.9	0.57
164	0.013	1.4	0.83	107	0.045	1.5	0.56
88	0.014	1.7	0.82	237	0.049	1.4	0.56
202	0.014	2.0	0.82	318	0.049	1.2	0.54
156	0.016	1.6	0.79	127	0.050	1.3	0.54

The highest spot volume fold-change detected on the pH 3-11 NL proteome map was for spot 33, which had a 2.6 fold-change and a p-value of 3.52E-04. A 2-D visualisation of the gel area containing the spot is shown in Figure 19 below. Spot 33 is clearly noticeable on gels from strain PV256 while absent in PV266.

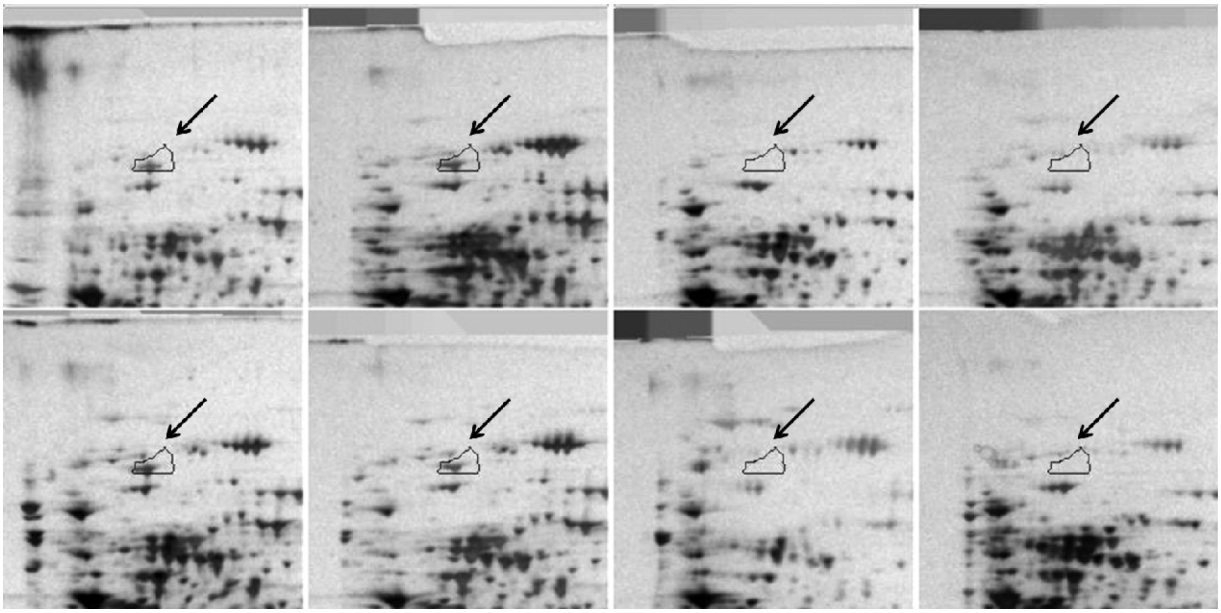

Figure 19. Example of differential protein expression in the pH 3-11 NL proteome map (spot 33). The 4 gels on the left belong to strain PV 256 while the 4 gels on the right to PV266.

Figure 20 below shows another example on differential expression from the pH 3-11 NL proteome map, this time in a 3-D visualisation. Spot 233 is clearly visible on the PV266 map while absent in strain PV256.

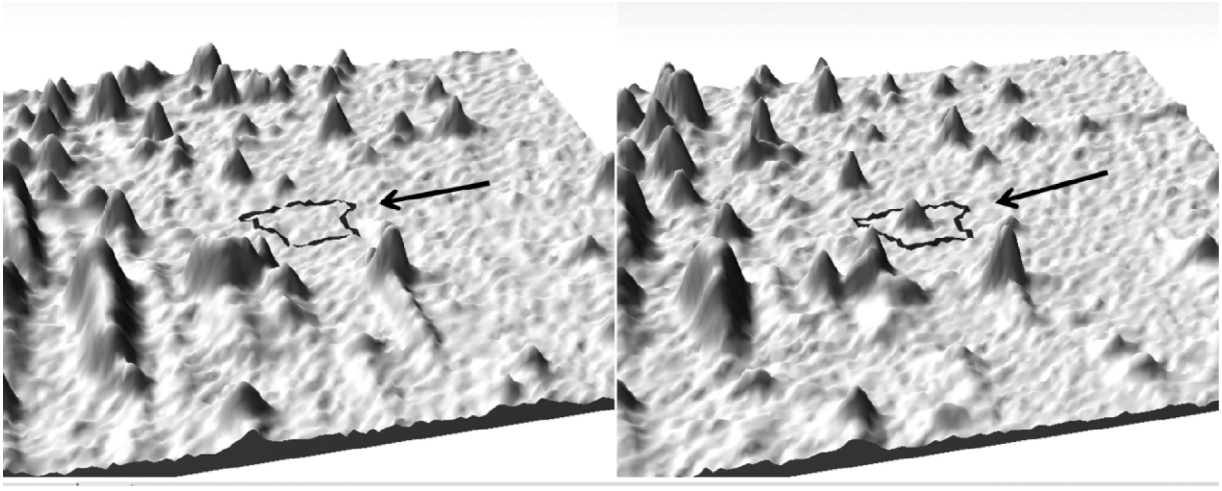

Figure 20. 3-D visualisation of differential protein expression, spot 233 of the pH 3-11 NL proteome map (p-value 0.005, 1.8-fold change). Protein spot 233 is present in PV266 (right) while absent in PV256 (left).

A PCA analysis was also performed for pH 3-11 NL proteome spots showing significant protein expression variation between strains PV2556 and PV266 (Figure 21). As in the case of the pH 4-7 proteome map, all-spot data clustering for the pH 3-11 NL proteome map was able to clearly differentiate between the two strains.

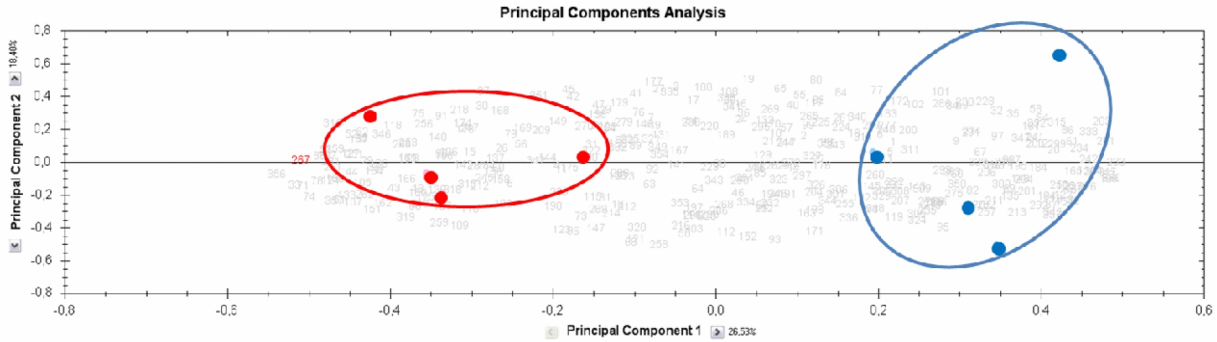

Figure 21. PCA analysis of all spots from pH 3-11 NL proteome map. Gels from strain PV256 are indicated by the red ellipsoid (left) while gels from PV266 are indicated in blue (right).

The PCA analysis performed only on the spots that showed statistically significant variation between the two strains is shown in Figure 22. This increased the amount of variation accounted for by PC1 to 67.89 % compared to 26.53 % in the first PCA which included all the spots (Figure 21). The degree of data clustering displayed by the two strains was similar. As in the case of the pH 4-7 proteome map, data clustering of both strains improved after the elimination of non-significant or random spot variations.

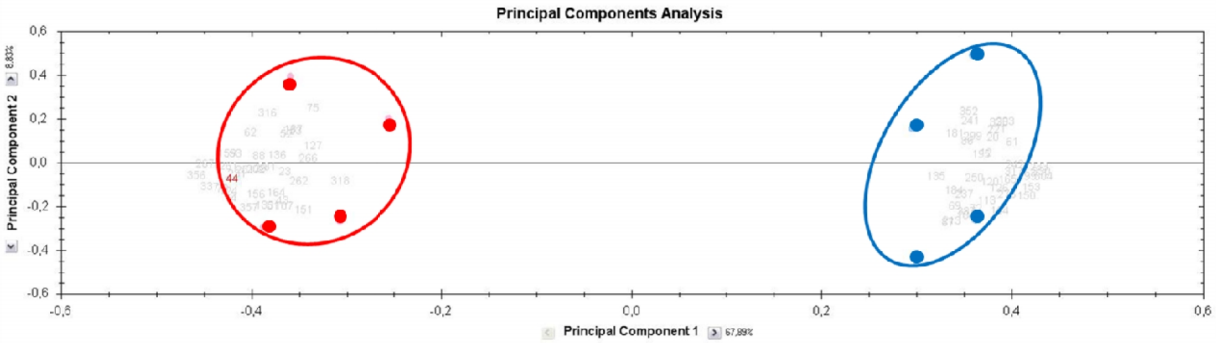

Figure 22. PCA analysis of spots from pH 3-11 NL proteome map with significant inter-strain expression variation ($p < 0.05$). Gels from strain PV256 are indicated by the red ellipsoid (left) while gels from PV 266 are indicated in blue (right).

3.4 Discussion

3.4.1 Effects of cell lysis treatment and IEF sample composition

Bacterial cell lysis is an important preliminary step in the protein extraction protocol for 2-DE. The 1-D SDS-PAGE gel images of protein samples obtained with 1 through 6 lysis cycles showed a small apparent increase in the amount of extracted protein for samples with 3 lysis cycles compared to those with 1 or 2 lysis cycles. As no further visible increase in the amount of proteins could be discerned for samples obtained with 4 through 6 lysis cycles, 3 lysis cycles were chosen as optimal for the protocol used in this study. A high number of lysis cycles that does not significantly augment the quantity of extracted protein is undesirable, as the prolonged sample treatment time increases the risk of unwanted changes in the state of the proteins (Westermeier et al. 2002). This may include thermal denaturation caused by the mechanical friction inherent to the glass bead stirring process (especially if the sample is not adequately cooled between lysis cycles) as well as proteolytic damage which can accelerate as cellular structure is broken up and proteins become increasingly vulnerable to protease action.

The IEF step is perhaps the most challenging stage of a 2-DE proteomic analysis. The composition of the IEF sample has to be adjusted to suit the specific characteristics of the experimental setup used as well as of the protein mixture under scrutiny in order to keep the proteins soluble during the procedure and ensure optimal separation (Brewis and Brennan 2010; Rabilloud et al. 2010). In this study, increasing the concentration of IPG buffer concomitant with the addition of DTT to complement TBP as a reducing agent resulted in considerable improvements in the efficiency of IEF (as indicated by the superior spot resolution and the higher number of spots detected in the gels subsequently obtained).

DTT is a reducing agent commonly used in IEF samples (Lopez 2007). It does however present a distinct drawback: the conditions demanded by the IEF process lead to the deprotonation of its thiol groups, thus causing the negatively charged form of DTT to migrate towards the anode. This phenomenon causes problems particularly for the proteins in the alkaline region of the IPG strip which becomes depleted in DTT and thus vulnerable to

oxidation effects (Westermeier et al. 2002). If the concentration of reducing agents in a region of the IPG strip drops below a certain threshold during the IEF process, proteins in that area may reform disulfide bonds. This in turn can cause some of the proteins to aggregate and precipitate resulting in diffuse rather than narrow bands on the strip. The poorly focused bands are subsequently converted after the SDS-PAGE step into horizontal smears on the 2-D gels. Compounding this problem, the reforming of intra- and intermolecular disulfide bonds tends to occur randomly and may end up linking peptide chains or proteins that were not originally bridged in the native sample (Herbert et al. 2001). This is one reason why the use of DTT is mandatory in the 1st step of the IPG strip equilibration stage (after IEF but before the SDS-PAGE stage) in order to ensure that all disulfide bridges are reduced. The 2nd step of the equilibration (alkylation with iodoacetamide) is meant to permanently block the thiol groups.

TBP has been proposed as a suitable replacement for DTT in the composition of IEF samples (Herbert et al. 1998). The main reason is that - being a nonionic molecule - TBP does not migrate under the influence of the electrical field and as such was suggested to preserve a stable reducing environment in the IPG strip. In this study, however, samples with 10 mM TBP performed considerably worse than those with 50 mM DTT and 4 mM TBP (as exemplified in Figure 23).

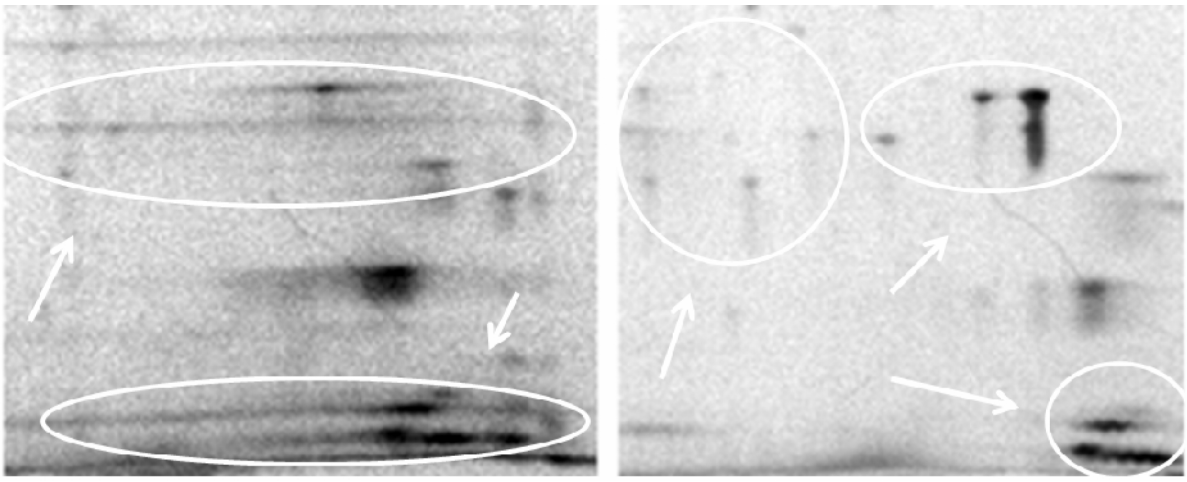

Figure 23. Comparison of corresponding areas in the alkaline, low-molecular mass region of the pH 3-11 NL proteome maps (magnified selection) obtained with composition A (10 mM TBP) – left image, and composition B (4 mM TBP and 50 mM DTT) – right image. Arrows point to areas of extended horizontal streaking (left), compared to superior spot resolution and significantly reduced streaking (right).

A potential explanation for the observed ineffectiveness of TBP may be found in its reported tendency to become unstable in the presence of high concentrations of urea (Hoving et al. 2002). It might be possible that the conditions imposed by the IEF protocol used in this study somehow affected the TBP and prevented it from fulfilling its role as a reducing agent. It also appears that the concentration of DTT used in composition B samples (50 mM) was high enough to prevent depletion in the alkaline region and maintain reducing conditions across the entire pH range. In fact, a number of workers have obtained good results using lower concentrations of DTT compared to this study (as low as 20 mM) and without including TBP at all (Izquierdo et al. 2009; Wu et al. 2009; Majumder et al. 2011). It therefore seems that DTT is a crucial component of the IEF sample composition, particularly in experiments using wide pH range strips (such as 3-11 NL) and cannot be replaced with TBP.

On a cautionary note, it should be mentioned that in this experiment the profile of the reducing agents was not the only parameter changed in the IEF sample. The concentration of IPG buffer was also altered between the two experimental runs. IPG buffer (i.e. carrier ampholytes) can also help to maintain proteins in a solubilized state, especially when used in optimal concentrations (Westermeier et al. 2002). As such, it is not clear whether the superior results of composition B samples were due solely to the addition of DTT, to the increase of IPG buffer concentration, or to the combined effect or perhaps synergistic interaction of the two.

3.4.2 Effect of IEF sample protein load and gel staining method

The amount of protein loaded in the IEF samples is known to affect the outcome of the IEF process (Westermeier et al. 2002). The higher protein-load (200 µg protein / sample) Coomassie-stained pH 4-7 gels obtained in this study were of considerably lower quality compared to the 40 µg protein / sample, silver-stained gels. A content of 200 µg protein / sample was not expected to cause IPG strip overload, as this type of strips have been used successfully in preparative runs with loads of 500 µg or even as high as 1 mg (Lopez 2007). It is however possible that some dominant proteins in the sample were present in quantities high enough to produce small, localized overloads which might partially account for the horizontal

streaking and relatively poor spot resolution that characterized the Coomassie-stained gels. The exact cause of the large spot resolution quality decrease in the higher protein-load gels remained however unclear.

The fact that the number of spots detected on the silver-stained gels was almost 40 % higher than on the Coomassie-stained gels is largely owed to the characteristics of the two staining methods. Silver staining is one of the most sensitive methods for the visualisation of protein spots on polyacrylamide gels, with detection limits in the low nanogram range (Chevallet et al. 2006). This is one or even two orders of magnitude better than the detection threshold of Coomassie dyes (Winkler et al. 2007). The main disadvantage of silver staining methods is their narrow dynamic range, with a linear response restricted between 0.02 and 0.80 ng protein / mm^2, compared to a range of 0.5 to 20.0 µg / mm^2 for Coomassie dyes (Lopez 2007). A particularly damaging feature of silver staining is the fact that the central regions of over-stained spots become lighter in color than the periphery of the respective spots, leading to what are somewhat informally called “donut” or “crater” spots (Winkler et al. 2007). The phenomenon occurs because the silver ions that bind to proteins decrease the binding of other silver ions in a process that is not yet fully understood (Chevallet et al. 2006). This type of artifact (“plateau staining”) cannot be managed by the spot detection software currently available and translates into an inability to provide quantitative measurements for higher protein concentrations. However, silver staining appears to be a more valuable tool than Coomassie staining for a particular category of differential studies that are aimed principally at the detection of protein expression differences, without necessarily requiring quantitative determinations. This was the case for the present study, where the goal was to obtain good spot resolution and detect the highest possible number of individual spots. The fact that silver staining detected 60 differentially expressed spots in the 4-7 pH range gels compared to just 18 spots for the Coomassie-stained gels supports the statement that silver staining was the superior method for this type of study.

3.4.3 Proteome maps and differential expression

The highest total number of protein spots in this study was detected on the pH 3-11 NL proteome map (356 spots). The pH 4-7 proteome map yielded a comparatively lower number of spots (305 spots). This finding was somewhat surprising due to the fact that, as observed in a previous study, the vast majority of *P. freudenreichii* proteins display an isoelectric point in the range 4-7 (Jan et al. 2001). As protein spots do not exhibit sharp boundaries but rather a Gaussian distribution of protein concentration and therefore of staining intensity, the "tails" of larger spots tend to overlap with smaller, neighboring spots if the they are packed too close together (Rehm 2006). When wide pH range IPG strips of equal length are used, the strip area corresponding to the pH 4-7 range is only a fraction compared to that on pH 4-7 IPG strips. This leads to spot over-crowding and the formation of saturated gel areas in the pH 4-7 range, thus canceling out the benefits of the extended pH range offered by the 3-11 NL strips. The number of alkaline proteins with pI > 7.0 detected tends to be lower than the number of pI 4-7 proteins masked through over-crowding. Indeed, pH 4-7 IPG strips have been by far the most commonly employed in proteomic studies focusing on *P. freudenreichii* (Leverrier et al. 2003; Anastasiou et al. 2006; Zhang et al. 2010; Dalmasso et al. 2012). One explanation for the lower number of spots detected in this study on the pH 4-7 map may have been the fact that the IEF sample composition used in the respective experimental run did not contain DTT. As previously discussed, the addition of DTT to the IEF sample composition resulted in considerably improved spot resolution, and this may have been a crucial advantage for the pH 3-11 NL gels in this study.

The type of staining used can influence the number of spots detected on a 2-D gel. A comparison of a parent strain and a genome-shuffled strain by Zhang et al. was the only proteomic study on *P. freudenreichii* to use silver staining for protein spot visualisation (Zhang et al. 2010). They were able to detect 180 protein spots on a pH 3-8 NL proteome map, of which 38 were found to be differentially expressed between the two strains. The fact that they used short IPG strips (7 cm as opposed to 11 cm in this study) probably accounts for the comparatively low total spot number.

Most of the other proteomic studies of *P. freudenreichii* to date have relied on a 2-DE protocol set up by Jan et al. who used autoradiography for the detection of protein spots (Jan et al. 2001). In these experiments, the bacterial cells were radioactively labeled using a [^{35}S] methionine / cysteine protein labeling system (Jan et al. 2002b; Leverrier et al. 2003; Anastasiou et al. 2006; Dalmasso et al. 2012). Autoradiography has the advantage of offering a highly linear response compared to chemical dyes (Anastasiou et al. 2006). This approach yielded good results, as proteome maps with a total number of spots between 700 and 900 were obtained. However, the decisive technical advantage of these experiments was most likely the length of the IPG strips used (18 cm or 24 cm, compared to 11 cm in this study). The use of longer IPG strips (implicitly resulting in a higher final 2-D gel area) prevents protein spot over-crowding and yields superior 2-D gel maps.

For both pH 4-7 and pH 3-11 NL maps, the PCA analysis performed on all the spots (which does not take into account the operator-set sample categories) yielded data clustering that clearly grouped the gels according to the strain of the biological replicate. No unusual outliers were noticeable in the 4-gel groups. This showed that analytical variance was sufficiently low to allow the clear visualisation of biological variance. The use of blocking in the 2-DE setup most likely aided the reduction of variance due to experimental variations. Removal of spots with p-value > 0.05 from the PCA analysis (i.e. spots showing insignificant or random variation) enhanced the separation of the sample groups corresponding to the two strains. This could be observed not only by the increase in the linear distance between the two groups on the graph, but also by the increase in the proportion of variance accounted for by the first principal component (reaching approx. 70 %). Together with the good levels of statistical power (> 0.55 for all significant spots, with around half of significant spots showing power > 0.80), the PCA analysis pointed to the experimental design used in this study as being appropriate for the detection of biological differences between the two *P. freudenreichii* strains under scrutiny. The potential for improvement was visible though, as the statistical power requirement usually set in proteomic studies is 0.80 for all significant spots. The power graphs shown in the Experimental Design section indicate the use of 5 biological replicates

instead of 4 - as in the present study - would have achieved this goal. Most of the previous proteomic studies on *P. freudenreichii* do not specify the statistical power of their spot data, although they do state that at least 3 biological replicates were analyzed (Jan et al. 2001). The drawback of using an increased number of biological replicates is the associated higher consumption of time and analytical resources. Therefore, the best course of improvement in this case would most likely be the use of longer pH 4-7 IPG strips (18 cm or 24 cm, instead of 11 cm) and of correspondingly larger gels that would greatly improve spot resolution and the coverage of the proteome maps. Future work on the *P. freudenreichii* proteome maps and inter-strain comparisons would also mandatorily include MS analysis and identification of the highest possible number of differentially expressed proteins in order to shine light on the causes of phenotypic differences between the subject strains.

4 CONCLUSIONS

This study examined the influence of the number of cell lysis cycles on *P. freudenreichii* protein extraction as well as the influence of the concentrations of DTT and IPG buffer on the efficiency of the IEF process. Two staining methods for protein spot visualisation were compared (i.e. silver staining and Coomassie staining) and the effect of the amount of protein loaded in the IEF samples was investigated. The main aim was to obtain proteome maps of the two strains of *P. freudenreichii* examined and detect differentially expressed proteins.

The effect of the number of cell lysis cycles on the amount of protein extracted was small, although a number of 3 lysis cycles appeared to provide optimal results.

The addition of 50 mM DTT alongside 4 mM TBP in IEF sample composition greatly improved the resulting protein spot resolution compared to samples that contained 10 mM TBP but no DTT. It appears that - particularly for wide range pH intervals (i.e. pH 3-11 NL) - DTT is a crucial component of the IEF sample composition and cannot be replaced with TBP. The increase in the concentration of IPG buffer from 0.5 % to 1.0 % may also have contributed to the observed improvement.

Silver staining of low protein load gels (40 µg / sample) yielded a 38.6 % higher total spot number compared to higher protein load (200 µg / sample) Coomassie-stained gels. The exact influence of the protein load on the efficiency of the IEF process remained undetermined. The number of differentially expressed spots detected on the silver-stained gel (60 spots) was also much higher than the one corresponding to the Coomassie-stained gels (18 spots). In spite of the inability to provide quantitative results, greater sensitivity meant that silver staining was the superior method for the construction of proteome maps aimed at maximizing the total number of spots.

This small scale study employing 11 cm IPG strips was able to obtain pH 4-7 and pH 3-11 NL proteome maps that displayed 305 spots, and 356 spots, respectively. 72 differentially expressed proteins were detected on the pH 3-11 NL proteome map compared to 60 on the pH

4-7 proteome map. However, the 3-11 NL proteome map displayed visible spot over-crowding in the pH range approximately 4-6. Moreover, the pH 4-7 proteome map was obtained using samples that did not contain DTT, and it is likely that the optimal setup would have involved the use of DTT-containing samples run on pH 4-7 IPG strips.

The multivariate analysis of data extracted from the comparison of the proteome maps of the two strains of *P. freudenreichii* showed that the analytical variance in the data set was sufficiently low to allow for the visualisation of relevant inter-strain biological variance. The number of biological replicates and the experimental blocking used thus appear to have been adequate. The statistical power obtained in this study was good (>0.55), although only approximately half of significant spots displayed power equal to or higher than the commonly used target of 0.80.

Both the coverage of the proteome maps and the statistical power of the data obtained would best be improved by a repeat of the study using silver-staining, longer IPG strips (18 cm or 24 cm instead of the 11 cm strips) and DTT in all IEF samples. The influence of the sample protein load and IPG buffer concentration on the efficiency of IEF should be further investigated. Finally, differentially expressed proteins should be excised and identified by MS analysis. This step would provide critical information allowing for the deciphering of specific pathways and uncovering the proteins responsible for inter-strain phenotypic variation in *P. freudenreichii*.

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