Genomic analysis of the regulation of sorbic acid-inducible protein expression in spoilage yeast

Vasso Makrantoni

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CONTENTS

Abbreviations

ABC	ATP-binding cassette
CAI	Codon Adaptation Index
CBP	Calmodulin binding peptide
CIP	Calf Intestinal Phosphatase
CPY	Carboxypeptidase Y
CTD	Carboxyl-terminal repeat domain
CV	Column volume
DTT	Dithiothreitol
2-D	2-Dimension
2,4-D	2,4-dichlorophenoxyacetic acid
ECL	Enhanced cheminumilescence
EDTA	Ethylenediaminetera-acetic acid
EGTA	Ethylene glycol bis(2-aminoethyl ether) -tetraacetic acid
ER	Endoplasmic reticulum
ES-MS	Electrospray mass-spectrometry
FFE	Fast Flow Electrophoresis
FK506	Calcineurin inhibitor
GFP	Green Fluorescent Protein
GST	Glutathione-S-Transferase
IEF	Isoelectric focusing
IPTG	Isopropyl-β-thiogalactopyranoside
LBMedium	Luria Bertani medium
mAb	Monoclonal antibody
MEN	Mitotic exit network
MFS	Major Facilitators Superfamily
OD_{600}	Optical density at 600 nm
ORF	Open Reading Frame
P-ATPase	Plasma membrane ATPase
PCR	Polymerase Chain Reaction
PDR	Pleiotropic drug resistance
PMSF	Phenylmethylsulphonylfluoride
PVC	Packed cell volume
SC	Synthetic Complete medium
SDS-PAGE	Sodium dodecylsulfate-poyacrylamide gel electrophoresis
SGD	Saccharomyces Genome Database
SPBs	Spindle pole bodies
TAP	Tandem Affinity Purification
TEV	Tobacco Etch Virus
UBX	Ubiquitin regulatory X subunit
V-ATPase	Vacuolar ATPase
WARE	Weak Acid Response Element
YEPD	Yeast Extract broth

Declaration

I, Vasso Makrantoni, hereby certify that this thesis, which is approximately 45,000 words in length, has been written by me, is a record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Abstract

The ability of yeasts to grow and adapt under extreme environmental conditions and in the presence of weak acid food preservatives enables them to spoil manufactured products causing major economic losses. Sorbic acid is used extensively in the food industry as a preservative largely to prevent the growth of yeasts and moulds. Understanding its effect on the yeast cells could lead to the development of specific and consumer-friendly preservatives. The aim of this study was to gain further fundamental insight into the mechanisms of adaptation to this compound and identify potential targets for new preservation strategies.

Current knowledge indicates that the main mechanisms of sorbic acid adaptation to weak acid stress requires function of the ABC transporter Pdr12p, regulated by the transcription factor War1p and an enhanced plasma membrane H^+-ATPase activity.

In this study, we have undertaken a systematic survey for genes that conferred increased sensitivity to sorbic acid. Taking advantage of the existing complete set of *S. cerevisiae* haploid deletion mutants, we performed a phenotypic screen of 4,847 non-essential genes, representing ~88 % of all viable haploid disruptants. The screen revealed 119 gene deletions with varied physiological roles and metabolic functions implicated in sorbic acid stress recovery, accounting for 2.5 % of the genes analysed. The screen was validated by the identification of many of the genes already known to influence sorbic acid tolerance. From the initial genomic screen we have identified a novel role for the cell cycle regulated serine-threonine protein kinase, Dbf2p in sorbic acid tolerance, which we chose as a focal point for further analysis.

We developed a new, fast and sensitive methodology in yeast, which combined affinity purification, 2D-PAGE and peptide mass fingerprinting for studying changes in the yeast phosphoproteome under sorbic acid stress. This approach provided a powerful genomic screening tool that can identify initial candidate phosphoproteins for further studies. We applied this methodology on the *Δdbf2* mutant strain and identified Vma2p, a component of the ATP-binding subunit of the vacuolar H^+-ATPase as a putative phosphoprotein. Importantly, deletion of *VMA2* resulted in notable sorbic acid

sensitivity. In this thesis with a combination of phosphoproteomics and biochemistry we present the first evidence that Dbf2p regulates the phosphorylation of the vacuolar H^+-ATPase (V-ATPase) non-catalytic subunit Vma2p via a novel, Pdr12p-independent signal transduction pathway. Therefore, we propose that a fully functional V-ATPase is crucial for sorbic acid adaptation in budding yeast *S. cerevisiae* by pumping up excess protons from the cytosol and storing them into the vacuole thus, resulting in weak acid adaptation.

In this study we propose that sorbic acid stress adaptation in *S. cerevisiae* is a multi-component stress response involving distinct mechanisms of tolerance. We have highlighted an important, novel mechanism of sorbic acid adaptation in yeast, which involves a fully functional V-ATPase regulated via the Dbf2p kinase. This knowledge may offer potential new routes to combat spoilage or better understanding of how it can be prevented.

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CHAPTER 1

1. Introduction

1.1 Food spoilage and the role of additives in food preservation

Food preservation techniques have a long history of use. Foods have been preserved by smoking, drying salting and microbial fermentation for centuries. The use of chemical preservatives others than salts, sugars, spices and vinegar only became widespread within the last 200 years (Thakur, 1994). Increased use of such additives appears to be primarily due to changes in the way food products are now processed and subsequently distributed. Today foods which are produced in one geographical area, are often transported to another area for processing and yet another for distribution. To accomplish the long-term storage and distribution time necessary for such a system together with greater consumer demand for more convenience foods, the need for more effective preservation systems has increased in recent times.

Although more sophisticated, many preservation methods nowadays are still based on older, empirically derived procedures. Preservatives are used to prevent or delay microbial growth, thus prolonging shelf life and safety of food products. Food spoilage is a complex event, in which a combination of microbial and biochemical activities may interact. The microbiology of food spoilage has over the years received considerable attention and the characterisation of the typical microflora, which develop on different types of foods during storage, has been well documented (Huis in 't Veld, 1996; Mossel, 1995).

Progress in the development of new preservation systems has been slow and despite the recognised need for additives their safety has been questioned on numerous occasions. Over the last years consumer demands for more 'natural' products and subsequent changes in legislation in many countries has caused a move away from chemical additions to foods. Thus, many chemicals known to exhibit strong antimicrobial properties have been abandoned, when undesirable physiological and biochemical properties were discovered (Thakur, 1994).

Amongst the most extensively used treatments are those that combine an antimicrobial acid with simultaneous reduction of pH values. The major weak organic acid food preservatives include acetic, propionic, sorbic and benzoic acids against spoilage microorganisms. Together with the inorganic sulfite and nitrite these compounds constitute the most widely used acid preservatives in industrial food and beverage production (Beales, 2004; Brul et al., 2002; Gould, 1996).

Other newer techniques of food preservation include: irradiation, application of high hydrostatic pressure, electroporation, ultrasonication combined with increased temperature and slightly raised pressure and addition of bacteriolytic enzymes. All these preservation systems act by direct inactivation of the target microorganisms (Gould, 1996). Yet, the major preservation techniques currently employed to prevent or delay food spoilage are reduction in temperature, pH and water activity (Loureiro, 1999).

Microbial spoilage of food causes losses of up to 40% of all food grown for human consumption worldwide. Yeast growth is a major factor in the spoilage of foods and beverages that are characterized by a high sugar content, low pH, and low water activity, and it is a significant economic problem (Thomas, 1993; Tudor, 1993). However, only a small fraction of the characterized yeast species are classified as 'spoilage yeasts' (Steels et al., 2000). They include a number of *Zygosaccharomyces*, as well as some isolates of *Saccharomyces cerevisiae*. *Z. rouxii* is considered the most important *Zygosaccharomyces* because of its ability to grow at very low a_w, while *Z. bailii* and *Z. lentus* are the most resistant to weak acid preservatives. The latter yeasts can sometimes grow in the presence of the highest levels of those acids allowed in food preservation, at pH values below the pK_a values of these acids (Fleet, 1992; Steels et al., 2000; Thomas, 1985). However, *S. cerevisiae* isolated from instances of food spoilage can frequently adapt to levels of sorbate and benzoate only slightly lower than the levels inhibitory to *Z. bailii* and *Z. lentus*.

While growth of spoilage yeasts such as *Z. bailii* and *S. cerevisiae* can usually be retarded by weak organic acid preservatives, the inhibition often requires levels of preservative that are near or greater than the legal limits and is limited to a cytostatic effect (Lambert and Stratford, 1999). Apart from a number of studies on the uptake and utilization of acetate by *Z. bailii* (Sousa et al., 1998; Sousa, 1996) comparatively little is

known about how *Z. bailii* and *Z. lentus* acquire their remarkable weak acid resistances. This study will focus on *S. cerevisiae* where considerably more is known about weak acid adaptation. Progress in the development of novel preservation systems is of great importance. A comprehensive understanding of the adaptive response to weak organic acids and the physiological requirements for these adaptations is a prerequisite for designing ways to overcome the resistance of food spoilage organisms to growth-inhibiting compounds.

1.2 Weak organic acid preservatives

Weak lipophilic acids such as sorbate, and benzoate can occur naturally in many fruits and vegetables. These have been widely used to maintain microbial stability in low pH foods including fruit juices, beverages, wines, pickled vegetables, mayonnaise and salad dressings (Pilkington and Rose, 1988; Restaino, 1982; Sofos, 1981) It has also become apparent that resistance to one of the more common preservatives is often associated with resistance to one or more of the others. In particular species resistant to sorbic acid appear to also exhibit some resistance to acetic acid (Warth, 1985).

1.2.1 Sorbic Acid

$CH_3CH=CHCH=CHCOOH$

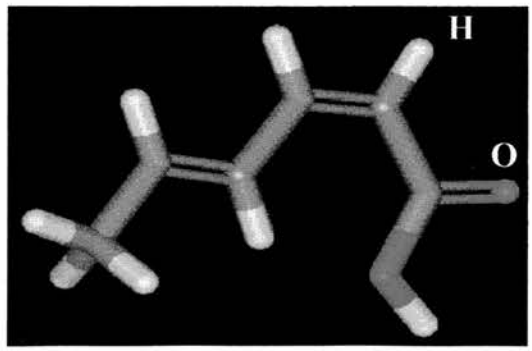

Figure1.1 Structureofsorbicacidmolecule. (http://www.chm.bris.ac.uk/webprojects2001/anderson/e200.htm).

Sorbic acid is a naturally occurring weak acid, appears as white crystals and is used either on its own or as the sodium, potassium or calcium salts known as sorbates. Sorbic acid is a straight chain trans-trans a, b-unsaturated monocarboxylic acid with the chemical formulae $CH_3CH=CHCH=CHCOOH$ a molar mass of 112.13 and a pKa of 4·76 (Figure 1.1).

It was first isolated from the oil of unripened Rowanberries by a German chemist A.W. Hofman during 1859 in London (Sofos, 1981) but it was not until the 1950's that sorbic acid became widely used as a preservative. Sorbic acid is an antimicrobial agent, which has been most intensively studied, tested and used, in a wide range of products. A survey of the literature demonstrates the extensive range of products for which sorbate has been used as a preservative. ((Sofos, 1981); Table 1.1).

Because of their physiological inertness, their effectiveness even in the weakly acid pH range and their neutral taste, sorbic acid and its salts have become the leading preservatives in the food sector throughout the world in the past 30 years. Sorbic acid is the only unsaturated organic acid currently permitted as a food preservative (Baird-Parker, 1980).

Table 1.1. Sorbate applications in food products (adapted from Sofos, 1981).

Dairy products	Cheeses, yogurt, sour cream, cheese dips and spreads
Bakery products	Cakes, pies, doughnuts, fudges, icings, toppings
Fruits and vegetable products	Wines, beverages, fruit juices, dried fruits, salads, fermented vegetables
Other food products	Dry sausages, mayonnaise, margarine, smoked and salted fish, salad dressings

The most commonly used products are sorbic acid itself (E200) and potassium sorbate (E202). In many countries sodium sorbate (E201) and calcium sorbate (E203) are also permitted. Sorbic acid is sparingly soluble in water, whereas its salts have much better solubility and are thus preferred when it is desired to use the preservative in liquid form or when aqueous systems are to be preserved (Lueck, 1990). Practical applications of sorbates include the preservation of human food, animal feed, pharmaceuticals and packaging material. As with all fatty acids, sorbic acid is metabolised completely by both humans and animals (Sofos, 1981).

Generally sorbic acid is more effective against yeast and moulds than against bacteria. In the case of the latter, certain selectivity has been reported. A general opinion is that catalase-positive organisms are less resistant to sorbate than catalase-negative ones

(Lueck, 1980; Sofos, 1981). Effective antimicrobial concentrations of sorbate in most foods are in the range 0.05 %-0.3 % (Sofos, 1981).

However, it is becoming clear that microorganisms possess a series of mechanisms whereby they can adapt rapidly to particular environments. This enables them to colonize and grow on diverse substrates and to adapt to a wide range of hostile conditions such as low pH environments, higher temperatures and weak acid conditions (Huis in 't Veld, 1996). These hostile conditions are commonly used in food preservation (Hill et al., 1995; Mager and Ferreira, 1993; Sanchez et al., 1992). One such microorganism, which exhibits adaptation to stress responses, is the yeast *S. cerevisiae.* It has become a model organism to study how eukaryotic cells respond to stress. Considerably more is known about weak acid adaptation in *S. cerevisiae*, in comparison to other yeast species involved in spoilage, as it is amenable to genetic analysis. This study will therefore focus mainly on *S. cerevisiae*, where the acquisition of weak organic acid resistance appears to involve a stress response quite distinct from other more widely studied stress responses, such as those induced by osmotic stress or heat shock.

1.3 Mode of action of weak organic acids

Acids generally inhibit molecular reactions essential to the microorganisms by increasing the hydrogen ion concentration, which results in decreased internal pH (pH_i) (Brown, 1991). This reduction in pH_i, below the normal physiological range, results in extended lag phase and inhibition of growth (Restaino, 1982). The pH of the environment and the dissociation constant (pK_a) of the weak acid determine the proportion of the hydrophobic (undissociated) form in the medium and thus the effectiveness of the weak acid (Eklund, 1983; Vasseur et al., 1999). The strength of an acid is defined by its dissociation constant (pK_a), namely the pH value when the dissociated and undissociated forms of the acid are in equal amounts. Weak acids have a higher pK_a value than strong acids such as hydrochloric acid.

In solution weak acid preservatives exist in a dynamic, pH-dependant equilibrium between the undissociated and anionic states (Holyoak et al., 1999). An acidic pH favours the undissociated, uncharged state, in which weak acid preservatives exert much stronger antimicrobial activity. Thus, at a low pH values of between 3 and 6 (the pH range for normal food) acetic (pK_a 4.75), sorbic (pK_a 4.76) and benzoic acid (pK_a 4.19) exist mainly in their undissociated state (RCOOH), by which they can enter the cell via passive diffusion. Once inside the cell, in the higher pH environment of the cytoplasm, weak acids dissociate generating protons and anions ($RCOO^-$). The acid anions tend to accumulate intracellularly to very high levels as, being charged, they cannot easily diffuse from the cell. Acidification of the cytosol can inhibit many metabolic processes (Krebs et al., 1983).

In early studies, the antimicrobial effects of weak organic acids at low pH have often been attributed to this intracellular acidification and anion accumulation (Krebs et al., 1983; Russell, 1991; Salmond et al., 1984). Yet, weak acid preservatives do not exert their effects on growth simply as a function of their excessive accumulation in the cytoplasm, there are more explanations for the antimicrobial activity of weak acids and not all of these acids are operating identically to inhibit growth. (Eklund, 1983) investigated the antimicrobial effects of dissociated and undissociated sorbic acid and found that the inhibitory action of the latter was 100 to 600 times greater. However, in

the same study it was found that the dissociated form of sorbic acid could also cause more than 50 % growth inhibition at a pH values higher than six.

Inhibition of growth by preservatives has been also proposed to be due to membrane disruption. Comparative experiments on the effects of acetate and sorbate on *S.cerevisiae* showed that the more hydrophobic sorbate is mainly inhibiting cells through disordering of membrane structure, whereas a similar effect was not observed with acetate (Bracey et al., 1998; Stratford and Anslow, 1996). High concentrations of acetic acid (80–150 mM) are needed to totally inhibit the growth of *S. cerevisiae* at pH 4.5. In contrast, only 1–3 mM of the more liposoluble sorbate, an acid with an identical pK_a and therefore degree of dissociation to acetate, can achieve this same level of inhibition (Piper et al., 1998; Stratford and Anslow, 1996). Also, while decreases in pH_i have been observed with the addition of acetate to *S. cerevisiae* (Arneborg et al., 2000), they are not seen with sorbate addition (Bracey et al., 1998). Instead, sorbate-stressed yeast may be suffering more from the effects of disruption to membrane organization and the oxidative stress caused by associated effects on respiratory chain function (Piper, 1999). A strong action of many acid preservatives on membranes is also apparent from the strong propensity of monocarboxylic acids to become more inhibitory as they become more lipophilic (Holyoak et al., 1999; Piper et al., 2001; Piper et al., 1998; Stratford and Anslow, 1998).

Sorbate- and benzoate-stressed *S. cerevisiae* and *Z. bailii* experience a severe energy depletion (Piper et al., 1997; Warth, 1991). In *S. cerevisiae* this energy crisis is partly caused by strong inhibitory effects of sorbate and benzoate on glycolysis, an inhibition exerted mainly at the phosphofructokinase (Pfk) reaction (Krebs et al., 1983; Pearce et al., 2001). The trehalose accumulation with sorbate treatment of *S. cerevisiae* is probably in response to this Pfkp inhibition (Cheng et al., 1999). In the presence of oxygen, this energy crisis is exacerbated still further by the severe influences of the more lipophilic weak acid preservatives on membrane transport processes and energy coupling. The associated mitochondrial electron transport chain dysfunction increases free radical formation, causing sorbate- and benzoate-treated *S. cerevisiae* to suffer an excessively high endogenous production of superoxide free radicals (Piper, 1999).

Generally it is in low pH cultures that the effects of weak organic acids are most apparent. At neutral pH, residues of acetic, sorbic or benzoic acids are essentially completely dissociated. As such, they pose a much smaller threat and may even provide

a potential carbon source. However, even at neutral pH high sorbate levels still exert some inhibitory effects on *S. cerevisiae* (Stratford and Anslow, 1996) and a strong transcriptional response to sorbate is still apparent (Martinez-Pastor et al., 1996; Piper et al., 1998).

The maintenance of pH_i within a normal physiological range is essential for growth, as an acidified cytoplasm is known to inhibit metabolism (Francois et al., 1986). Change in pH_i has been reported as being important in the control of the cell cycle and the inherent proliferative capacity of cells (Anand and Prasad, 1989). Concentrations of 20-120 mM acetic acid induce a programmed cell death (PCD) response in *S. cerevisiae.* Similar to what has been recently described for *S. cerevisiae* acetic acid induces in *Z. bailii* either an apoptotic or a necrotic death process, depending on the dose. However, in *Z. bailii* the PCD process was found to occur at higher acetic acid concentrations (320–800 mM) (Ludovico et al., 2003), which is consistent with the known higher resistance of *Z. bailii* compared to that of *S. cerevisiae*. Maybe the lower plasma membrane permeability to the acid of *Z. bailii* as compared to that of *S. cerevisiae*, and the ability of *Z. bailii* to metabolise acetic acid under aerobic conditions (Sousa et al., 1998) are physiological traits that underlie the higher acetic acid concentration required to trigger apoptosis in *Z. bailii*.

The observation that acetic acid-induced PCD can occur not only in *S. cerevisiae* but also in *Z. bailii* further reinforces the concept of a physiological role of the PCD in the normal yeast life cycle and raises the possibility of this mode of cell death being more generalised in yeast than previously considered (Ludovico et al., 2003; Ludovico, 2001). Moreover, advances in the understanding of the mechanisms of death induced by acid stress in yeast will allow the development of new categories of preservatives for food and beverages with the consequent reduction of economic losses in the food industry. Uncoupling of active transport systems has also been suggested as a possible mechanism by which yeasts are inhibited under anaerobic conditions.

1.4 Resistance Mechanisms

Inhibition of growth by preservatives has been proposed to be due to a number of actions, including membrane disruption (Bracey et al., 1998), inhibition of essential metabolic processes (Krebs et al., 1983), stress on intracellular pH homeostasis (Bracey et al., 1998; Cherrington et al., 1991; Salmond et al., 1984) and finally the accumulation of toxic anions (Eklund, 1985).

Yeasts strive to maintain their pHi around neutrality (Serrano, 1991), which is essential for optimal activity of many important cellular processes. Warth first discovered in 1977 that resistance of *Z. bailii* to weak acids was due to the induction of an energy requiring system for transport of the preservative anion out of the cell.

1.4.1 The plasma membrane H^+-ATPase

The plasma membrane H^+-ATPase of *S. cerevisiae*, encoded by *PMA1*, is a proton pump that serves to create an electrochemical proton gradient necessary for cell growth and development. This proton gradient drives the uptake of nutrients by secondary active transport systems and regulates the intracellular pH (reviewed in Ambesi et al., 2000; Morsomme et al., 2000; Portillo, 2000). The H^+-ATPase is one of the most abundant proteins in the yeast plasma membrane (Serrano et al., 1986). An additional gene, *PMA2*, encodes a nearly identical protein, but this homologue is only weakly expressed and not essential for viability or growth (Viegas et al., 1994).

The H^+-ATPase belongs to a large family of highly conserved transporters known as the P-type cation pumps (Dunbar and Caplan, 2001; Lutsenko and Kaplan, 1995; Lutsenko and Kaplan, 1996). The yeast H^+-ATPase is structurally and functionally related to the P-type Na^+, K^+-, H^+ and Ca^{2+} -ATPase of mammalian cells and the H^+-ATPases of plant cells. It shares with them a characteristic reaction mechanism in which ATP is split to ADP and inorganic phosphate (P_i) via a covalent β-aspartyl phosphate intermediate (reviewed in Ambesi et al., 2000). Alignment of the amino acid sequences of all these enzymes suggested a common evolutionary origin for all P-type ATPases (Axelsen and

Palmgren, 1998; Fagan and Saier, 1994) and allowed the identification of several highly conserved motifs (Serrano, 1988; Taylor and Green, 1989).

Secondary structure prediction studies suggested a consensus structure with two large hydrophilic domains and ten transmembrane segments in all eukaryotic P-ATPases (Serrano and Portillo, 1990; Wach et al., 1992). This model is in agreement with recent data from cryoelectron microscopy of the *Neurospora crassa* H^+-ATPase (Auer et al., 1998) and sarcoplasmic reticulum Ca^{2+}-ATPase (Toyoshima et al., 2000; Zhang et al., 1998). The yeast ATPase is composed of a single, 100 kDa polypeptide, firmly embedded in the lipid bilayer by 10 hydrophobic α-helices (Figure 1.2). The membrane embedded part of the protein contains 10 membrane-spanning segments with the N and C termini located in the cytoplasm (Ambesi et al., 2000; van der Rest et al., 1995). The central region protrudes into the cytoplasm and contains strongly conserved motifs for ATP binding and phosphorylation (reviewed in Ambesi et al., 2000; Morsomme et al., 2000). The amino terminus is highly variable among P-type ATPases, both in length and amino acid sequence (Moller et al., 1996). The carboxyl-terminus constitutes the main regulatory domain involved in activation of ATPases. However, recent results suggest that the amino terminus may also play a subtle role in modulating ATPase activity (Hasper et al., 1999; Mason et al., 1998).

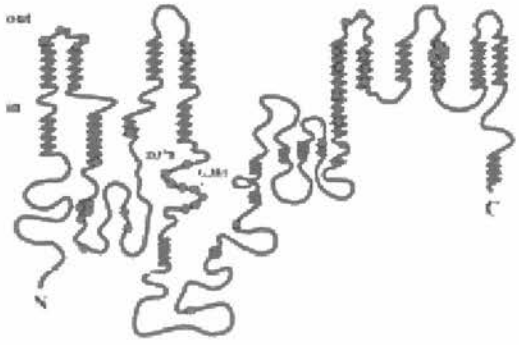

Figure 1.2 Topological model of the yeast plasma membrane H^+-ATPase, based on two- and three-dimensional crystallography of related ATPases. N and C termini are located in the cytoplasm. *Zig-zag lines* represent regions predicted to have α-helical secondary structure; *red circles* mark positions at which mutations have been found to disrupt protein folding, block biogenesis, and/or lead to a dominant lethal phenotype (Ferreira et al., 2001).

The ATPase is estimated to consume 10 to 15% of the ATP produced during cell growth and has a reaction stoichiometry of 1 H^+ proton translocated per ATP hydrolysed (Lutsenko and Kaplan, 1995; Lutsenko and Kaplan, 1996).

In recent years *PMA1* has been the focus of extensive mutagenesis studies, giving an increasingly detailed picture of the role of individual amino acid residues and motifs throughout the protein. Of the 918 amino acid residues that make up the yeast Pma1p H^+-ATPase (Serrano et al., 1986), 212 have now been mutated and analysed (reviewed in Ferreira et al., 2001; Morsomme et al., 2000), providing insights into the structural requirements for proper folding and trafficking of the H^+-ATPase (DeWitt et al., 1998; Harris et al., 1994; Nakamoto et al., 1998; Portillo, 1997).

1.4.1.1 The plasma membrane in weak acid adaptation

The activity of the *PMA1* encoded H^+-ATPase is regulated at both transcriptional and post-translational levels to match the requirements for nutrient uptake, ion homeostasis and stress tolerance (reviewed in Portillo, 2000). Two main factors are responsible for this regulation, glucose and acid pH.

Glucose increases both expression of *PMA1* encoded H^+-ATPase (Rao et al., 1993; Serrano, 1983) and catalytic activity of the enzyme, as required by the high growth rate induced by this preferred carbon source (Eraso and Portillo, 1994; Estrada et al., 1996; Portillo et al., 1991; Serrano and Portillo, 1990; Venema and Palmgren, 1995). The mechanism of glucose activation is known to rely upon inactivation of an inhibitory domain at the C-terminus of the ATPase, which interacts with the active site to inhibit enzyme activity (Portillo, 2000; Portillo et al., 1989). It is suggested that this activation is due to phosphorylation of the C-terminal region at several sites (Chang and Slayman, 1991; Portillo, 2000). In particular, Ser-899 is conspicuous, since introduction of a negatively charged amino acid residue at this position yields a Km similar to that of the activated enzyme (Eraso and Portillo, 1994; Portillo, 2000).

Recently the Ptk2p kinase was identified for activation of Pma1p via phosphorylation at its C-terminus (Goossens et al., 2000). Ptk2p belongs to a group of kinases controlling plasma membrane transporters and is required for the full activation of Pma1p by glucose. It is likely that the Ptk2p-mediated activation of the Pma1p generates an increased membrane potential that stimulates uptake of cations by various transport systems. Another kinase that has the potential of modulating cation homeostasis by

controlling Pma1p activity is the casein kinase I (CKI) (Robinson et al., 1992). Notably, CKI has been reported to down regulate Pma1p activity by phosphorylation (Estrada et al., 1996). The signals controlling the activity of CKI have not been identified.

The plasma membrane H^+-ATPase has also been shown to be an important determinant in several stress responses. Thus, the enzyme activity is increased by different environmental stresses, such as the presence of ethanol (Monteiro et al., 1994; Rosa and Sa-Correia, 1991; Rosa and Sa-Correia, 1996) supraoptimal temperatures (Coote et al., 1994; Viegas et al., 1995), heat shock (Piper et al., 1997) and deprivation of nitrogen source (Benito et al., 1992), while glucose removal and low temperature reduced its activity (Mason et al., 1998). Upon exposure to weak acids the plasma membrane H^+-ATPase that regulates pH_i homeostasis in yeast cells is activated and plays an essential role in optimal adaptation to preservatives (Holyoak et al., 1996; Piper et al., 1997; Viegas et al., 1998; Viegas and Sa-Correia, 1991). Measurement of H^+-ATPase activity of purified plasma membranes in weak-acid stressed cells revealed markedly high activation levels (Holyoak et al., 1996; Piper et al., 1998; Piper et al., 1997; Viegas and Sa-Correia, 1991). In addition, reduced expression of *PMA1* resulted in increased sensitivity of cells to weak acids (Holyoak et al., 1996).

Because the membrane H^+-ATPase has been shown to consume up to one-quarter of cellular ATP (Ambesi et al, 2000) this adaptive mechanism is energetically expensive, resulting in the depletion of intracellular ATP (Bracey et al., 1998; Holyoak et al., 1996; Piper et al., 1997). Mutants with reduced expression of key glycolytic enzymes and, thus with a reduced ability to generate ATP, were unable to adapt optimally to weak acid stress (Holyoak et al., 1996).

1.4.2 General stress response

The heat shock response is a universal phenomenon observed in all organisms. Exposure to elevated temperatures or, other types of stress including: ethanol, anoxia, weak acids or starvation leads to the synthesis of a small group of proteins known as the heat shock proteins (HSPs). HSPs are divided into two major class sizes of 70-100 kDa and 17-30 kDa (Hohmann, 2003). They predominantly function as protein molecular chaperones, involved in protein folding, stabilization, activation, trafficking, and degradation (Hahn and Thiele, 2004). In *S. cerevisiae* at least 52 different proteins are induced by a temperature shift from 25°C to 38°C (Boy-Marcotte et al., 1999).

In budding yeast, the classic heat shock response is primarily governed by the heat shock transcription factor (HSF), encoded by a single-copy essential gene, *HSF1* (Estruch, 2000; Morimoto, 1996; Wiederrecht et al., 1988). Yeast Hsf1p is an evolutionary conserved protein that contains a helix-turn-helix DNA-binding domain and coiled-coil hydrophobic repeat domain, which mediates the trimerisation of Hsf1p (Harrison et al., 1994; Nieto-Sotelo et al., 1990). *HSF* binds to arrays of a five base pair heat shock element (HSE), nGAAn, (where n is any nucleotide) in the promoters of target genes and activates transcription of these genes in response to heat shock (Figure 1.3). The overall fundamental structure of HSF and its HSE consensus-binding site are conserved from yeast to humans (Hahn and Thiele, 2004). Regulation of the heat shock response, especially through Hsf1p remains an enigmatic area. How does Hsf1p sense heat so rapidly-is the mechanism entirely based on protein chaperone interactions with an unfolding proteome or do the multiple phosphorylation events play a role in activation? Are signals generated by physical perturbation of membranes? The heat shock response program in yeast is clearly a multifaceted one, involving multiple transcription factors governing the expression if a large number of gene products.

Another regulatory system involved in gene induction in response to heat stress is the transcription factors Msn2p and Msn4p (Msn2/4p), which are known as the 'General' Stress Response because they are activated by very different forms of stress to induce the transcription of a large battery of genes (Estruch, 2000). Both Msn2p and Msn4p bind to a nearly invariant five base pair sequence element (CCCCT) called the stress

response element or STRE (Estruch, 2000; Ruis and Schuller, 1995; Sorger, 1991) (Figure 1.3). This element was first identified as an Hsf1p-independent mediator of heat shock induction and has since been found in 200 genes (Kobayashi and McEntee, 1993; Moskvina et al., 1998; Treger et al., 1998). STREs are activated by multiple stress factors like nitrogen starvation, osmotic, oxidative stress, low external pH, weak organic acids and ethanol stress (reviewed in Ruis and Schuller, 1995).

Most HSP genes characterised to date are regulated solely by Hsf1p and contain one or more sets of HSE repeats in their promoters. Importantly a number of HSP genes, including *HSP12*, *HSP26* and *HSP104*, also contain STREs in their promoter regions (Amoros and Estruch, 2001; Varela et al., 1995).

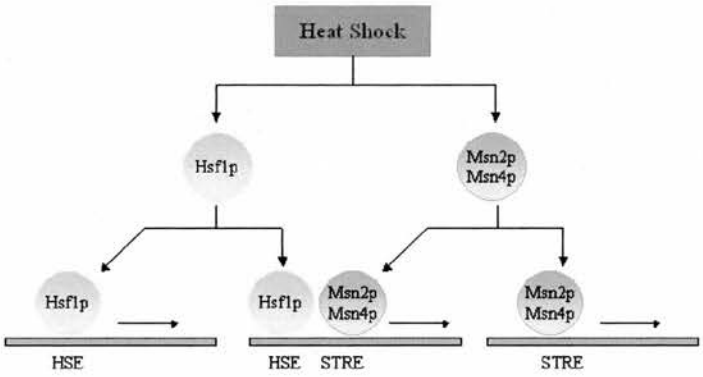

Figure 1.3 Schematic of stress sensing and gene control by Hsf1p and Msn2p/Msn4p. The transcription factors Hsf1p and Msn2/4p respond to heat shock stimuli by activating gene expression through their DNA binding sites. Most characterised heat shock genes contain either heat shock element (HSE), or stress response element (STRE) or both in their promoter regions and are induced upon heat shock (adapted from (Hohmann, 2003).

1.4.2.1 The Heat Shock Protein Hsp30 is induced by sorbic acid stress

S. cerevisiae has a single integral plasma membrane heat shock protein, encoded by *HSP30*. Hsp30p is a highly hydrophobic protein induced by several stresses, including heat shock, ethanol exposure, severe osmostress, weak organic acid exposure and glucose limitation (Panaretou and Piper, 1992; Piper et al., 1997; Piper et al., 1994; Regnacq and Boucherie, 1993; Schuller et al., 1994; Seymour and Piper, 1999). A previous study showed that Hsp30p functions to regulate, either directly or indirectly, the activity of the plasma membrane H^+-ATPase (Braley and Piper, 1997). *Δhsp30* mutants show increased H^+-ATPase activity induced by heat shock or by weak organic acid stress (Piper et al., 1997).

The plasma membrane H^+-ATPase is known to consume a substantial fraction of the ATP generated by the cell, a usage that will be increased by the H^+-ATPase stimulation occurring with several Hsp30p-inducing stresses. Hsp30p might therefore provide an energy conservation role, limiting excessive ATP consumption by plasma membrane H^+-ATPase during prolonged stress exposure (Piper et al., 1997). Consistent with this role of Hsp30p, cultures of *Δhsp30* mutants exhibit lower final biomass yields. They also have lower ATP levels, due to the excessive H^+-ATPase activity, at the glucose exhaustion stage of batch fermentations (diauxic lag), when Hsp30p is normally induced. However, loss of Hsp30p does not affect several stress tolerances but it extends the time needed for cells to adapt to growth under several stressful conditions where the maintenance of homeostasis will demand an unusually high usage of energy. *HSP30* is the first yeast gene identified as both weak organic acid-inducible and assisting the adaptation to growth in the presence of these acids (Piper et al., 1997). Because Hsp30p is strongly induced by treatments that increase the membrane fluidity (elevated temperatures, ethanol, and weak organic acids) it is possible that *HSP30* is responding to a membrane-fluidity-sensing system that stimulates a signal transduction pathway acting on *HSP30* and other genes (Seymour and Piper, 1999).

In a recent study *HSP30* expression was found not to be under the control of either of the two, so far identified stress regulatory elements, HSE and STRE. This suggested that a third system of activation of genes by heat shock must exist in yeast (Seymour and Piper, 1999). Stress activation of *HSP30* appears therefore to involve an as yet

unknown mechanism. In a more recent study, using a genome-wide microarray analysis 21 genes, including *HSP30* were sorbate-induced during short-term stress (Schuller et al., 2004). These findings provide evidence that HSP30p maybe part of a larger, as yet unknown regulon, independent from the Msn2/4p and Hsf1p (Schuller et al., 2004). Interestingly, several genes of this regulon are involved in processes on the level of the plasma membrane. Hsp30p is the only membrane-bound heat shock protein therefore, it could be involved in sensing membrane damage as exerted by weak organic acid exposure. Four genes coding for polyamine transporters (*TPO1,2,3,4*) were also activated (Albertsen et al., 2003). Furthermore an *HSP30* homologue *YRO2* (Wu et al., 2000) as well as *CIT2*, encoding a nonmitochondrial citrate synthase that participates in the glyoxylate cycle (Kim et al., 1986), were also controlled by this unknown regulon.

In another study on sorbic acid adapted response, more heat shock proteins were identified. In this study two major technologies were used; microarrays and proteomics. A number of heat shock proteins were upregulated in the presence of sorbic acid (de Nobel et al., 2001). Hsp26p is a temperature regulated molecular chaperone, forming large oligomeric complexes, which dissociate at heat shock temperatures. *Δhsp26* mutants showed marked sensitivity to sorbic acid whereas, *HSP26* showed significant increase in transcript level upon exposure to sorbic acid (de Nobel et al., 2001). The role of Hsp26p in resistance to sorbic acid could be to prepare denatured protein for degradation or refolding (de Nobel et al., 2001). In the same study Hsp70p isoforms, Ssa1/2p, Ssb1/2p and Hsp42p and Hsp12p showed increased expression upon exposure to sorbic acid (de Nobel et al., 2001).

1.4.3 Yeast Multidrug resistance: the PDR Network

In analogy to multidrug resistance (MDR) phenomena observed in all cells from bacteria and archaea to mammalian (Miyauchi et al., 1992; Nikaido, 1994), the budding yeast *S. cerevisiae* displays a similar phenotype known as pleiotropic drug resistance (PDR) (Mamnun et al., 2002). The most important resistance mechanisms ubiquitous from bacteria to man, which lead to multidrug resistance is the overexpression of membrane associated transporters that extrude drugs from the cell (Kolaczkowska and

Goffeau, 1999). The ATP binding cassette (ABC) superfamily and major facilitators superfamily (MFS) drug transporters are the two most prominent contributors to MDR in yeasts (Prasad et al., 2002; Yamamoto et al., 2005). The first family couples hydrolysis of ATP to the transport of compounds across the membrane (Decottignies and Goffeau, 1997) and the second couple the energy contained in the transmembrane proton gradient to transport (Prasad et al., 2002).

The *S. cerevisiae* genome harbours 31 genes encoding ATP-binding cassette (ABC) proteins (Decottignies and Goffeau, 1997). Expression of several yeast ABC transporters mediates the pleiotropic drug resistance (PDR) phenotype (Wolfger et al., 2001). Notably, each cellular compartment, except for the endoplasmic reticulum (ER) and nuclear membrane, appears to have at least one ABC transporter (Figure 1.4). Some membranes like the plasma membrane carry several different ABC transporters. Based on phylogenetic tree analysis, ABC proteins have been classified into six distinct subfamilies ((Taglicht and Michaelis, 1998) and (reviewed in Bauer et al., 1999)). These families are defined as the MDR (mitochondrial function), the PDR, the MRP/CFTR, the ALDp, the YEF3 and the RLI subfamilies (reviewed in Bauer et al., 1999).

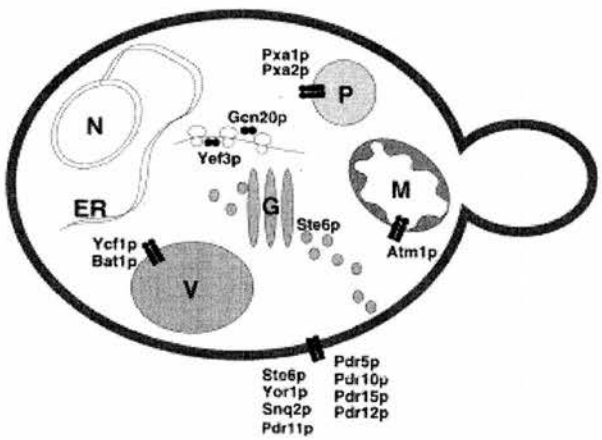

Figure 1.4 Subcellular localization of *S. cerevisiae* ABC transporters. ER, endoplasmic reticulum; G, Golgi apparatus; M, mitochondrion; N, nucleus; P, peroxisomes; and V, vacuole. Ste6p is localized in Golgi vesicles, endosomes as well as in the plasma membrane (Bauer et al., 1999).

The PDR subfamily is the largest in yeast comprising 10 members. Amongst them Pdr5p, a highly abundant and well studied plasma membrane pump with broad substrate specificity (Egner et al., 1998; Kolaczkowski et al., 1998; Mahe et al., 1996; Mamnun et al., 2004), Snq2p (Servos et al., 1993) and Yor1p (Katzmann et al., 1994) cause enhanced efflux of hundreds of xenobiotics (Kolaczkowski et al., 1998). The Pdr5p

drug efflux pump has two additional homologues, Pdr10p and Pdr15p (Bissinger and Kuchler, 1994; Parle-McDermott et al., 1996) the latter being involved in cellular detoxification (Wolfger et al., 2004). Pdr12p is involved in resistance to weak acid preservatives (Piper et al., 1998).

Transcriptional regulation within the PDR network was previously shown to require the PDRE (PDR responsive element) consensus motif, which is present in varying numbers in the promoters of PDR responsive genes (Delahodde et al., 1995; Katzmann et al., 1994). Transcription of *PDR5*, *SNQ2*, *PDR10*, *PDR15*, *YOR1* is controlled by the zinc finger regulators ($Zn(II)_2Cys_6$), Pdr1p and Pdr3p (Balzi et al., 1987; Delaveau et al., 1994) and Yrr1p (Cui et al., 1998)that can bind to the PDREs (Mamnun et al., 2002). However, *PDR12* induction is not influenced by the Pdr1p/Pdr3p master regulators of the PDR Network and is independent of the general stress response regulators Msn2p/Msn4p.

1.4.4 The Pdr12p transporter is required for weak acid resistance

Exposure of *S. cerevisiae* to sorbic acid strongly induces two plasma membrane proteins. One is the aforementioned Hsp30p, a heat shock protein that is also induced by heat and ethanol stress (Piper et al., 1997), the other is a 171 kDa membrane ABC transporter, Pdr12 (Piper et al., 1998). So strong is this Pdr12p induction in sorbate-stressed cells, that levels of this transporter in the plasma membrane approach those of the most abundant plasma membrane protein, the plasma membrane H^+-ATPase, whereas in unstressed cells its present at very low levels (Piper et al., 2001; Piper et al., 1998).

Pdr12p appears to be acting as an efflux pump for weak organic carboxylate anions and is required for the development of weak organic acid resistance by mediating the energy-dependent extrusion of water-soluble carboxylate anions from the cell (Figure 1.5) (Piper et al., 1998). *In vivo* studies have shown that it lowers the intracellular levels of benzoate and fluorescein diacetate by catalysing an active efflux of these compounds from the cell. Weak acid resistance is severely compromised in Δ*pdr12*

mutants that cannot catalyse this efflux (Holyoak et al., 1999; Piper et al., 1998). Located in the plasma membrane, Pdr12p appears to confer resistance to those carboxylic acids that partition into both the lipid bilayer and aqueous phases (Hatzixanthis et al., 2003), especially water-soluble, monocarboxylic acids with chain lengths of from C_{1-7} but not to more lipophilic C_{8-10} acids (Holyoak et al., 1999). Maximal Pdr12p induction was seen with the C_{5-7} acids in both pH 4.5 and pH 6.8 cultures (Hatzixanthis et al., 2003).

Strains lacking the Pdr12p transporter are hypersensitive to water-soluble monocarboxylic acids of relatively short aliphatic carbon chain length. Thus, *PDR12* is strongly induced by sorbate, benzoate and certain other moderately lipophilic carboxylate compounds (Hatzixanthis et al., 2003; Holyoak et al., 1999; Piper et al., 2001). Δ*pdr12* mutants are also sensitive to short-chain alkanols (n-propanol, n-butanol and n-pentanol), without significant resistance this stress factor. However, Δ*pdr12* cells are neither sensitive to dicarboxylic acids, nor to the highly lipophilic, long-chain fatty acids and alcohols. Their toxic effects are attributed mainly to detergent disruption effects on membranes (Holyoak et al., 1999; Weber and de Bont, 1996). Therefore, Pdr12p imparts resistance to those organic acids or alcohols that are reasonably soluble in both lipid and aqueous phases (Holyoak et al., 1999; Holyoak et al., 2000).
In a recent study it was found that Pdr12p does not confer resistance to acetate (Bauer et al., 2003) and in contrary to what was believed in the past (Piper et al., 1998), Pdr12p and another membrane transporter Azr1p show hypersensitivity to acetate when the cells have auxotrophic requirements for aromatic amino acids (Bauer et al., 2003). Loss of Pdr12p does not render cells hypersensitive to the known substrates for other well-characterized *S. cerevisiae* ABC transporters, drug pumps such as Pdr5p or Snq2p (Bauer et al., 1999).

How does Pdr12p confer sorbic acid resistance in adapted cells? According to the model presented (Figure 1.5) in both unadapted (A) and adapted (B) cells the uncharged form of the weak acid (XCOOH) enters the cells freely by diffusion (reviewed in Piper et al., 2001). There, in the higher pH environment of the cytoplasm the XCOOH dissociates giving anions ($XCOO^-$) and protons (H^+), which being charged accumulate inside the cell, the latter resulting in a cytoplasmic acidification. In unadapted cells this weak acid-induced cytoplasmic acidification can be counteracted by increased Pma1p catalysed

proton extrusion. Thus, weak acid influx in (A) will act to dissipate the ΔpH, though not the charge (Z) component of this gradient.

In weak acid-adapted cells (Figure 1.5B) Pdr12p-catalysed anion extrusion will reduce both intracellular organic acid levels and facilitate Pma1p-catalysed proton extrusion by balancing the charge movement. Combined effort of the H^+-ATPase pump and Pdr12p are crucial for restoring homeostasis to the point where substantial metabolic activity and cell growth can resume. This mechanism is extremely expensive since for every weak acid molecule that enters the cell two molecules of ATP are consumed. This high energy requirement is reflected in the dramatic reductions in biomass yield of cultures grown in the presence of weak acids (Piper et al., 1997; Stratford and Anslow, 1996; Verduyn et al., 1992; Viegas and Sa-Correia, 1991).

However, induction of Pdr12-catalysed acid anion extrusion alone would be pointless without simultaneous limitation to the diffusional uptake of the undissociated acid (Piper et al., 1998). Without this limitation, acid could potentially diffuse in the cytosol at the same rate as Pdr12p pumps it out in a futile cycle that, would not only be highly energy demanding but would also cause substantial influx of protons (Figure 1.5). How this weak acid diffusion is restricted in adapted cells is at present unknown. One explanation could be via the cell wall or alteration of the cell membrane. It has been reported that cell wall mannoproteins can reduce the porosity of the yeast cell wall (de Nobel and Barnett, 1991). Interestingly, different yeast species exhibit different abilities and efficiencies in adapting to weak acid stress. Previous studies have shown that benzoic acid adapted *S. cerevisiae* and *Z. bailii* cells can maintain an intracellular versus an extracellular distribution of benzoic acid that is not in equilibrium. This suggests that adapted cells restrict diffusional entry of weak acids across the membrane (Henriques et al., 1997; Piper et al., 1998).

Interestingly, an inverse correlation between benzoic uptake rates in different yeast species and their resistances has been observed (Warth, 1989). Thus, although published evidence indicates that Pdr12-mediated anion efflux is essential for weak acid adaptation (Piper et al., 1998), it is likely that Pdr12 is not the only component of this adaptation mechanism.

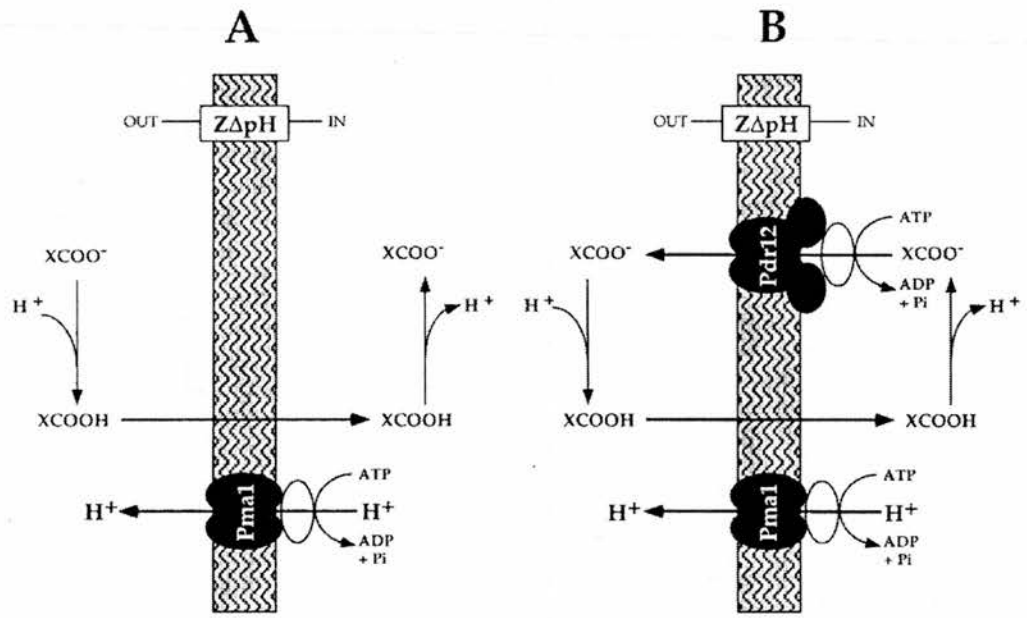

Figure 1.5 Schematic model of the effects of weak acids on unadapted and adapted yeast cells. In both unadapted (A) and adapted (B) cells the uncharged form of the weak acid (XCOOH) enters the cell by diffusion. The electrochemical potential difference across the plasma membrane (ZΔpH) is controlled by proton extrusion by Pma1p. The main mechanism of weak acid adaptation in yeast is regulated via the Pdr12p transporter (Piper et al., 1998).

1.4.5 The *PDR12* transporter is regulated by *WAR1*

Although the *PDR12* gene is induced very strongly by weak organic acid stress, it is relatively unresponsive to a wide range of other stresses (heat shock, ethanol, osmostress, oxidative stress) (Piper et al., 1998), suggesting the existence of a specific weak acid stress sensing system. Consistent with this, the *PDR12* sorbate induction is independent of Pdr1p/Pdr3p, Yap1p, Msn2p/Msn4p transcription factors that direct the induction of several stress genes as well as a number of the other yeast ABC transporters (Bauer et al., 1999; Piper et al., 1998).

Concurrent with this study, (Kren et al., 2003) discovered that the *PDR12* promoter contains a cis-acting weak acid response element (WARE), which is required for its induction. WARE is the major target of a novel nuclear Zn_2Cys_6 zing finger transcription factor, War1p, as the only mediator of Pdr12p stress induction (Kren et al., 2003) (Figure 1.6). War1p occupies the site in both presence and absence of the stress, demonstrating constitutive DNA binding, and suggesting that weak acid stress, such as that produced by sorbate or benzoate, triggers phosphorylation and possible activation of War1p (Kren et al., 2003). War1p is rapidly and extensively phosphorylated in response to weak acid stress (Kren et al., 2003). Its' phosphorylation strictly requires stress conditions and is quickly lost after stress relief. Taken together, these data suggest that War1p phosphorylation must be tightly linked to the activation of War1p (Kren et al., 2003).

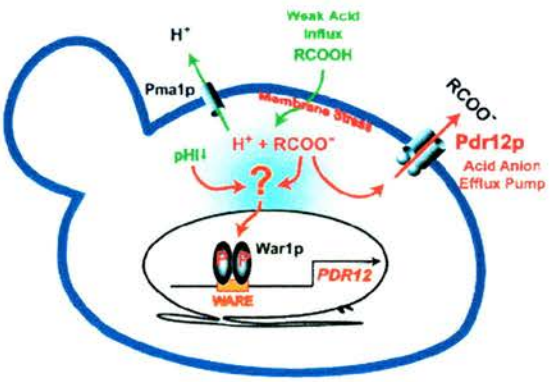

Figure 1.6 Schematic model of War1p-mediated weak acid stress adaptation. Uncharged weak acids first enter the cell and dissociate in the cytoplasm. The resulting protons are extruded from the cell by Pma1p and the anions effluxed by induced Pdr12p in cells adapting for sorbate. The acid anions can directly or indirectly activate War1p, which is constitutively bound to the PDR12 promoter. War1p is phosphorylated upon sorbic acid stress and Pdr12p is induced to high levels (Kren et al., 2003).

In turn, War1p activation allows the induction of *PDR12* through a novel signal transduction event that elicits weak organic acid stress adaptation through the active efflux of weak organic acids from the cytosol (Kren et al., 2003). Loss of War1p abolishes *PDR12* induction and results in hypersensitivity to various weak organic acids (Kren et al., 2003).

Kren and co-workers showed that weak acids elicit a novel stress response via War1p. However, the actual signal transduction mechanism that triggers War1p activation is not clear and the upstream components of the signalling cascade mediating weak acid stress

tolerance remain elusive (Figure 1.6). Since War1p activation is highly specific Kren and co-workers proposed that the upstream sensor, whether intracellular or membrane bound, could also be highly specific. Since War1p is constitutively bound to the WARE element, stress induced phosphorylation could signal War1p activation. Otherwise War1p activity could be modulated directly by organic monocarboxylate anions (Figure 1.6). Their binding to War1p could induce conformational changes or modify interaction with other proteins, including the kinase that phosphorylates War1p (Kren et al., 2003). Notably, in a more recent study from global transcriptome analysis, *PDR12* was found to be the only major target gene of War1p, mediating ATP-dependent efflux of weak acids (Schuller et al., 2004).

1.5 Global approaches to elucidate weak acid response

The budding yeast *Saccharomyces cerevisiae* has been studied thoroughly by systematic genomic and proteomic technologies. Its ~6000 predicted ORFs have been analyzed for expression under a multitude of conditions (DeRisi et al., 1997; Horak and Snyder, 2002). Approximately 5000 ORFs have been individually deleted and the resulting strains phenotypically characterized (Giaever et al., 2002; Winzeler et al., 1999) whereas protein interactions have been detected by both biochemical methods, mass spectrometry (Gavin et al., 2002; Ho et al., 2002) and two-hybrid approaches (Ito et al., 2001; Uetz et al., 2000). Furthermore many of the proteins have been localized by indirect immunofluorescence or by fusion to green fluorescent protein (GFP) (Huh et al., 2003; Kumar et al., 2002; Ross-Macdonald et al., 1999).

Currently genome wide approaches offer insights into novel interactions between proteins involved in the same biological function. (Schuller et al., 2004) have recently combined a genome wide phenotypic screening using a commercial deletion strain collection with global transcriptome microarray analysis to identify and characterize important components for weak acid stress defence. The functional genetic analysis revealed a multitude of different cellular activities involved in weak acid stress response (Schuller et al., 2004). Comparison with a concurrent study showed that the stringency

of the selection (*ie* amount of sorbate used) affected the number of candidate genes implicated in stress response (Mollapour et al., 2004). Microarray studies of cells treated with 8 mM sorbate for 20 min in the early exponential phase have shown that except for a small group of six genes including *PDR12* (*TFB2*, *FUN34*, *ECM39*, *YOL032w*, *YGR048w*) that their induction was War1p-dependent, the induction of most of the genes was highly dependent upon Msn2p/Msn4p (Schuller et al., 2004). Finally a third regulon independent of War1p and Msn2p/Msn4p was found, namely the plasma membrane heat shock protein Hsp30p (Schuller et al., 2004). Interestingly several genes of this regulon were involved in processes on the plasma membrane, suggesting that Hsp30p could be sensing membrane damage as exerted by weak acid stress. However, the pattern of sorbate induced genes during short-term stress was distinct from the one reported in an earlier study on sorbate adapted cells (de Nobel et al., 2001). The above independent studies have provided sufficient evidence of existence of multiple mechanisms that contribute to at least three distinct sorbate-inducible regulons (War1p, Msn2p/Msn4p and Hsp30p).

1.6 Aim of this study

The purpose of this research study is to gain further fundamental understanding of how spoilage yeasts adapt to preservative stress at the molecular level and identify other, novel components that contribute to detecting preservative stress. This knowledge may offer potential new routes to combat spoilage or better understanding of how it can be prevented

CHAPTER 2

2. Materials & Methods

2.1 Chemicals and Suppliers

General laboratory chemicals were of analytical grade and purchased from Sigma-Aldrich UK Ltd., Fisher Scientific UK Ltd. and Melford Laboratories UK Ltd. All polymerase chain reaction (PCR) DNA oligonucleotide primers were synthesized by MWG Biotech AG. Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), *Taq* polymerase and DNA ladder (1 kb) were purchased either from Promega, UK or Helena Biosciences, UK. *Pfu* polymerase and TEV enzyme were made by Dr. Cubeddu and Mark Dorward respectively. Plasmid DNA extraction kit (QIAprep® Spin Miniprep), PCR purification kit (QIAquick™) and gel extraction kit (QIAquick™) were from Qiagen Ltd. Ribonuclease A (RNAse A), lyticase and sheared herring testes carrier DNA were from Sigma-Aldrich UK Ltd. Complete protease inhibitor cocktail was purchased from Roche Diagnostics UK Ltd or Sigma-Aldrich UK Ltd. γ-[^{32}P] ATP and thrombin were purchased from Amersham Pharmacia Biotech, Buckinghamshire, UK.

Prestained SDS-PAGE standards broad range, precast 12.5 % Tris-HCl polyacrylamide gels, 10X TGS (Tris/Glycine/SDS) electrophoresis buffer were from Bio-Rad, UK. MagicMark™ western protein standards were purchased from Invitrogen, UK. Immobiline™ DryStrip strips, IPG buffer, DeStreak™ rehydration solution and Hybond™-ECL™ Nitrocellulose membrane were purchased from Amersham Pharmacia Biotech, Buckinghamshire, UK. SuperSignal® West Pico or Femto Chemiluminescent Substrate for detection of HRP, were from Pierce Biotechnology, Inc. USA.

All disposable plasticware was obtained from Greiner Bio-One Ltd. PCR tubes were from Abgene, UK and concentrators were from Vivascience AG. Purified agar was purchased from Helena Biosciences, UK. All Bacto branded media components were obtained from Difco Laboratories (BD Biosciences, UK). IPTG was purchased from Melford

Laboratories, UK. Silver stain kits and SYPRO® Ruby stain were obtained from Genomic Solutions, MI, USA. Pro-Q Diamond Phosphoprotein gel stain was purchased from Molecular Probes, Ore., USA).

2.2 Strains and Growth media

All growth media, additions and stock solutions were sterilized by autoclaving at 121 °C for 20 min at 15 PSI. Where autoclaving was not possible, 0.22 μm Millex GP disposable filters (Milipore, Co, Ireland) were used. Media, buffers and solutions were prepared using deionised water at 18.2 MΩ (Milli-Q® water) unless otherwise stated.

2.2.1 Yeast strains and Growth media

The main *S. cerevisiae* strain used in this study was BY4741*a* (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Research Genetics, Huntsville, USA). All gene deletions in this study were from Research Genetics BY4741 *MAT*a haploid genome deletion set, which contains 4,847 yeast strains with all nonessential Open Reading Frames (ORFs) disrupted by the *KanMX* cassette (http://www-sequence.stanford.edu/). All yeast strains used in this study are listed in Table 2.1.

Cells were grown either in complete YEPD, pH 5.8 liquid medium (1 % yeast extract, 1 % bacto-peptone, 2 % glucose) or in Synthetic Complete liquid medium (SC) (0.67 % YNB without amino acids, 2 % D-glucose) (ForMedium, UK) supplemented with appropriate amino acids and bases to a final concentration of 20 μg/ml. For solid media, 2% micro-agar was added prior to autoclaving. When weak acids (sorbic, benzoic and acetic acid) were added to media, the pH was adjusted to 4.5. Other stresses used in this study (hydrogen peroxide, ethanol, salt) were added at the appropriate concentrations. Growth was measured by change in optical density (OD_{600}).

Table 2.1. Yeast strains used in this study

Strains	Genotype*	Source
BY4741a	*MAT***a** ***his3Δ1 leu 2 Δ0 met 15 Δ0 ura 3 Δ0***	Research Genetics
BY-Deletions	*GEN::kanMX4* deletions of 4,847 gene	Research Genetics
TAP-Vma2	*MAT***a** ***his3Δ1 leu 2 Δ0 met 15 Δ0 ura 3 Δ0 VMA2-TAP***	Open Biosystems
TAP-Dbf2	*MAT***a** ***his3Δ1 leu 2 Δ0 met 15 Δ0 ura 3 Δ0 DBF2-TAP***	Open Biosystems
VMY100	*MAT***a** BY4741a *DBF2::kanMX4 his*::pRS313-*DBF2*	This study
VMY101	*MAT***a** BY4741a *VMA2::kanMX4 his*::pRS313-*VMA2*	This study
VMY102	*MAT***a** BY4741a *VPS15::kanMX4 his*::pRS313-*VPS15*	This study
VMY103	*MAT***a** BY4741a *CTK1::kanMX4 his*::pRS313-*CTK1*	This study
VMY104	*MAT***a** BY4741a *BUB1::kanMX4 his*::pRS313-*BUB1*	This study
VMY105	*MAT***a** BY4741a *ROX3::kanMX4 his*::pRS313-*ROX3*	This study
VMY106	*MAT***a** BY4741a *REG1::kanMX4 his*::pRS313-*REG1*	This study
VMY107	*MAT***a** BY4741a *DBF2::kanMX4 his*::pRS423-*DBF2*	This study
VMY108	*MAT***a** BY4741a *VMA2::kanMX4 his*::pRS423-*VMA2*	This study
VMY109	*MAT***a** ***his3Δ1 leu 2 Δ0 met 15 Δ0 ura 3 Δ0 DBF2-Myc13- kanMX6***	This study
VMY110	*MAT***a** BY4741a *VMA2::kanMX4 his*::pRS423-*DBF2*	This study
VMY111	*MAT***a** BY4741a *DBF2::kanMX4 his*::pRS423-*VMA2*	This study

* All strains are in BY4741a background

2.2.2 *Escherichia coli* strains and Growth media

E. coli strains DH5α, DH5αF' IQ and Top10 (Invitrogen, UK) were used for all DNA cloning procedures and transformations. For protein expression, the *E. coli* BL21 (DE3)/PlYS strain (Novagen, UK) was used for production of $(His)_6$-tagged proteins. All *E. coli* strains used in this study are listed in Table 2.2.

Table 2.2. *E. coli* strains used in this study

Name	Genotype	Source
DH5α	F- φ80*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (rk-, mk+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *pho*A	Invitrogen
DH5αF'IQ	F′ proAB+ *lac*IqZΔM15 zzf::Tn5 (KmR) φ80*lacZ*ΔM15 Δ(*lacZ*YA-argF) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 λ-thi-1 *gyr*96 *rel*A1	Invitrogen
Top10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(ara-leu)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
Rosetta	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm lacY1* pRARE2[2] (Cm^R) pAR5615 (Ap^R)	Novagen

2.3 Growth conditions

2.3.1 Yeast growth conditions

Single colonies of yeast strains were picked from plates with sterile toothpicks and inoculated into 5 ml of YEPD or SC containing the appropriate supplements. Overnight incubation at 30 °C in an orbital incubator at 200 rpm provided starter cultures, which were used for inoculation. Cells were then incubated according to individual protocols.

For short-term storage, cells were streaked to single colonies on to either YEPD or SC agar, containing the appropriate supplements, and stored at 4 °C for up to two months. For long-term storage, sterile glycerol was added to a liquid culture to yield a final concentration of 15 % (v/v), vortexed and stored at –80 °C.

2.3.2 *E. coli* growth conditions

All bacterial cells were grown using standard procedures (Sambrook, 1989). Briefly, strains were grown in Luria-Bertiani (LB) Media (1% Bacto-peptone, 0.5% Bacto-yeast extract, 1% NaCl) for liquid media. For solid media, 2% Bacto-agar was added prior to autoclaving. For plasmid maintenance and selection, the appropriate filter-sterilized antibiotics were added to medium after autoclaving. Single *E. coli* colonies were picked from a fresh LB-ampicillin or LB-Kanamycin (LB-Amp, LB-Kan) plate, inoculated into 10 ml of medium, and grown to stationary phase overnight at 37 °C in an orbital incubator at 200 rpm.

For short-term storage, strains were streaked onto LB plates, supplemented with appropriate antibiotics, to obtain single colonies. For long-term storage, sterile glycerol was added to a final concentration of 15 % (v/v) to an overnight culture, vortexed and stored at –80 °C.

2.4 Yeast Growth assays

2.4.1 Screening the 'disruptome'

A growth test was developed to screen the entire *S. cerevisiae* genome gene deletion set (Research Genetics, Huntsville, USA) for sensitivity or resistance to sorbic acid. First, using a 48-pin replica plater, individual yeast mutant strains were inoculated from the original strain collection stored in 96-well plates into sterile 96-well plates containing fresh YEPD medium. After overnight incubation at 30 °C, growth was monitored by optical density, and approximately 5 µl of the individual cultures was spotted, at an optical density at 600 nm (OD_{600}) of 1.0, using 48-pin replica plater, onto YEPD agar medium containing either 0 or 1.75 mM sorbic acid plates, pH 4.5. Colony growth was inspected after 48 h of incubation at 30 °C and compared with the reference plate containing the wild type strain BY4741 and the *Δpdr12* positive control.

More-detailed sensitivity assays were performed by spotting 10-fold serial dilutions of an exponentially growing culture at a starting OD_{600} of 0.1 onto YEPD, pH 4.5 agar with increasing concentrations of sorbic acid (0 to 2.5 mM). Colony growth was inspected as described above. The yeast mutant strains tested with this assay were all protein kinases, phosphatases and transcription factors, classified according to the MIPS protein Classes index (http://mips.gsf.de/genre/proj.yeast.index.jsp). The aforementioned sensitivity assay was also used for all the different stresses used in this study.

2.4.2 Growth assays of *S. cerevisiae* strains

Yeast strains were inoculated at OD_{600} of 0.005 in 1 ml YEPD, pH 4.5 liquid cultures and grown aerobically by orbital shaking 200 rpm, at 30 °C in a Universal Microplate Spectrophotometer PowerWave XS (Bio-Tek Instruments, INC, USA) for 48 hrs. Cells were treated with different concentrations of various stresses (sorbic, benzoic, acetic acids, ethanol, hydrogen peroxide and heat). Controls consisted of the wild type BY4741a strain.

All treatments were performed in triplicate. Growth was monitored by optical absorbance at 600 nm every 15 min.

2.5 Cloning

All yeast growth media for genetic manipulations are described in section 2.1. General molecular biology methods were performed according to standard protocols (Sambrook, 1989). Yeast strains were transformed by the lithium acetate method as previously described (Gietz et al., 1992). The sequence of the constructs was verified by DNA sequence analysis.

2.5.1 *S. cerevisiae* gene cloning for complementation studies

For complementation studies the *S. cerevisiae* genes: *DBF2, VPS15, BUB1, CTK1, REG1, ROX3* were cloned under the control of their own promoters first into pGEM-T^{easy} (Promega) and afterwards into the single-copy pRS313 vector (Sikorski and Hieter, 1989) according to standard methods (Sambrook, 1989). The aforementioned genes were amplified from *S. cerevisiae* BY4741a strain genomic DNA with Taq Polymerase (Promega, UK). All oligonucleotides used in this study are listed in Table 2.3. These oligonucleotides introduced restriction sites at both sites and digestion with appropriate enzymes (Promega, UK) allowed cloning into the pRS313 vector. All vector maps are shown in Appendix 1. Gene sequence analysis was performed by the fluorescent dideoxynucleotide method, using ABI PRISM^{TM} 377 DNA Sequencer (Perkin-Elmer Life Sciences Ltd., UK) at the DNA Sequencing Unit of the University of St. Andrews or at the Sequencing Service of Dundee University (http://www.dnaseq.co.uk).

2.5.2 Cloning of *S. cerevisiae VMA2* and *DBF2* for overexpression studies

Both *VMA2* and *DBF2* were cloned under the control of their own promoters into the 2μ-based multicopy vector pRS423 (Sikorski and Hieter, 1989) according to standard methods (Sambrook, 1989). Restriction sites and oligonucleotides used are listed in Table 2.3. The pRS423 vector map is shown in Appendix I.

2.5.3 Cloning *DBF2* by homologous recombination in *S. cerevisiae*

One-step PCR cloning by homologous recombination was used for tagging of chromosomal *DBF2*, as described elsewhere (Longtine et al., 1998). For C-terminal tagging of full-length Dbf2p protein, pFA6a-13Myc-His3MX6 plasmid containing a 13Myc tag was used as template for the PCR along with appropriate target-gene-specific primer pairs (Table 2.3). The gene-specific sequence of the forward primer was chosen to end just upstream of the stop codon of *DBF2*, preserving the reading frame of the tag, whereas those of the reverse primer were chosen to end just downstream of the stop codon (Figure 2.1). *DBF2* was amplified with Taq Polymerase (Promega) [program used: 1 cycle at 94°C for 2 min, 30 cycles at 94°C, 30 sec, 55°C, 45 sec and 72 for 2.5 min and 1 cycle at 72°C for 7 min]. The products from PCR reactions were purified using QIAgen PCR Clean up Kit (Qiagen, UK) according to manufacturer's instructions. The resulting concentrated DNA was transformed into *S. cerevisiae* according to standard protocols (Gietz et al., 1992). Transformants were selected on SC-his agar plates and confirmed by PCR and immunoblotting

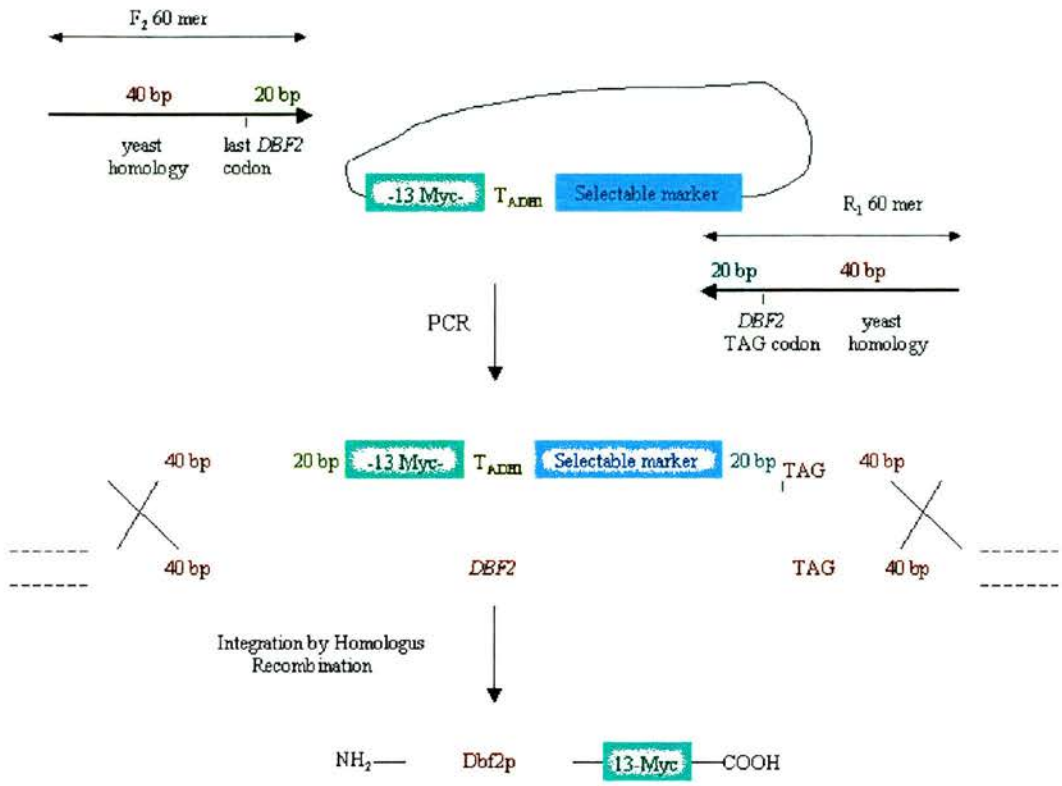

Figure 2.1 Cloning by homologous recombination. In the PCR reaction, two 60-mer oligonucleotides (F_2 and R_1) with 40-mer ORF specific homology were used, increasing the targeting specificity during mitotic recombination. The resulting Dbf2p is C-terminally 13*Myc*-tagged.

2.5.4 Cloning of *VMA2* and *DBF2* for bacterial expression

The *S. cerevisiae* 1554 bp *VMA2* and 1719 bp *DBF2* genes were amplified from genomic DNA by PCR using Pfu thermostable polymerase and the primers listed in Table 2.3. The presence of restriction sites for *Bam*HI and *Xho*I on the primers allowed for ligation into the pET28c expression vector (Novagen). This would give IPTG inducible expression of the proteins under the control of T7 polymerase. The full-length proteins were cloned and sequenced prior to expression of the protein in *E.coli*. The pET28a-c vector map is shown in Appendix 2.

Table2.3 Oligonucleotide primers used in gene cloning and recombinant protein construction.

Target DNA	bp	Direction	Length (nt)	Tm (°C)	Sites	Nucleotide sequence (5'- 3')
pRS313						
*DBF2**	2369	Fwd	27	69.5	*Xho*I	CCG CTC GAG GCA GTA GGA GCT ACA TCA
		Rev	26	71.1	*Bam*HI	CGC GGA TCC ATC GCG GCG AAT GCA AG
VPS15	5015	Fwd	29	69.5	*Xho*I	CCG CTC GAG GTA CTC GGT TTC ATC AAG GA
		Rev	29	66.7	*Bam*HI	CGC GGA TCC CGC ATT TAG AAT AAA GAA GC
BUB1	3766	Fwd	28	69.5	*Xho*I	CCG CTC GAG ATC GAC GCT GTA CGA AGA A
		Rev	28	72.4	*Bam*HI	CGC GGA TCC GTC ACC TAT GCG GGA GAT G
CTK1	2337	Fwd	28	72.4	*Xho*I	CCG CTC GAG CTT CGA CAA CTG CGC TGG A
		Rev	29	70.9	*Bam*HI	CGC GGA TCC ACC TTC TAT TCA CTA GGC CG
REG1	3695	Fwd	29	72.3	*Xho*I	CCG CTC GAG CAG CCC TGA GAT TGT GTG AC
		Rev	29	69.5	*Eco*RI	CCG GAA TTC GCA CTG ATC CAC ACT ACC TG
ROX3	1333	Fwd	27	69.5	*Xho*I	CCG CTC GAG GCG TAT TAC CTT CTG CTG
		Rev	27	68.0	*Eco*RI	CCG GAA TTC GCT GAT ATG CGA TGG CGT
*VMA2**	2204	Fwd	25	63.0	*Xho*I	CCG CTC GAG CAG ATA TGT AGA CAT T
		Rev	29	66.7	*Bam*HI	CGC GGA TCC CGG ACA AAA TAA AAA AAG CC
pET28c						
DBF2	1719	Fwd	26	58.4	*Bam*HI	CGG GAT CCA TAT GCT ATC AAA ATC AG
		Rev	29	54.5	*Xho*I	CCG CTC GAG CTA GTA AAA GGT TGA AAA GG
VMA2	1554	Fwd	31	68.2	*Bam*HI	CGG GAT CCG AAT GGT TTT GTC TGA TAA GGA G
		Rev	32	68.2	*Xho*I	CCG CTC GAG TTA GAT TAG AGA TTC TTC TTG GC
Homologous recombination						
13MycDBF2		Fwd	60	>75		CAA CGG ACT GGA ACA CTC AGA CCC CTT TTC AAC CTT TTA CCG GAT CCC CGG GTT AAT TAA
		Rev	60	74.2		TTA TAT CGC GGC GAA TGC AAG ACA AGA ATT CAT TTT TAC GGA ATT CGA GCT CGT TTA AAC

* The same primers were used for cloning into the pRS423 multicopy vector

2.6 Protein analysis

2.6.1 Antibodies

The antibodies used in this study are shown below (Table 2.4). Antibodies were used in immunoblotting or immune precipitation, as described in the following sections.

Table 2.4 Antibodies and target proteins

Antibody	Target protein	Source
Anti-Pdr12p (mAb)	Pdr12p	Kren *et al.*, 2003
8B1 anti-H^+- ATPase (mAb)	Vma2p	Molecular Probes
Clone 4A6	Dbf2p.13myc	Upstate
Clone 9E10	Dbf2p.13myc	Santa Cruz Biotechnology
Anti-phosphoserine (mAb)	Total lysate	Sigma (clone PSR-45)
Anti-phosphotyrosine (mAb)	Total lysate	Sigma (clone PT-66)
Anti-phosphothreonine (mAb)	Total lysate	Sigma (clone PTR-8)
ACT1 (C4)	Act1p	MP Biomedicals
CBP	TAP-Vma2p, TAP-Dbf2p	Upstate
CAB1001	TAP-Vma2p, TAP-Dbf2p	Open Biosystems

2.6.2 Protein analysis and concentration determination

Bio-Rad Criterion 12.5% polyacrylamide minigels (Bio-Rad, UK) were used for SDS-PAGE analysis of proteins according to manufacturer's instructions. Determination of sample protein content for western blotting was carried out using the Pierce BCA protein assay kit (Pierce, Rockford, IL). To ensure that equal concentrations of protein were loaded on 2-D PAGE gels, the samples were pre-run on 1-D PAGE gels and visualized by staining with colloidal Coomassie blue (Bio-Rad). The concentrations of proteins were determined empirically and adjusted accordingly.

2.6.3 Proteome analysis by two-dimensional electrophoresis (2-D Electrophoresis)

Protein was extracted from duplicate mid-exponential-phase BY4741a and *Δdbf2* cultures at an OD_{600} of 0.6 (200ml) grown in YEPD, pH 4.5 medium either in the presence or absence of 1.5 mM sorbic acid. Cells were pelleted and washed twice in ice-cold, double distilled water. Cell pellets were resuspended in 1.5 ml of lysis buffer {8 M urea, 2 M thio-urea, 4% 3-[(3-cholamido-propyl)- dimathylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiotheitrol-, 40 mM Tris base, 1% Pharmalyte 3-10 (Amersham Pharmacia Biotech), fungal protease inhibitor cocktail (Sigma)}, followed by the addition of an equal volume of 425-to 600-µm-diameter glass beads (Sigma). Cells were lysed using a mini-bead beater (Biospec, Bartlesville, Okla) four times for 1 min each time, with 1 min between bead beating on ice. The lysate was removed and transferred to 1.5 ml microcentrifuge tube. Cell debris was removed by centrifugation at 4 °C for 10 min and the resulting protein preparation was stored at –80 °C.

Comparative two-dimensional electrophoresis was performed using a MultiphorII apparatus for isoelectric focusing (IEF) (Amersham Pharmacia Biotech) as previously described (Qi et al., 1996) and a Hoefer-DALT apparatus (Amersham Pharmacia Biotech) for the second dimension. Immobilized pH gradient 18-cm strips, pH 4.5 to 5.5, pH 4 to 7, and pH 6 to 11 (Amersham Pharmacia Biotech), were used for IEF. SDS-10 % polyacrylamide gels (22 cm by 25 cm) (10 % Duracryl [Genomic Solutions, Ann Arbor, Mich], 0.4 M Tris [pH 8.8], 0.1% SDS, 0.1% ammonium persulfate, 0.01% *N,N,N',N'*-tetramethylethylenediamine [TEMED]) were used for the second dimension. Protein samples were first run on analytical gels (with approximately 0.1 mg of total protein) and were visualized using a silver stain kit (Genomic Solutions). Silver-stained gels were scanned using an Image Scanner II (Amersham Pharmacia Biotech). Protein spots showing obvious and reproducible changes in expression (present on both gel sets from duplicate experiments) were catalogued. Subsequently, identical protein samples were run on preparative gels (with approximately 1 mg of total protein) and stained with SYPRO® Ruby stain according to manufacturer's instructions (Genomic Solutions). The SYPRO® Ruby gels were scanned

using a FLA-5000 scanner (Fuji Photo Film Europe GmbH) at an excitation wavelength of 473 nm and emission of 618 nm. The protein spots with altered expression catalogued previously were excised from these gels for identification by peptide mass fingerprinting. Images were exported to Microsoft PhotoDraw 2000 version 2.0 for annotation and presentation. The isoelectric point and molecular weight of the proteins of interest were calculated from protein migration.

2.6.4 Phosphoproteome analysis by 2-D Electrophoresis and affinity chromatography

Affinity capture of phosphoproteins was performed using the PhosphoProtein Purification Kit from Qiagen Ltd., Crawley, UK, according to the manufacturer's instructions with some alterations (Makrantoni et al., 2005). Protein was extracted from duplicate, late-exponential-phase cultures at an OD_{600} of 0.8 (500 ml) grown in YEPD, pH 4.5, either in the presence (approximately 6 h exposure), or absence of 1.5 mM sorbic acid. Cells were pelleted (2000 g) and washed in ice-cold double distilled water. Cell pellets were resuspended as per manufacturer's instructions. The resuspended cells were then added to an equal volume of 425-600 µm-diameter glass beads (Sigma) in a 1.5 ml tube. Cells were lysed as previously described (section 2.6.3). The resulting protein preparation was stored at –80 °C.

Approximately 25 mg of total protein was adjusted to a concentration of 0.2 mg/ml in Phosphoprotein Lysis Buffer containing 0.25 % CHAPS (Qiagen) prior to loading onto the column. After the entire sample had passed through, the column was washed twice and the bound proteins were eluted. For the alkaline phosphatase 2-D gels the lysate was treated with 50 mM Calf Intestinal Phosphatase (CIP, Sigma) for 1 h at 30 °C prior to loading onto the column. Comparative 2-D electrophoresis was performed using a MultiphorII apparatus for isoelectric focusing (IEF) (Amersham Pharmacia Biotech) and a Hoefer-DALT apparatus (Amersham Pharmacia Biotech) as previously described (section 2.6.3). Immobilized pH gradient 18 cm strips of only one pH range 4.0 to 7.0 (Amersham

Pharmacia Biotech), were used for IEF. The second dimension was performed as previously described (section 2.6.3). Protein samples were run on preparative gels (0.15 mg total protein) and stained with SYPRO® Ruby, as described by the manufacturer (Genomic Solutions). Protein spots showing reproducible changes in protein abundance (present on both gel sets from duplicate experiments) were catalogued as described in section 2.6.3. Pro-Q Diamond Phosphoprotein gel stain (Molecular Probes) was also used to visualize phosphorylated proteins using a FLA-5000 scanner according to the manufacturer's instructions. The protein spots with altered abundance were catalogued as previously described.

2.6.5 Mass spectrometry

Protein spots of interest were excised from the gels and digested in the gel with trypsin (Promega) using an Investigator Progest digestion robot (Genomic Solutions) as previously described (Lawrence et al., 2004). Half the sample was desalted and concentrated using a micro C_{18} column (0.2 µl of ZipTip) (Millipore, Gloucestershire, UK) according to the manufacturer's instructions. The peptides were eluted directly from the tip onto the target in 1.5 µl α-cyano-4-hydroxycinnamic acid (saturated stock prepared in acetonitrile-0.2 % trifluoroacetic acid [60:40]). Spectra were obtained on a Micromass TofSpec 2E instrument (Micromass, Manchester, U.K.), equipped with a 337 nm wavelength laser and operated in reflectron mode. The data were calibrated using the tryptic peptides of β-galactosidase (Sigma) and lock mass corrected using a Glu-Fibrinopeptide B spike. Monoisotopic peptide masses were selected using BioLynx ProteinProbe (Micromass) and submitted for peptide mass matching against the MSDB database using the Mascot search engine (URL: http://www.matrixscience.com/search_form_select.html).

2.6.6 2-D gel shifts in the p*I* of Vma2p

Protein was extracted in Lysis buffer A [50 mM Tris-HCl, pH 7.5, 10 % glycerol, 1 % Tritox-100, 0.1 % SDS, 150 mM NaCl, 5 mM EDTA, complete protease inhibitors (Roche)] from duplicate mid-exponential-phase cultures of BY4741a and Δ*dbf2* strains at an OD_{600} of 0.6 (200ml) as described before (section 2.6.3). Strains were grown in YEPD, pH 4.5 medium either in the presence or absence of 1.5 mM sorbic acid. For the calf intestinal alkaline phosphatase (CIP) treatments, 40 µg of total lysate was treated with 50 CIP (Sigma) for 1 h at 30 °C prior to IEF. Comparative 2-D electrophoresis was performed as previously described (section 2.6.3) with minor alterations. A MultiphorII apparatus was used for IEF (Amersham Pharmacia Biotech) and a CRITERION™ CELL apparatus (BioRad) for the second dimension. Immobilized pH gradient 11-cm strips, pH 4 to 7 (Amersham Pharmacia Biotech), were used for IEF and the 40 µg of total lysate was diluted in a total of 200 µl of lysis buffer (section 2.6.3). The IEF programme used was: 1 h at 150 V, 2 m A, 5 W; 1 h at 300 V, 2 mA, 5 W; 90 min at 3500 V, 2 mA, 5 W; and 12,5 h at 3500 V, 2 mA, 5 W. For the second dimension, 12.5 % Tris-HCl, 1.00 mm precast polyacrylamide gels (Bio-Rad Criterion) with exactly 11 cm IPG comb were used.

Proteins were then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) presoaked in transfer buffer (25 mM Tris, 0.192 M glycine), and run at 400 mA for 30 min using a Hoefer SemiPhor semi-dry transfer unit (Amersham Pharmacia Biotech), assembled according to manufacturer's instructions. Western blot analysis for detection of phosphorylated Vma2p was conducted using 8B1 anti-H^+- ATPase monoclonal antibody (Molecular Probes; Table 2.3) at 1:4000 dilution, as described in section 2.7. Following washes in PBS-Tween, the blots were incubated in horseradish peroxidase-labeled antimouse polyclonal antibody at 1:1000 dilution (Amersham Pharmacia Biotech). Membranes were developed using enhanced-chemiluminescence system (Supersignal West Femto chemiluminescence kit, Pierce). Blots were compared for Vma2p p*I* changes. All sets of blots representing Vma2p pI changes in the presence and absence of sorbic acid in both control BY4741a and Δ*dbf2* strains were exposed for the same time and developed in parallel using a Luminescent Image Analyser LAS-1000plus (Fujifilm, Co, Ltd. Japan).

2.7 Western blotting analysis

Protein was extracted in extraction buffer [50 mM Tris-HCl, pH 7.5, 10 % glycerol, 1 % Tritox-100, 0.1 % SDS, 150 mM NaCl, 5 mM EDTA, complete protease inhibitors (Roche)]. Following sample analysis by SDS-PAGE, proteins were transferred to nitrocellulose membranes presoaked in transfer buffer (25 mM Tris, 0.192 M glycine), and run at 400 mA for 30 min as previously described (section 2.6.6). Following electroblotting, the membrane was incubated in blocking buffer [(5 % (w/v) skimmed milk powder, 0.1 % (v/v) Tween 20 in PBS (PBS-T) or 5 % BSA in PBS-Tween for phosphorylated proteins)] for 1 h to block nonspecific protein binding sites. The protein of interest was subsequently detected with primary antibody (Table 2.3) by incubating the membrane in antibody suspension (according to manufacturer's instructions) for 2 hrs or overnight at 4 °C. In order to detect bound antibodies the membrane was washed (three 10 min washes in PBS-T on a rocking platform) and incubated in appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h. The membrane was then washed as above and further washed in water before bands were visualized by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Pierce). Images were taken with a Luminescent Image Analyser LAS-1000plus (Fujifilm, Co, Ltd. Japan).

2.7.1 Immunoblot analysis of eluate and flow-through fractions of Qiagen phosphocolumns

Cell extracts for immunoblotting were prepared exactly as described in section 2.6.4. Equal concentrations of phosphorylated (eluate fraction) and unphosphorylated (flow-through fraction) protein were loaded. Cell lysates were resolved by SDS-PAGE using 12.5 % polyacrylamide minigels (Bio-Rad Criterion) as previously described (Bardin et al., 2003) and transferred to nitrocellulose membranes. Western blot analysis for detection of phosphorylated proteins was conducted using the following antibodies: monoclonal anti-phosphoserine (1:500 dilution; Table 2.3), monoclonal anti-phosphotyrosine (1:1000 dilution; Table 2.3) and monoclonal anti-phosphothreonine (1:1000 dilution; Table 2.3)

(Makrantoni et al., 2005). Supersignal West Femto chemiluminescence substrate was used for detection (Pierce).

2.8 Protein expression

Proteins of interest were expressed either in yeast or in *E. coli*. In yeast the tandem affinity purification (TAP) method was used for rapid purification under native conditions with the added advantage that proteins were expressed at their natural levels. In *E.coli*, proteins of interest were expressed as recombinant proteins under an IPTG inducible promoter with a $(His)_6$ tag at the C-terminus using the pET28c vector (Invitrogen).

2.8.1 Tandem Affinity Purification (TAP)

Tap tagged strains were obtained from Open Biosystems (Table 2.1) and the TAP strategy was used for the purification of both Vma2p and Dbf2p. The TAP method involves the fusion of the TAP tag to the target protein and the introduction of the construct to the host cell or organism, maintaining the expression of the fusion protein at, or close to, its natural levels (Rigaut et al., 1999).

The TAP tag consists of two immunoglobulin-binding domains of protein A from *Staphylococcus aureus*, a cleavage site for the tobacco etch virus (TEV) protease, and the calmodulin–binding peptide (CBP) (Figure 2.2) (Puig et al., 2001; Rigaut et al., 1999). Cell extracts for TAP purification were prepared as described elsewhere (Puig et al., 2001) with minor alterations. Briefly, extracts were prepared from 2 L of yeast cells grown to late log phase ($OD_{600} \sim 2$-3).

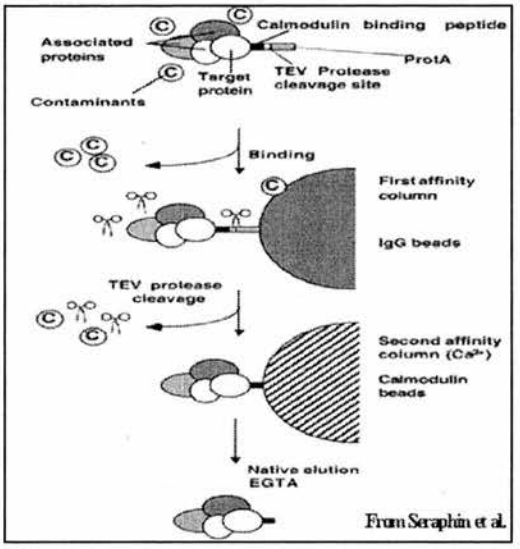

Figure 2.2. Schematic representation of the TAP purification strategy (Rigaut et al., 1999).

Cell pellets were washed once with water and pelleted again in 50 ml polypropylene tubes. The packed cell volume (PCV) was measured and the tube was frozen in liquid nitrogen and stored in –80 °C. One PCV of Buffer D [(20 mM K-Hepes pH 7.9, 50 mM KCl, 0.2 mM EDTA pH 8.0, 0.5 mM DTT, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, 4 μM chymostatin, protease inhibitors tablet (Roche)] was added to the pellet which was rapidly thawed and kept at 4 °C. All subsequent steps were performed at 4 °C with precooled buffers and equipment. Cells were lysed using a mini-bead beater (Biospec, Bartlesville, Okla.) six times for 30 sec, with 30 sec between bead beating on ice. Extracts were centrifuged at 25,000g for 30 min. The supernatant was then filter-sterilized with 0.2 μm filters into new tubes. All the binding and elution steps were performed in 0.8 x 4-cm Poly-Prep columns (Bio-Rad, Hercules, CA).

Two hundred microliters of IgG agarose bead suspension (Sigma) were transferred into the column. The beads were subsequently washed with 10 ml IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Igepal). Prior to loading onto the column, the composition of the extract buffer was adjusted to 10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 0.1% Igepal. Extracts were then rotated with the IgG beads for 2 h at 4 °C. Elution was performed by gravity flow and the beads were washed three times with 10 ml of IPP150and once with TEV buffer (IPP150 adjusted to 0.5 mM EDTA and 1 mM DTT). Cleavage was achieved

with in the same column by adding 1 ml of TEV cleavage buffer and approximately 100units of TEV protease. The beads were rotated for another 2 h at 16 ºC or overnight at 4 ºC and the eluate was recovered by gravity flow.

Two hundred microliters of calmodulin bead suspension (Stratagene, CA) were transferred into a new column and washed with 10 ml of IPP150 calmodulin binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1 % Igepal). Three milliliters of IPP150 calmodulin binding buffer and 3 µl of 1 M $CaCl_2$ were added to the 1 ml of eluate recovered after TEV cleavage. This solution was then transferred to the column containing the washed calmodulin beads and rotated for 1 h at 4 ºC. After the beads were washed with 30 ml of calmodulin binding buffer, the bound proteins were eluted with 1 ml IPP150 calmodulin elution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1 % Igepal, 2 mM EGTA). Five elution fractions of 200 µl each were collected. The fractions were further concentrated using 5,000 moleculat weight cut-off membrane (5,000 MWCO PES) concentrators (Vivascience) prior to loading. Following sample analysis by SDS PAGE, gels were stained with SYPRO® Ruby, proteins of interest were excised and identified by mass fingerprinting as previously described (section 2.6.5).

2.8.2 Establishment of bacterial recombinant expression

Several colonies from each *E. coli* transformation were screened for protein expression. Briefly, 2 ml of LB containing the appropriate antibiotics (ampicillin at 50 µg/ml) was inoculated with a transformant colony and grown for 16 h at 37 ºC. A 100 µl aliquot of this culture was then used to inoculate fresh LB (5 ml) and grown to an OD_{600} 0.4-0.6 (~ 2 h) before induction with IPTG (0.5 mM). Cellular protein fractions (pre- and post-induction) were analyzed after a further 5 hours post-induction growth.

2.8.3 Affinity purification of *S. cerevisiae* recombinant Dbf2p and Vma2p

For production of recombinant Dbf2p and Vma2p proteins, high yielding clones (in pET28c plasmids) were grown for 16 h at 37 °C in LB containing appropriate antibiotics. A 1:100 dilution of overnight culture was used to inoculate 1 L of fresh LB media in 2 L Erlenmeyer flasks. Protein expression was induced by the addition of IPTG to a final concentration of 0.2 mM, when the culture reached an OD_{600} of 0.5-0.6. After 4-5 h, the cells were harvested by centrifugation (3,000g, 4 °C, for 15 min). At this stage it was possible to snap freeze the whole cell pellet for storage at -80 °C, if required. To promote the formation of soluble recombinant protein, some cultures were grown at 21 °C for approximately 16 h after induction of protein expression.

Cells were lysed in Buffer A [20 mM Tris, pH 8.0, 50 mM NaH_2PO_4, 100 mM NaCl, 10 mM imidazole, protease inhibitor cocktail (Roche)]. The recombinant protein was mechanically lysed either with a French Press or by bead-beating as previously described (section 2.6.3). The supernatant (soluble fraction) was clarified by centrifugation (40,000g, 4 °C, 30 min) and then passed through a 0.2 µm filter.

As both Dbf2p and Vma2p after SDS PAGE were only seen in the insoluble fraction a solubilization protocol was attempted from the insoluble inclusion bodies. Insoluble material recovered after cell lycis was suspended in Solution A (containing 8M urea, 10 mM imidazole and 10% (w/v) glycerol) and then clarified at 15,000 g at room temperature for 30 min. The clarified supernatant was filtered and applied on a pre-equilibrated in Solution A 5 ml HisTrap Chelating HP column (Amersham Biosciences) charged with Ni. Refolding of the proteins on the column was achieved with sequential washings of successively lower concentrations of urea. The column was first washed with 10 column volumes (CV) of Solution A containing 8 M urea, 10 mM imidazole and 10% (w/v) glycerol, followed by sequential washes with 5 CV of Solution A containing 6, 3, 1 and O M urea. Recombinant proteins were then eluted in 5 CV of Solution A containing 250 mM imidazole and 5% (w/v) glycerol. Fractions were analyzed by SDS PAGE gel

electrophoresis. The fractions corresponding to the recombinant proteins were combined and concentrated to a final volume of ~10 ml.

2.8.4 Size exclusion chromatography

Concentrated Dbf2p and Vma2p fractions purified from Ni column were further purified by preparative gel-filtration using a size-exclusion column (26/70-gel filtration column) and resolved using buffer C (20 mM Tris, pH 8.0, 50 mM NaH_2PO_4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT) at 2 ml/min. Protein gel analysis was used to show essentially homogenous Dbf2p and Vma2 protein was obtained. Purified protein concentrations were estimated from the absorbance of the protein solution at 280 nm (A_{280}) using the extinction coefficients obtained from ProtParam analysis of amino acid composition (http://ca.expasy.org/tools/protparam.html/).

2.9 Protein-protein interactions

Protein interactions under sorbic acid treatment were identified by using the TAP-tag methodology as previously described (section 2.8.1) for identifying interacting partners for Dbf2p and Vma2p. For kinase assays TAP-Dbf2p, TAP-Vma2p and a 13Myc-Dbf2p (section 2.5.5) were used.

2.9.1 Immunoprecipitations

The preparation of cell extracts for immunoprecipitation and kinase assays were performed as described elsewhere (Toyn and Johnston, 1994) with minor alterations. A 13Myc-Dbf2p strain (Table 2.1) was grown to an OD_{600} of 0.8 in YEPD, pH 5.8. Cell pellets were resuspended in ice-cold lysis buffer [(20 mM Tris pH 7.4, 100mM NaCl, 10mM EDTA, 1 % Triton X-100, 5 % glycerol, 1 mM each of NaF, β-glycerophosphate, Na_3VO_4, EGTA and sodium pyrophosphate and complete protease inhibitors cocktail (Roche)]. Cells were lysed using a mini-bead beater (Biospec, Bartlesville, Okla) and sufficient acid-washed beads (0.5 mm diameter, Sigma) four times for 1 min each time, with 1 min between bead beating on ice. Protein extracts were stored at –80 °C without loss of kinase activity.

Four microlitres of clone 4A6 anti-Myc antibody (Upstate Ltd, UK; Table 2.3) or 9E10 (Santa Cruz Biotechnology) were added to yeast extract containing 500 µg of total protein (extracted as described above) and incubated at 4 °C for 1 h with rotation. Twenty microlitres of 1:1 slurry of prewashed with PBS protein G beads (Protein G Sepharose P3296, Sigma) were added and incubation was continued with mixing for a further 4 h at the same conditions or overnight at 4 °C. The beads were then washed twice by rounds of mixing and centrifugation successively in 1 ml lysis buffer and lysis buffer containing 1 mM NaCl. The proteins in the immune complexes were dissociated by heating (100 °C, 5 min) in gel electrophoresis sample buffer (0.05 M Tris-HCl pH 7.0, 0.2 % SDS, 5% 2-mercaptoethanol and 5 % glycerol) and subjected to SDS-PAGE as described before.

Analysed proteins were subsequently fixed and stained with SYPRO® Ruby, as described by the manufacturer (Genomic Solutions). Interacting proteins were identified by peptide mass fingerprinting (section 2.6.5).

2.9.2 Kinase assay

Preparation of protein extracts and immunoprecipitation were performed as described above. Beads were then washed in kinase assay buffer [25 mM 3-[N-morpholino] propanesulfonate (MOPS) pH 7.2, 60 mM b-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 0.1 mM Na_3VO_4, 10 µM ATP and protease inhibitor cocktail (Roche)] and resuspended in 10 µl of assay buffer. The kinase reaction was initiated by the addition of 10 µCi of [γ-^{32}P] ATP and 5 µg of calf thymus H1 histone (type III-S, Sigma). Reaction mixtures were incubated at 30 °C for 30 min. Reactions were terminated by addition of 15 µl SDS-PAGE boiling sample buffer. Proteins were separated by SDS-PAGE. After electrophoresis, the gel was exposed to film and bands were visualized using a FLA-5000 scanner according to the manufacturer's instructions.

2.10 Detection of Vacuole Acidification by Quinacrine Fluorescence

Vacuolar acidification was examined by quinacrine accumulation and fluorescence using modifications of published procedures (Roberts, 1991). Yeast cells were grown in YEPD, pH 4.5 to an OD_{600} 0.8-1.0 and 1.5 ml was harvested and washed twice in uptake buffer (YEPD buffered to pH 7.6 with 100 mM HEPES). The cell pellet was resuspended in 100 µl uptake buffer and 0.5 µl of a fresh 40 mM quinacrine stock (Sigma) was added to a final concentration of 200 µM. Cells were incubated at 30 °C for 10 min and held on ice for another 5 min, after which they were washed 3 times with ice-cold wash buffer (100 mM HEPES, pH 7.6, 2% glucose) with 5-sec pulse spins. Cell pellets were resuspended in 100

µl wash buffer, 2-4 µl was placed on a microscope slide, mixed with an equal volume of 1% low melting agarose and covered with a coverslip. Accumulation of quinacrine into the vacuoles was monitored within 1 h after quinacrine staining and images were taken, using fluorescence microscopy with excitation at 490 nm and 435 nm and emission of 503 nm (Olympus IX70 Delta Vision). Images with direct interference contrast optics for Normarski were also taken as controls.

2.11 Computer Analysis

Commonly used programs and databases are listed below

Saccharomyces Genome Database (SGD): http://www.yeastgenome.org/
NCBI BLAST Home Page: http://www.ncbi.nlm.nih.gov/BLAST/
FatiGO Data mining with Gene Ontology: http://fatigo.bioinfo.cnio.es/
NetPhos 2.0 Server: http://www.cbs.dtu.dk/services/NetPhos/
ExPASy Proteomics Server: http://us.expasy.org/
ProtParam: http://ca.expasy.org/tools/protparam.html/
Pfam: http://www.sanger.ac.uk/Software/Pfam/index.shtml
EUROSCARF: http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html
Yeast GFP Fusion Localisation Database: http://yeastgfp.ucsf.edu/
GRID Database: http://biodata.mshri.on.ca/grid/servlet/Index
MIPS Database: http://mips.gsf.de/genre/proj/yeast/index.jsp
S. cerevisiae Phosphorylome Database: http://networks.gersteinlab.org/phosphorylome/

All URLs referenced in this and other sections were confirmed as current and functional on the 13th of September 2005.

CHAPTER 3

3. Global identification of sorbic acid sensitive genes via screening the yeast genome non-essential deletion library

3.1 Introduction

Weak organic acids, such as sorbic acid, are widely used in food and beverage preservation. The mode of action for weak acids is well known (reviewed in Piper et al., 2001) and several genes have been implicated in the sorbic acid stress response and adaptation (de Nobel et al., 2001; Holyoak et al., 1999; Kren et al., 2003; Piper et al., 1998; Schuller et al., 2004). However, whilst we know much about the actual mechanisms of adaptation to sorbic acid in *Saccharomyces*, the stress-signaling pathway that induces these adaptive responses remains elusive.

In this study we present the results of a genome-wide screen in order to identify potential target genes and signaling components implicated in sorbic acid resistance. This was achieved using the complete non-essential yeast deletion strain collection, which had been generated through an international effort to systematically delete every yeast open reading frame (Winzeler et al., 1999). In this study the *S. cerevisiae* BY4741a based non-essential deletion collection was used (Research Genetics, USA). The collection screened with 1.5 mM sorbic acid at pH 4.5, uncovered several new genes required for sorbate resistance in yeast. Functional categories of genes that were sensitive upon exposure to sorbic acid stress included: metabolism, mitochondrial function, vacuolar protein sorting, transcription and cell cycle regulation.

More specifically, we wanted to identify genes involved in signaling or regulation of sorbic acid stress responses. Thus, a more detailed screen of all deletion mutants encoding protein kinases, phosphates and transcription factors was performed, resulting in seven regulatory proteins being sorbate hypersensitive. To confirm that the phenotypes observed were specifically due to the deletion of each gene, complementation studies were performed. To

further investigate whether the sensitivity to sorbic acid was specific, the deletion mutants identified were subjected to other stresses.

S. cerevisiae provides a relatively simple model system that is very well understood genetically and has been at the forefront of recent advances in functional genomics technologies (Blackburn and Avery, 2003; Giaever et al., 1999; Higgins et al., 2002; Raamsdonk et al., 2001; Winzeler et al., 1999). Thus, genome-wide screens like the one performed in this study allow for the identification of potential novel gene functions on a global scale. We exploited the yeast non-essential deletion strain collection, which provides an outstanding resource for addressing important biological questions and we managed to catalogue genes that are required for sorbic acid resistance and to identify interesting candidates for further studies. New candidates can give novel insights into the signaling mechanisms by which sorbic acid stress may be perceived by the yeast cell.

3.2 Screening the 'disruptome' for sensitivity to sorbic acid

To identify key genes required for growth in the presence of sorbic acid a phenotypic screen of all the 4,847 non-essential gene deletions in *S. cerevisiae* BY4741 *MAT***a** of the Research Genetics collection (section 2.2.1; Table 2.1) was performed. Yeast strains were grown overnight in 96-well plates in liquid YEPD, pH 4.5, and then spotted onto YEPD, pH 4.5 agar plates, containing 1.5 mM sorbic acid. As a control, YEPD, pH 4.5 plates lacking sorbic acid were used. Following incubation for 48 hrs at 30 °C, sorbic acid sensitivity was scored (Figure 3.1). Of the 4,847 nonessential gene deletions tested, 119 strains showed impaired growth in the presence of sorbic acid Table 3.1.

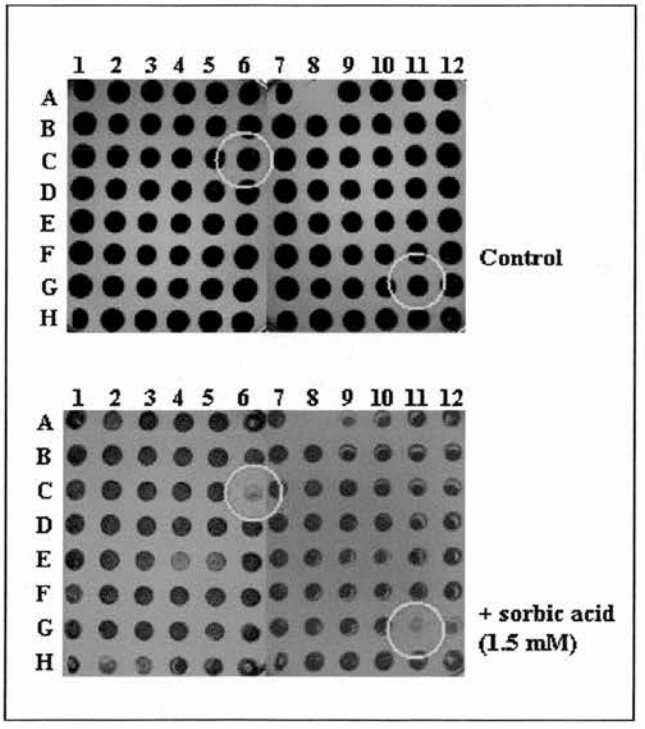

Figure 3.1 Screening for sorbic acid sensitivity using the *S. cerevisiae* deletion strain collection. Strains were cultured in liquid YEPD, pH 4.5 medium in a 96-well format and replica plated onto YEPD, pH 4.5 agar with and without sorbic acid (1.5 mM). Circles highlight strains that exhibited sensitivities to sorbic acid relative to their growth on the control plate. Empty inocula on the control plate correspond to positions at which essential open reading frames were originally deleted, producing nonviable mutants.

Table 3.1 Screening the yeast 'disruptome' identified genes required for optimal growth of *S. cerevisiae* in the presence of 1.5 mM sorbic acid at pH 4.5.

ORF	*Gene*	Function
METABOLISM		
Lipid, fatty acid and isoprenoid metabolism		
YER019w	*ISC1*	Inositol phosphoSphingolipid phospholipase C
YMR202w	*ERG2**	C-8 sterol isomerase
YLR056w	*ERG3**	C-5 sterol desaturase
YGL012w	*ERG4*	Sterol C-24 reductase
YML008c	*ERG6**	*Delta (24)-sterol C-methyltransferase*
YDR297w	*SUR2*	*Sphingosine hydroxylase*
YGR036c	*CAX4*	pyrophosphate phosphatase
Amino acid metabolism		
YBR249c	*ARO4*	2-dehydro-3-deoxyphosphoheptonate aldolase
YDR127w	ARO1	3-dehydroquinate synthase
YPR060c	ARO7	Chorismate mutase
YGL148w	ARO2*	Chorismate synthase
YDR007w	TRP1*	Phosphoribosylanthranilate isomerase
YKL211c	TRP3	Anthranilate synthese component II
YER090w	TRP2*	Anthranilate synthase component I
YLR027c	AAT2	Aspartate aminotransferase, cytosolic
Carbohydrate metabolism		
YPL002c	*SNF8*	Involved in glucose derepression
YOR136w	*IDH2*	Isocitrate dehydrogenase (NAD+) subunit2
YLR025w	*SNF7*	Involved in glucose derepression
YHR183w	*GND1*	Phosphogluconate Dehydrogenase
YLR354c	*TAL1*	Transaldolase, enzyme in the pentose phosphate pathway
YPR160w	*GPH1*	Glycogen phosphorylase; Releases glucose-1-phosphate from glycogen
YBL058w	*SHP1*	Glycogen metabolism; potential regulatory subunit for Glc7p
YOL086c	*ADH1**	Alcohol dehydrogenase; fermentation
YDR028c	*REG1*	Regulator of phosphatase Glc7p, involved in glucose repression
PtdIns metabolism		
YDR173c	*ARG82**	Dual specificity inositol 1,4,5-trisphosphate 6-kinase/inositol 1,4,5,6-tetrakisphosphate 3-kinase
YKL212w	*SAC1**	Phosphoinositide phosphatase
YLR240w	*VPS34*	Phosphatidylinositol 3-kinase
Mitochondrial metabolism; organization; repair		
YHR120w	*MSH1*	Involved in mitochondrial DNA repair
YHR147c	*MRPL6*	Mitochondrial large ribosomal subunit
YCR003w	*MRPL32*	Mitochondrial large ribosomal subunit
YOR211c	*MGM1*	GTP-binding domain protein related to dynamin
YBR122c	*MRPL36*	Mitochondrial large ribosomal subunit
YNL055c	*POR1*	Mitochondrial porin (voltage-dependent anion channel)
YCR028c	*RIM1*	DNA binding protein, essential for mitochondria
YGR101w	*PCP1*	Mitochondrial serine protease
YGR096w	*TPC1*	Mitochondrial thiamine pyrophosphate transporter
YOR158w	*PET123*	Mitochondrial ribosomal protein of the small subunit

YPL059w	*GRX5**	Thiol-disulfate exchange intermediate

CELLULAR TRANSPORT AND TRANSPORT MECHANISMS

ABC Transporters

YPL058c	*PDR12**	Plasma membrane weak-acid-inducible ATP-binding cassette (ABC) transporter, required for weak organic acid resistance

Vacuolar protein sorting

YMR183c	*SSO2**	Post-Golgi t-SNAREs
YOR036w	*PEP12*	Target membrane receptor (t-SNARE)
YDR069c	*DOA4**	Ubiquitin hydrolase ;vacuolar biogenesis and osmoregulation
YDR136c	*VPS61*	Vacuolar Protein Sorting
YKL041w	*VPS24*	Involved in secretion
YPL065w	*VPS28*	Involved in vacuolar protein targeting
YKR001c	*VPS1*	Involved in vacuolar protein sorting and normal organization of intracellular membranes
YKR020w	*VPS51*	Forms a tetramer with VPS52, VPS53, and VPS54
YJL029c	*VPS53*	Hydrophilic protein that is peripherally associated with the late Golgi and forms a stable complex with Vps52p and Vps54p
YDR495c	*VPS3*	Vacuolar Protein Sorting
YLR396c	*VPS33*	Vacuolar sorting protein essential for vacuolar morphogenesis and function
YBR097w	*VPS15*	Myristoylated Serine/threonine protein kinase involved in vacuolar protein sorting
YDR484w	*VPS52*	Golgi associated retrograde protein complex
YLR417w	*VPS36*	Vacuolar Protein Sorting
YGL054c	*ERV14*	14 kDa protein found on ER-derived vesicles
YKL002w	*DID4*	Class E vacuolar-protein sorting and endocytosis factor
YCL008c	*STP22*	CVT pathway; cytoplasm to vacuole targeting
YBR127c	*VAM6*	Protein involved in vacuolar morphogenesis
YLR148w	*PEP3**	Vacuolar peripheral membrane protein; required for vacuolar biogenesis

Vacuolar H^+-ATPase

YEL051w	*VMA8*	H^+-ATPsynthase V_1 domain 32 KD subunit
YEL027w	*CUP5*	H^+-ATPase V_0 domain 17 KD subunit
YHR060w	*VMA22**	Vacuolar ATPase assembly protein
YKL080w	*VMA5*	H^+-ATPase V_1 domain 42 KD subunit
YPR036w	*VMA13*	V1 domain subunit H (54 kDa)
YBR127C	*VMA2**	H^+-ATPase V_1 domain 60 KD subunit
YHR026w	*PPA1*	H^+-ATPase 23 KD subunit

TRANSCRIPTION

YKL139w	*CTK1*	Kinase subunit of RNA polymerase II carboxy-terminal domain kinase I
YML112w	*CTK3*	RNA polymerase II C-terminal domain kinase gamma subunit
YER068w	*MOT2*	Component of the CCR4-NOT transcription regulatory complex
YAL021c	*CCR4*	Transcriptional regulator for some glucose-repressed genes including ADH2
YNR052c	*POP2*	RNase of the DEDD superfamily, subunit of the Ccr4-Not complex
YML076c	*WAR1**	Transcription factor that mediates response to sorbic acid stress

YGR063c	*SPT4**	Transcriptional regulator zinc finger protein
YHR041c	*SRB2*	RNA polymerase II holoenzyme/mediator subunit
YGR104c	*SRB5**	Subunit of RNA polymerase II holoenzyme/mediator complex
YNL236w	*SIN4*	RNA polymerase II holoenzyme/mediator subunit involved in positive and negative regualtion of transcription
YBL093C	*ROX3*	RNA polymerase II holoenzyme component; repressor Of hypoXic genes
YCR081w	*SRB8**	RNA polymerase II mediator complex subunit; suppressor of RNA polymerase B
YDR432w	*NPL3*	RNA-binding protein that carries poly(A)+ mRNA from the nucleus into the cytoplasm
YKL057c	*NUP120*	Nuclear pore complex subunit
YJL140w	*RPB4**	RNA polymerase II fourth largest subunit
YDR162c	*NBP2*	Nap1 Binding Protein
YBR081c	*SPT7*	Subunit of the SAGA transcriptional regulatory complex
YDR469w	*SDC1*	Required in transcriptional silencing near telomeres; nuclear chromatin
YLR403w	*SFP1*	Split zinc finger protein
YDR443c	*SSN2*	Component of RNA polymerase II holoenzyme
	RIC1	Involved in transcription of ribosomal protein genes and ribosomal RNA

CELL CYCLE AND DNA PROCESSING

YGR188c	*BUB1*	ser/thr protein kinase
YGR092w	*DBF2*	ser/thr protein kinase
YLR226w	*BUR2*	Cyclin-dependent protein kinase
YDR295c	*PLO2*	Histone DeAcetylase
YDL047w	*SIT4*	SIT4 suppress mutations in DBF2; type 2A related protein phosphatase
YDL227c	*HO*	HOmothallic switching endonuclease

CYTOSKELETON

TLR337c	*VRP1*	cytoskeletal organization and cellular growth
YBR266c	*SLM6*	role in actin cytoskeleton organization

CELLULAR TRANSPORT AND TRANSPORT MECHANISMS

Vesicular transport (Golgi etc)

YAL026c	*DRS2*	P-type ATPase, potential aminophospholipid translocase
YDR137w	*RGP1*	Subunit of a Golgi membrane exchange factor
YLR262c	*YPT6*	Transport from late Golgi to endosome
YGR167w	*CLC1*	Clathrin light chain
YDL226c	*GCS1*	Involved in ER-Golgi transport

CONTROL CELLULAR ORGANISATION

Cell wall

YKL046c	*DCW1*	Required for cell wall biosynthesis
YGR229c	*SMI1*	Beta-1,3-glucan synthesis protein
YGL038c	*OCH1*	Alpha-1,6-mannosyltransferase
YJL184w	*GON7*	Involved in the transfer of mannosylphosphate groups onto N-linked oligosaccharides
YER083c	*RMD7**	Cell wasll organization and biosynthessis

RIBOSOME BIOGENESIS

YKL009w	*MRT4*	Involved in mRNA turnover and ribosome assembly

YJL190c	*RPS22A*	Protein component of the small (40S) ribosomal subunit
YDR450w	*RPS18A*	Protein component of the small (40S) ribosomal subunit
PROTEIN FATE		
YAL039c	*CYC3*	cytochrome c heme lyase; attaches heme to apo-Cyc1p in the mitochondrial intermembrane space
YJL121c	*PRE1*	20S proteasome beta-type subunit
INTERACTION WITH THE CELLULAR ENVIRONMENT		
Oxidative stress		
YJR104c	*SOD1*	Cu, Zn superoxide dismutase
UNKNOWN FUNCTION		
YBR052c		ORF, Uncharacterized
YBR030w		hypothetical protein
YBR051w		hypothetical protein
YBR054w	*YRO2*	putative plasma membrane protein of unknown function
YDR008c		hypothetical protein
YDR521w		hypothetical protein
YGL149w		hypothetical protein
YGR064w		hypothetical protein
YIL029c		hypothetical protein
YJL175w		hypothetical protein
YJR118c	*ILM1*	protein of unknown function; may be involved in mitochondrial DNA maintenance
YJL120w		hypothetical protein
YLR261c	*VPS63*	dubious open reading frame
YNR029c		hypothetical protein
YNL080c		ORF, Uncharacterized
YOR235w *		hypothetical protein
YKL118w		Involved in meiotic nuclear division

* Genes involved in sorbate sensitivity identified by Schuller et al, 2004
In gray: genes involved in sorbate sensitivity identified by Mollapour et al, 2005

3.2.1 Mutants indicating the importance of lipid, fatty acid, amino acid and carbohydrate metabolism to sorbic acid resistance

The mutant strains identified by the phenotypic analysis are involved in diverse cellular functions (Table 3.1). A major group belongs to energy metabolism (lipid, fatty acid and isoprenoid metabolism, amino acid and carbohydrate metabolism) including genes such as phosphogluconate dehydrogenase (*GPH1*), glycogen metabolism (*SHP1*), and alcohol dehydrogenase (*ADH1*). Several genes of the ergosterol biosynthesis pathway (*ERG2, ERG3, ERG4, ERG6*) were also identified as sorbate sensitive. Another group are involved in aromatic amino acid biosynthesis (*ARO1, ARO2, ARO4, ARO7*, *TRP1, TRP2* and *TRP3*). The importance of maintaining the glycolytic pathway has also been highlighted by the identification of many sorbic acid sensitive genes (*SNF8*, *SNF7*, *GND1*, *GPH1*, *SHP1*and *REG1*).

3.2.2 Mutants indicating the importance of the vacuolar H^+-ATPase pump, vacuolar protein sorting and phosphoinositide metabolism

The screen identified a number of deletion strains encoding the constituent proteins of the vacuolar H^+-ATPase pump (*vma)*, the vacuolar protein sorting mechanism (*vps*) and phosphoinositide metabolism. Mutants defective in the vacuolar membrane H^+-ATPase (Δ*vma2*, Δ*vma5*, Δ*vma8*, Δ*vma13*, Δ*vma22*, Δ*cup5*, Δ*ppa1*) were markedly sorbate-sensitive, suggesting that the acidified vacuole plays an important role in sorbic acid resistance. Defects in phosphoinositide (PtdIns) metabolism (Δ*arg82*, Δ*sac1*, Δ*vps34*) resulted in sorbate sensitivity. Sorbate sensitivity has also been associated with loss of Vps34p, a PtdIns 3-kinase activity required for CPY pathway trafficking between the Golgi apparatus and vacuole (Stack et al., 1995) and for macroautophagy (Kiel et al., 1999; Kihara et al., 2001).

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3.2.3 Mutants indicating the importance of transcription in sorbic acid resistance

A number of deletion mutants that are part of the RNA polymerase subunit II were identified in this study as sorbic acid sensitive (Table 3.1). Deletion of *CTK1* and *CTK3* resulted in sorbate sensitivity. These two genes encode proteins that form part of the CTDK-I complex, a protein kinase complex that specifically and efficiently hyperphosphorylates the carboxyl-terminal repeat domain (CTD) of RNA polymerase subunit II (Sterner et al., 1995).

Loss of some components of the CCR4 transcriptional apparatus (Mot2p, Ccr4p, Pop2p and Dbf2p) resulted in sorbic acid sensitivity. Deletion of *WAR1*, a novel transcription factor resulted in prominent sensitivity to sorbic acid. Concurrently with this study, War1p was identified as the major stress regulator of Pdr12p, the main ABC transporter, through a signal transduction mechanism that remains as yet unknown (Kren et al., 2003). Interestingly, deletion of *ROX3* and *SIN4*, a yeast global transcription factor and a mediator subunit respectively, resulted in notable sensitivity to sorbic acid. Nuclear transport between the nucleus and the cytoplasm occurs through the nuclear pore complex of the nuclear envelope. Loss of some nuclear pore subunits (Nup120p and Npl3p) also resulted in sorbic acid sensitivity.

3.2.4 Mutants indicating sensitivity associated with the cell cycle, cytoskeleton and DNA processing

Sorbic acid sensitivity (Table 3.1) is associated with defects in the actin organization (*Δvrp1*), as well as with defects in the spindle checkpoint and the cell cycle (*Δbub1, Δdbf2*). Loss of a cyclin-dependant protein kinase, Bur2p, which affects transcription via chromatin-mediated effects (Yao and Prelich, 2002), also resulted in sorbic acid sensitivity. Sit4p, a suppressor of Dbf2 protein kinase, exhibited apparent sorbic acid sensitivity.

3.2.5 Mutants involved in protein synthesis, cellular transport, and control of cellular organization

Several genes involved in cellular transport have been identified as sorbic acid sensitive, such as genes encoding for ribosomal proteins (Rps22ap, Rps18ap) and others involved in ER-Golgi transport (Gcs1p, Ypt6p and Rgp1p). This implies that the ribosome might be important for adaptation to sorbic acid stress. Pdr12p (Piper et al., 1998) was also identified as expected, thus validating the screening methodology used in this study.

Another group of sorbic acid sensitive genes belongs to mitochondrial metabolism and organization. Loss of components of the mitochondrial large ribosomal subunits (*MRPL*) generated sorbate sensitivity suggesting that the mitochondrial integrity is crucial for sorbic acid resistance in yeast. Notably, loss of a mitochondrial porin, Por1p also gave a clear sensitive phenotype. Another sensitive mutant was the mitochondrial glutaredoxin, Δ*grx5*, and Δ*tcp1*, a transporter that catalyzes the uptake of the essential cofactor thiamine pyrophosphate (ThPP) into mitochondria and is located in the mitochondrial membrane (Marobbio et al., 2002).

An intact cell wall seems to play an important role in sorbic acid resistance, since its main function is to preserve the osmotic integrity of the cell. Loss of five proteins required for cell wall biosynthesis and organization (Dcw1p, Smi1p, Och1p, Gon7p and Rmd7p) resulted in notable sensitivity to sorbic acid stress. Only one sensitive mutant involved in oxidative stress, Δ*sod1*, was identified.

In summary, this functional screen has revealed many genes with different cellular activities, involved in adaptation to sorbic acid stress. This demonstrates the complexity of the impact that weak acids exert on yeast cells. Although most of the identified genes are well characterized, some are of unknown function making their future study even more interesting.

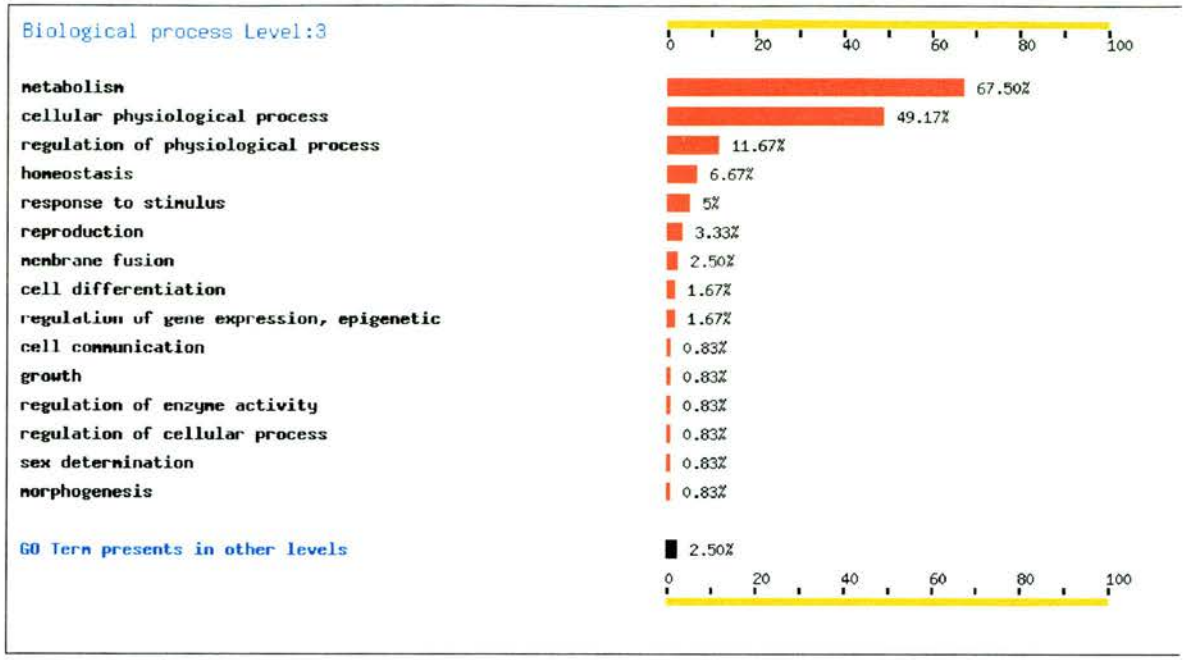
3.3 Gene Ontology (GO) of sorbic acid sensitive genes

The Gene Ontology (GO) project (http://www.geneontology.org/) is a method of gene product classification based on three fundamental properties: a) biological process, the biological objective to which a protein contributes; b) cellular component, the place in the cell where a protein is active; and c) molecular function, the biochemical activity (Hazbun et al., 2003). The Gene Ontology (GO) facilitates the exchange of information between groups studying similar processes in different model organisms (Ashburner et al., 2000). GO is classified according to different levels (level 1-3) based on the stringency of the acquired classification with level 3 being the highest and level 1 the lowest.

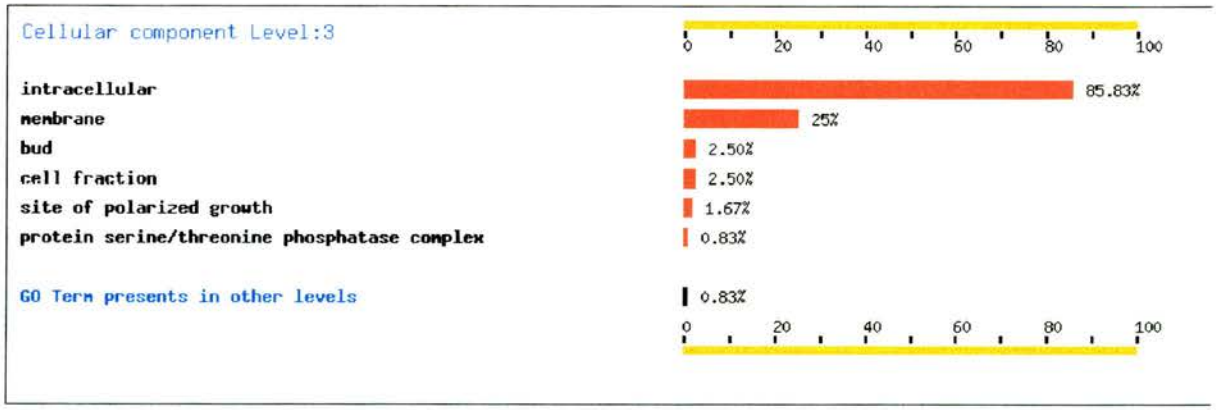
Using the yeast GO database (http://fatigo.bioinfo.cnio.es/), the functional categories of sorbic acid-sensitive gene deletions were further analyzed. Of the total of 120 genes, 105 had GO at level 3 (biological process), which resulted in the following GO profile: 67.50% metabolism, 49.17% cellular physiological process, 11.67% regulation of physiological process, 6.67% homeostasis, 5% response to stimulus, 3.33% reproduction, 2.50% membrane fusion, 1.67% cell differentiation, and 6.65% GO terms present at other levels (Figure 3.2).

Of the total 120 genes, 107 had GO at level 3, cellular component, which resulted in 85.83% intracellular, 25% membrane, 2.50% each bud and cell fraction and another 2.50% at other levels as shown in figure 3.2b. On a molecular function profile, 87/120 genes had GO at level 3 with higher scores at hydrolase (19.17%) and transferase activity (13.33%), following oxidoreductase activity (7.50%) and 6.67% kinase activity, ion transporter activity and protein binding for each. The remaining percentage was found at other levels (Figure 3.2).

A


Biological process Level:3
0
20
40
60
80
100
metabolism
67.50%
cellular physiological process
49.17%
regulation of physiological process
11.67%
homeostasis
6.67%
response to stimulus
5%
reproduction
3.33%
membrane fusion
2.50%
cell differentiation
1.67%
regulation of gene expression, epigenetic
1.67%
cell communication
0.83%
growth
0.83%
regulation of enzyme activity
0.83%
regulation of cellular process
0.83%
sex determination
0.83%
morphogenesis
0.83%
GO Term presents in other levels
2.50%
0
20
40
60
80
100

B


Cellular component Level:3
0
20
40
60
80
100
intracellular
85.83%
membrane
25%
bud
2.50%
cell fraction
2.50%
site of polarized growth
1.67%
protein serine/threonine phosphatase complex
0.83%
GO Term presents in other levels
0.83%
0
20
40
60
80
100

C

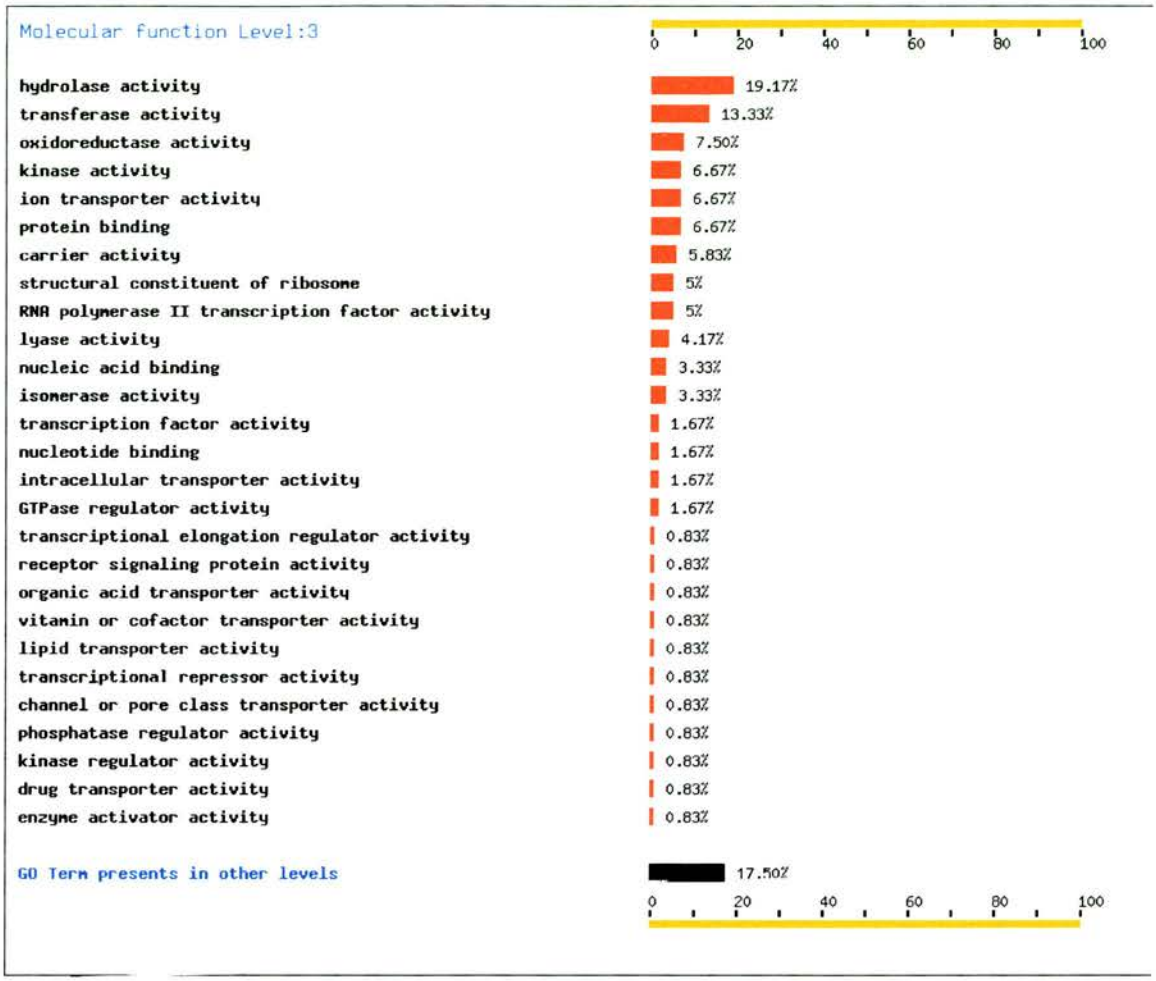


Figure 3.2 Schematic of functional categories of sorbic acid sensitive deletion mutants. Sorbate-sensitive deletion strains as illustrated in Table 3.1 and analysed by using the Gene Ontology (GO) database are classified according to: **A** biological process, **B** cellular component and **C** molecular function.

3.4 Detailed screening of protein kinases, phosphatases and transcription factors

Following the screen of the 'disruptome' (Table 3.1) we decided to undertake another, more detailed screen of deletion mutants encoding only regulatory proteins, as described in section 2.4.1. This screen consisted of 101 ORFs encoding nonessential protein kinases, 47 ORFs encoding protein phosphatases and 163 ORFs encoding transcription factors according to the MIPS protein Classes Index (http://mips.gsf.de/genre/proj/yeast/).

The growth of all regulatory deletion mutants under different sorbic acid stress conditions was assayed by spotting serial dilutions onto YEPD, pH 4.5 plates containing sorbic acid ranging from 0 mM to 2 mM (Figure 3.3). Individual deletions of genes encoding four protein kinases (Dbf2p, Vps15p, Ctk1p and Bub1p), two transcription factors (Rox3p and War1p) and one phosphatase (Reg1p) were identified as sorbic acid sensitive (Figure 3.3). Growth of the wild type BY4741a and Δ*pdr12* strains was also monitored. The latter was used as a positive control as it is well known from previous work to be hypersensitive to sorbate at low pH (Piper et al., 1998).

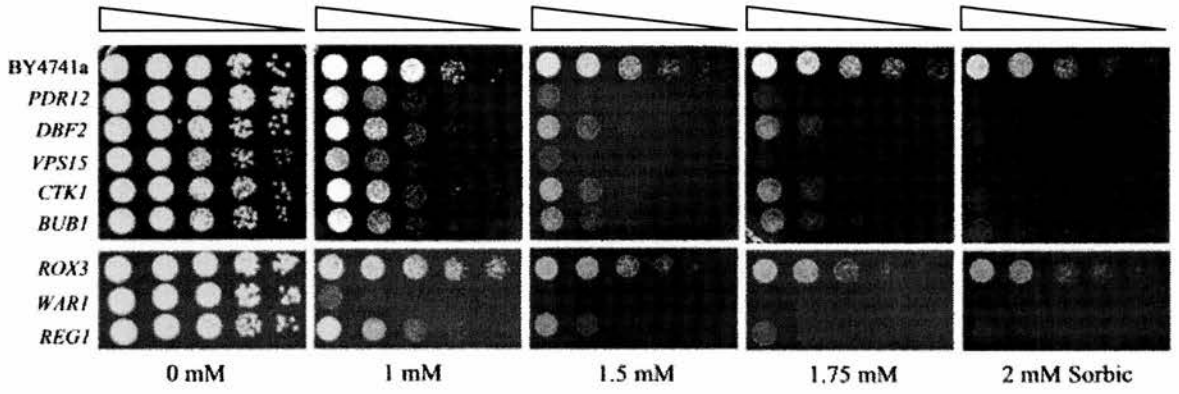


Figure 3.3 Growth of regulatory mutants under sorbic acid stress. Growth of wild type (BY4741a), deletion mutants (Δ*dbf2*, Δ*vps15*, Δ*ctk1*, Δ*bub1*, Δ*rox3*, Δ*war1* and Δ*reg1*) and Δ*pdr12* that served as a positive control. Overnight cultures were subjected to five-fold serial dilutions (indicated by triangle) and spotted on YEPD, pH 4.5 agar plates without sorbic acid or onto YEPD plates containing 1 mM, 1.5 mM, 1.75 mM and 2.5 mM sorbic acid. Plates were scanned after two days incubation at 30°C.

Further growth assays in liquid YEPD, pH 4.5 cultures were conducted to confirm the results of the spotting assay (Figure 3.3). Significant reduction of growth in sorbic acid concentrations of 1 mM was observed with two deletion mutants, Δ*war1* and Δ*dbf2*, compared to the wild type. Deletion of *WAR1*, a transcription factor, exhibited a marked hypersensitivity to 1 mM sorbic acid compared to the wild type strain (Figure 3.4 and 3.5). Deletion mutants Δ*ctk1*, Δ*bub1* and Δ*reg1* exhibited significant growth inhibition, whereas Δ*rox3* and Δ*vps15* showed slight growth inhibition relative to the BY4741a parent strain with 1 mM sorbic acid treatment (Figure 3.4). Analytical growth curves of each deletion mutant are shown in Figure 3.5. Taken together, the above results suggest that all the regulatory proteins tested are implicated in the sorbic acid resistance mechanism. It remains to be seen whether these regulatory proteins are involved in the same or different signaling pathways.

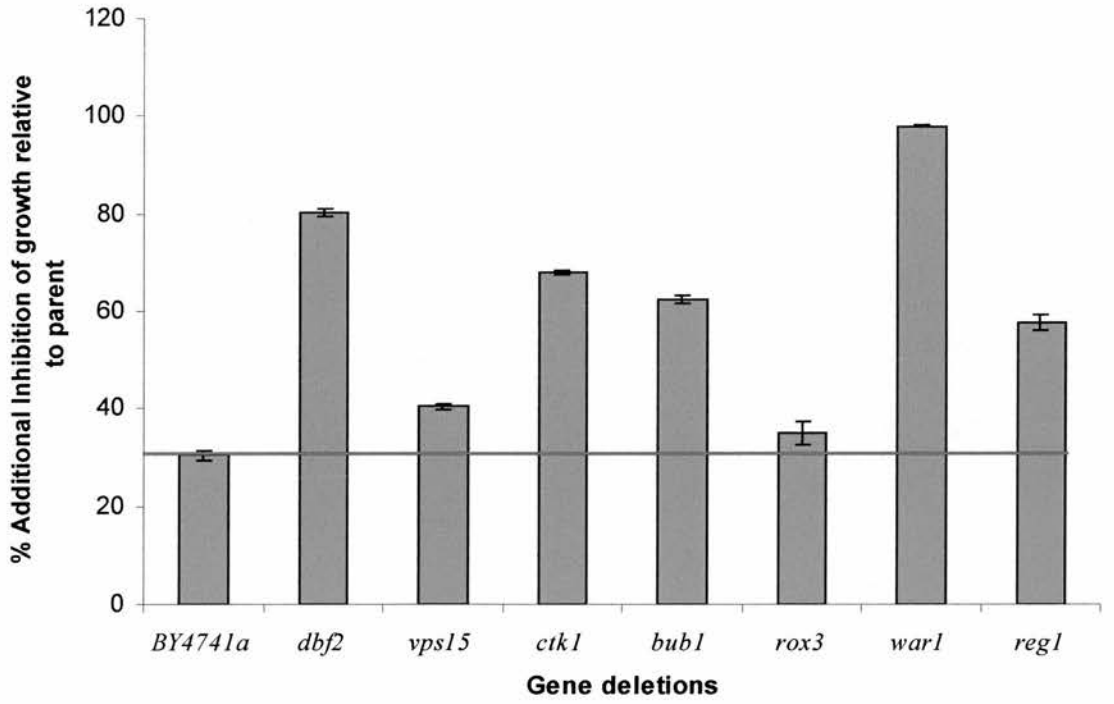


Figure 3.4 Growth inhibition by sorbic acid of wild type BY4741a strain versus single deletion mutants. A. Cultures were grown to late exponential phase in YEPD, pH 4.5 medium in the presence and absence of 1 mM sorbic acid. For each strain growth inhibition is expressed as a percent inhibition of growth rate. This was calculated by measuring the growth rate in the presence or absence of sorbic acid and expressing the additional inhibition seen in the sorbic acid-treated culture relative to the untreated culture as a percentage. Three replicate experiments were performed using a plate reader. The values are shown as mean ± standard deviation (error bars) of replicate experiments.

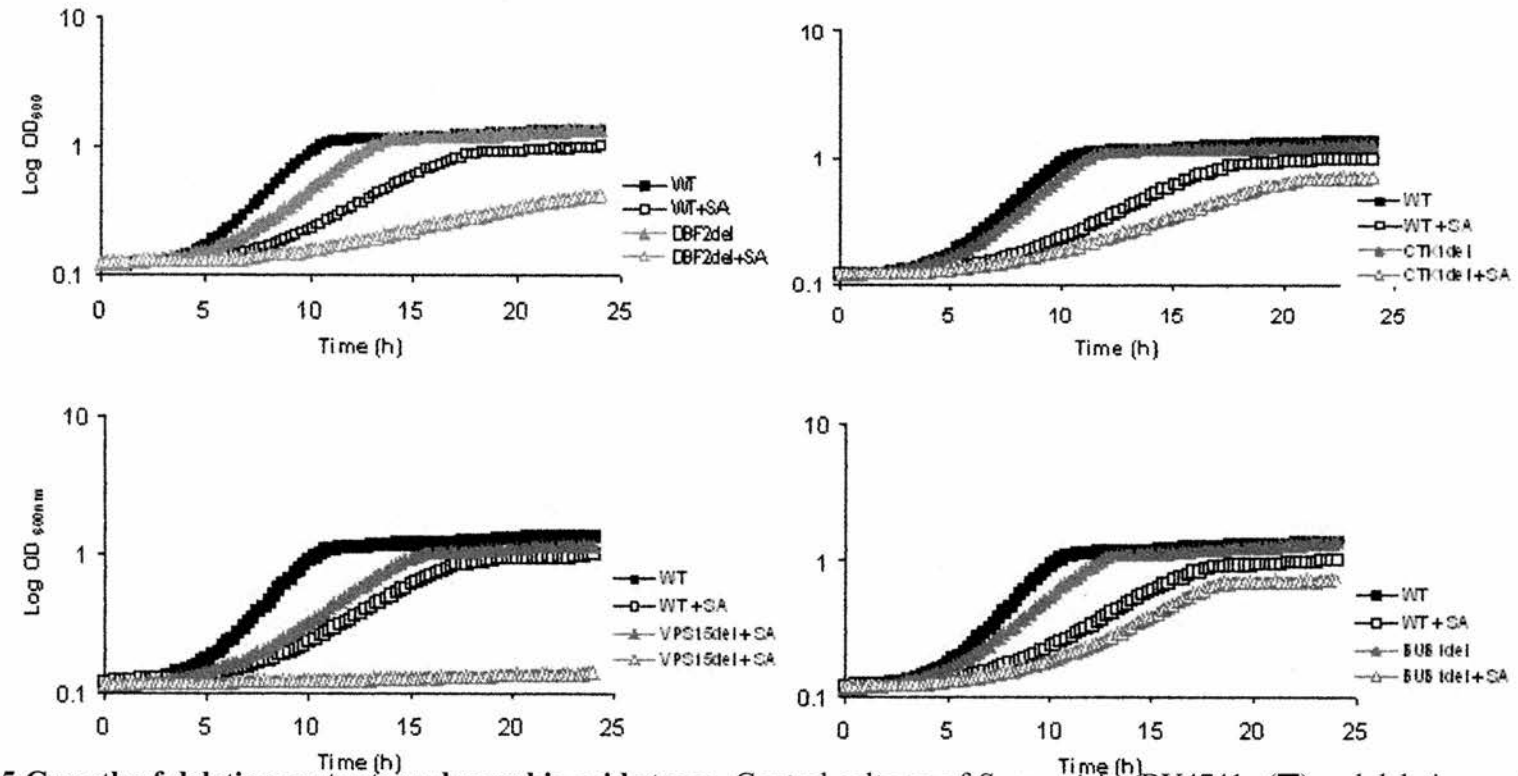


Figure 3.5 Growth of deletion mutants under sorbic acid stress. Control cultures of *S. cerevisiae* BY4741a (■) and deletion mutants (▲) were grown to late exponential phase in YEPD, pH 4.5 at 30°C. Identical cultures BY4741a (□) and deletion mutants (△) were grown to late exponential phase in the presence of 1 mM sorbic acid. Growth was performed in a PowerWave ™ x S Universal Microplate Spectrophotometer (BIO-TEK® Instruments, GmbH). Readings were taken every 30 min. Data shown is representative of three independent experiments for each deletion mutant.

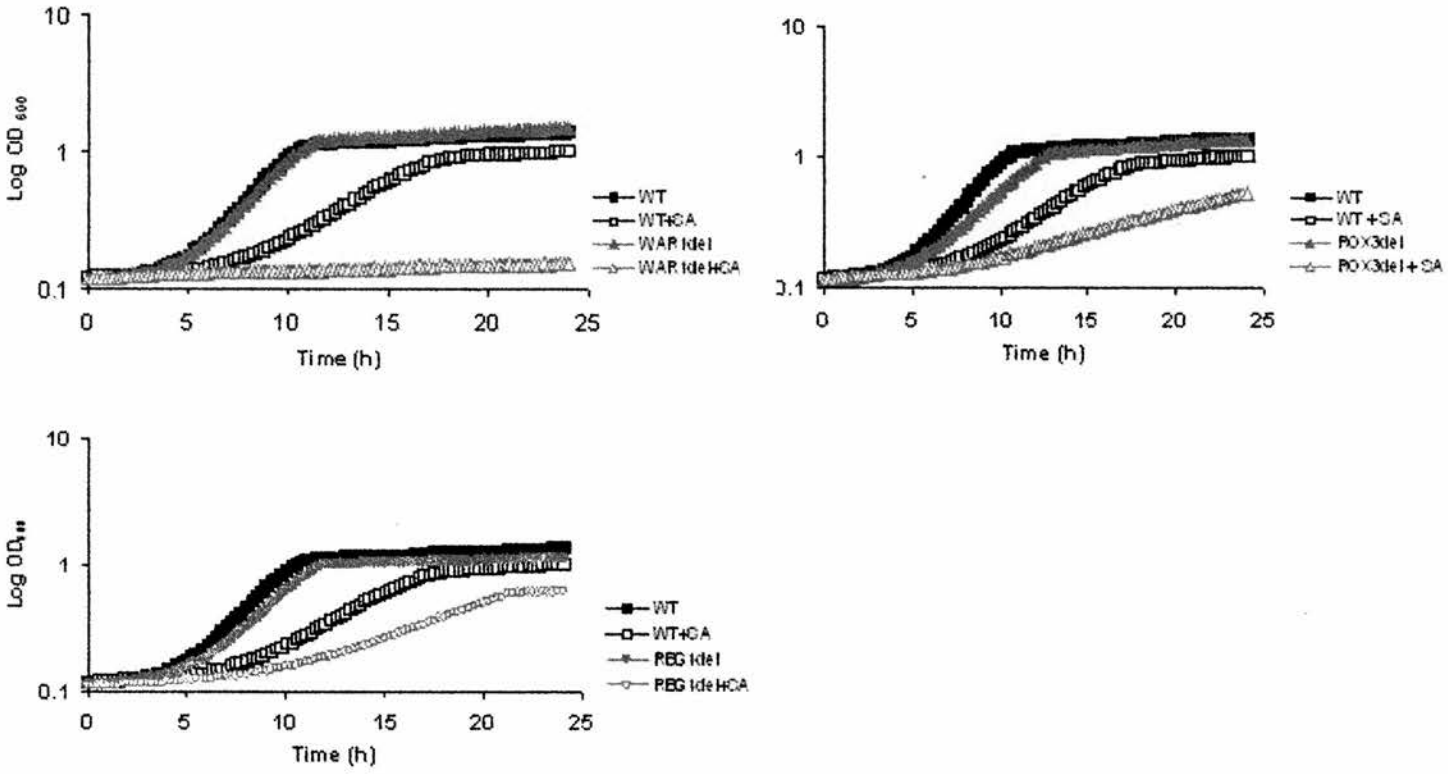
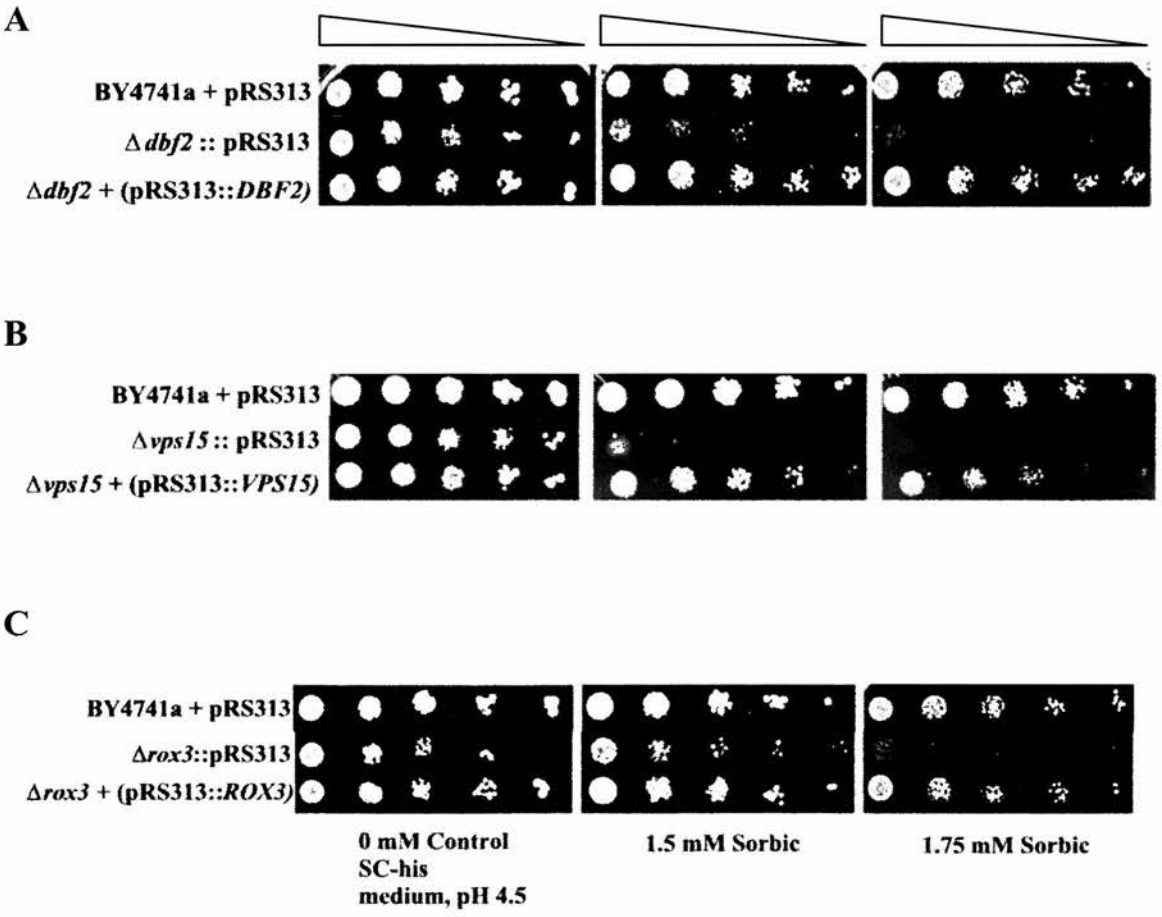


Figure 3.5 Growth of deletion mutants under sorbic acid stress. Control cultures of *S. cerevisiae* BY4741a (■) and deletion mutants (▲) were grown to late exponential phase in YEPD, pH 4.5 at 30°C. Identical cultures BY4741a (□) and deletion mutants (△) were grown to late exponential phase in the presence of 1 mM sorbic acid. Growth was performed in a PowerWave ™ x S Universal Microplate Spectrophotometer (BIO-TEK® Instruments, GmbH). Readings were taken every 30 min. Data shown is representative of three independent experiments for each deletion mutant.

3.5 Complementation studies

To confirm that the sorbic acid sensitive phenotypes observed in the deletion mutants (*Δdbf2*, *Δvps15*, *Δctk1*, *Δbub1*, *Δrox3*, *Δwar1* and *Δreg1*) were specifically caused by the disruption of the corresponding genes, complementation studies were performed. *WAR1* complementation was shown in a concurrent study (Kren et al., 2003). Initially the above genes were amplified and cloned into the pGEM-Teasy vector (Promega) for quick and easy further amplification (section 2.5.1). Genes were then cloned into the shuttle vector pRS313 (Sikorski and Hieter, 1989), transformed back into the deletion strain and tested on selective synthetic complete medium (SC-his, pH 4.5) in the presence and absence of sorbic acid. All genes were successfully complemented (Figure 3.6).



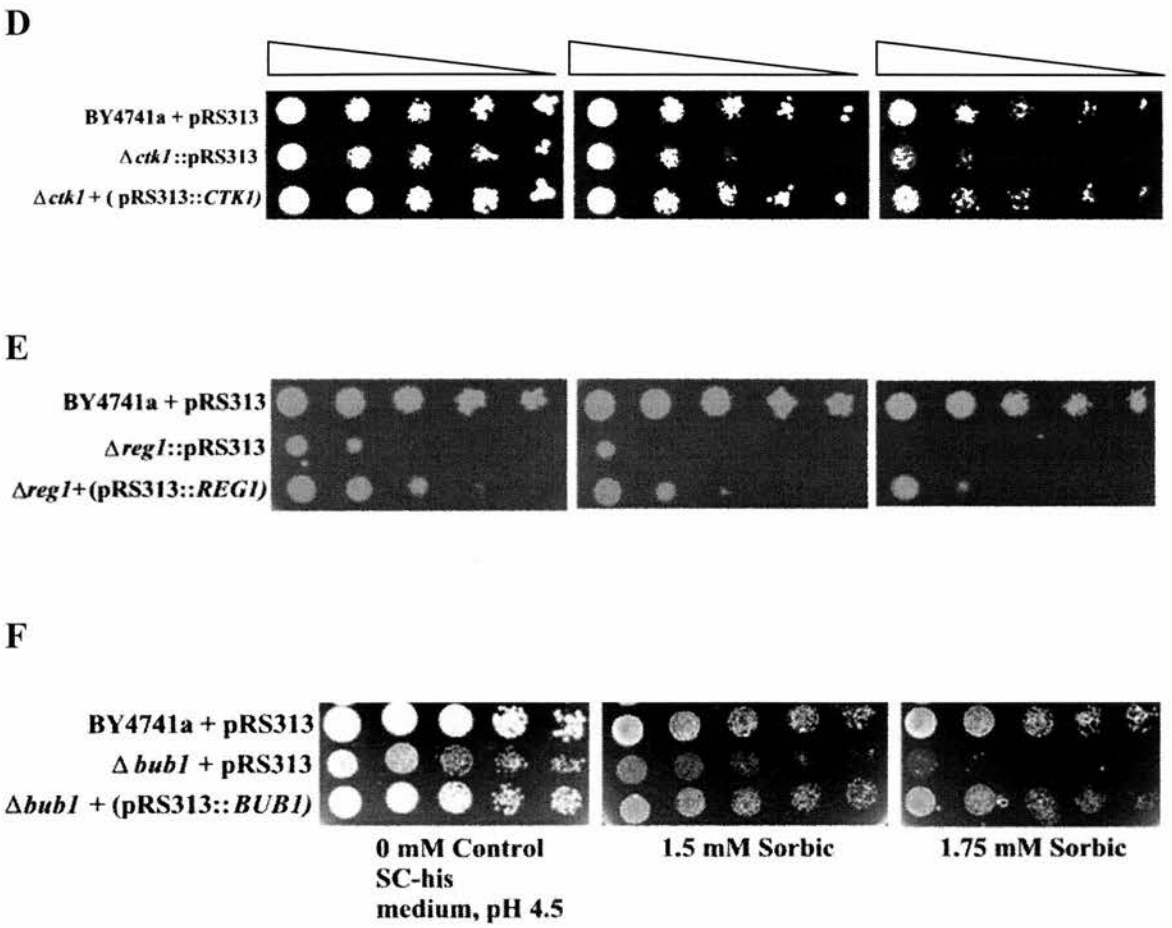


Figure 3.6 Complementation studies of sorbic acid-sensitive deletion mutants. For each deletion mutant strain (Δ*x*), deletion mutant carrying complementary gene (X) on plasmid (pRS313::*X*) was spotted against deletion strain carrying empty vector (Δ*x*::pRS313) and control wild type BY4741a strain also carrying empty vector (BY4741a + pRS313). Complementation of all genes (A: *DBF2*, B: *VPS15*, C: *ROX3*, D: *CTK1*, E: *REG1* and F: *BUB1*) is shown on SC-his medium at pH 4.5 in the presence of sorbic acid. Plates were imaged after two days incubation at 30°C. A representative result is shown.

3.6 Other stress factors

To investigate if any of the seven sensitive deletion mutants encoding regulatory proteins were involved in other stress responses a range of other stresses were tested. Thus, other weak organic acids (benzoic, acetic acid), organic alcohols (ethanol), high osmolarity (1.5 mM NaCl), oxidative stress (H_2O_2), and heat shock were tested both on YEPD, pH 4.5 agar and in liquid cultures, as previously described (section 2.4.1).

3.6.1 Benzoic acid

The growth inhibitory spectrum of benzoic acid, another food preservative although similar to that of sorbate (Lambert and Stratford, 1999; Piper et al., 1998) is quite distinct in the following mutants. Increasing concentrations of this food preservative, gave a marked hypersensitive phenotype in both Δ*pdr12* and Δ*war1* cells (Figure 3.7). Only a slight inhibitory effect on Δ*vps15* at concentrations higher than 2 mM was observed. No discernible sensitivity to benzoic acid was evident in the Δ*dbf2*, Δ*ctk1*, Δ*bub1*, Δ*rox3* and Δ*reg1* mutants compared to the BY4741a parent strain (Figure 3.7).

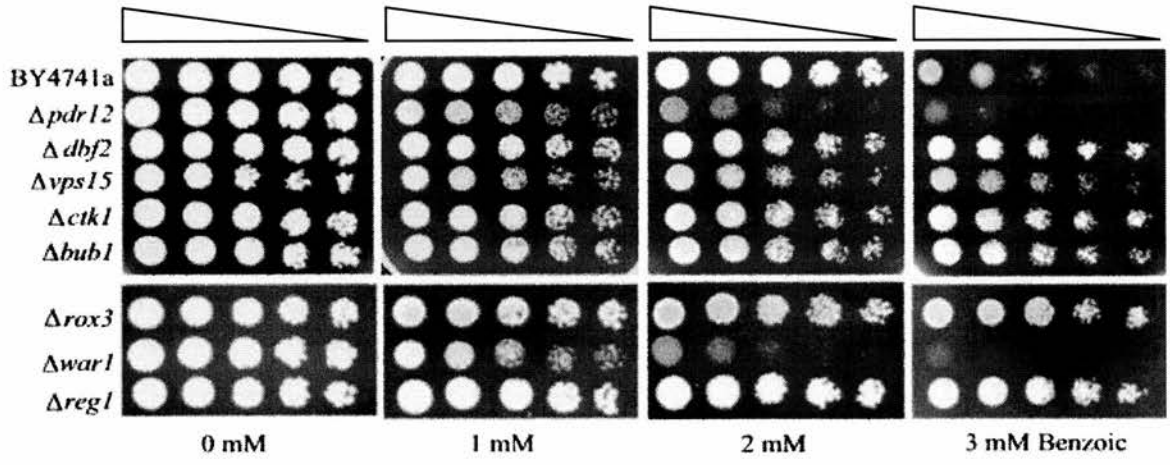


Figure 3.7 Growth under benzoic acid. Wild type (BY4741a), deletion mutants (Δ*dbf2*, Δ*vps15*, Δ*ctk1*, Δ*bub1*, Δ*rox3*, Δ*war1*, Δ*reg1*) and Δ*pdr12* (positive control) were subjected to five-fold serial dilutions (denoted by triangles) and spotted on YEPD, pH 4.5 containing 1, 2, and 3 mM benzoic acid. Plates were imaged after two days incubation at 30 °C. A representative result is shown.

3.6.2 Acetic acid

Another weak acid stress tested was acetic acid. At concentrations of 50 mM or higher at pH 4.5 there was almost complete growth inhibition of the Δ*vps15* mutant. At 75 mM concentration, acetic acid abolished growth in Δ*dbf2* and Δ*reg1* deletion mutants. Non-discernable growth inhibition of the Δ*ctk1* mutant was observed at 75 mM. Δ*bub1*, Δ*rox3* and Δ*war1* deletion mutants showed no sensitivity to acetic acid (Figure 3.8). Only mutants with a notable growth inhibition were further tested in liquid cultures. Surprisingly, no difference in growth between the Δ*dbf2* deletion mutant and the wild type strain was observed when strains were grown in liquid cultures (Figure 3.9).

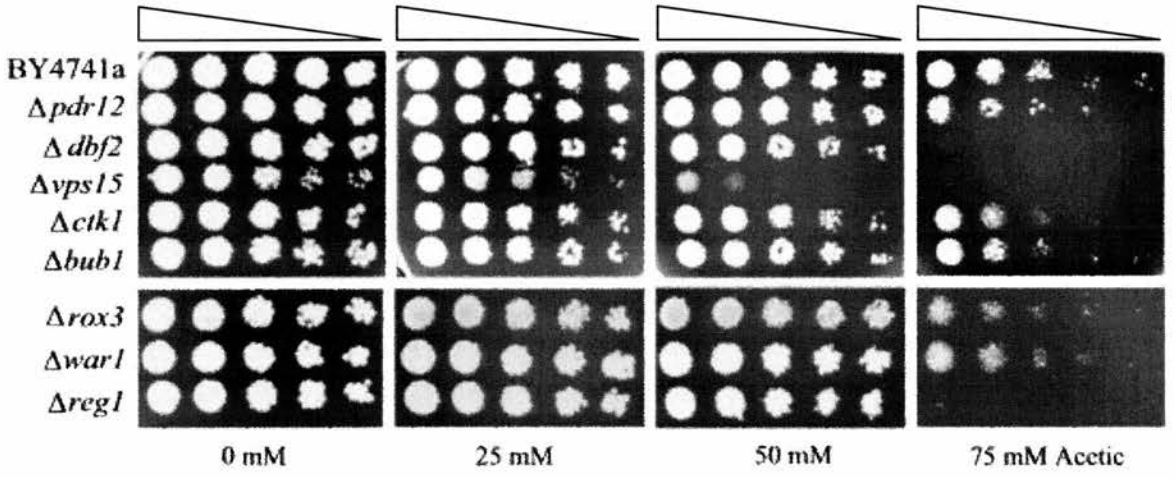


Figure 3.8 Growth under acetic acid. Wild type (BY4741a), deletion mutants (Δ*dbf2*, Δ*vps15*, Δ*ctk1*, Δ*bub1*, Δrox3, Δ*war1* and Δ*reg1*) and Δ*pdr12* (positive control) were subjected to five-fold serial dilutions (denoted by triangles) and spotted on YEPD, pH 4.5 plates containing 25, 50 and 75mM acetic acid. Plates were imaged after two days incubation at 30 °C. A representative result is shown.

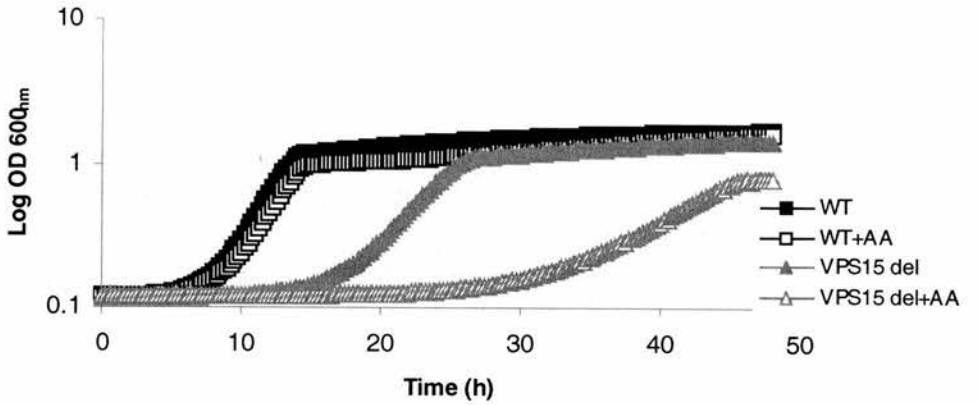


Figure 3.9 Growth under acetic acid. Wild type BY4741a (WT) and Δ*vps15* deletion mutant were grown in YEPD, pH 4.5 containing 50 mM acetic acid (AA) in a PowerWave ™ x S Universal Microplate Spectrophotometer (BIO-TEK® Instruments, GmbH). Readings were taken every 30 min. Plates were imaged after two days incubation at 30 °C. A representative result is shown.

3.6.3 Hydrogen peroxide stress

The mutants were also subjected to peroxide stress. Loss of the *VPS15* gene resulted in slight growth inhibition at 3 mM concentration of peroxide and a stronger inhibition at 4 mM concentration compared to the parent strain. Growth of Δ*rox3*, Δ*war1* and Δ*reg1* mutants was completely inhibited at 5 mM peroxide. Yet, at this concentration growth of the parent strain was also severely impaired (Figure 3.10). Both Δ*vps15* and Δ*reg1* deletion mutants were tested in liquid cultures. Consistent with the spotting assay data, growth of Δ*vps15* following incubation with 3 mM hydrogen peroxide was completely inhibited (Figure 3.11).

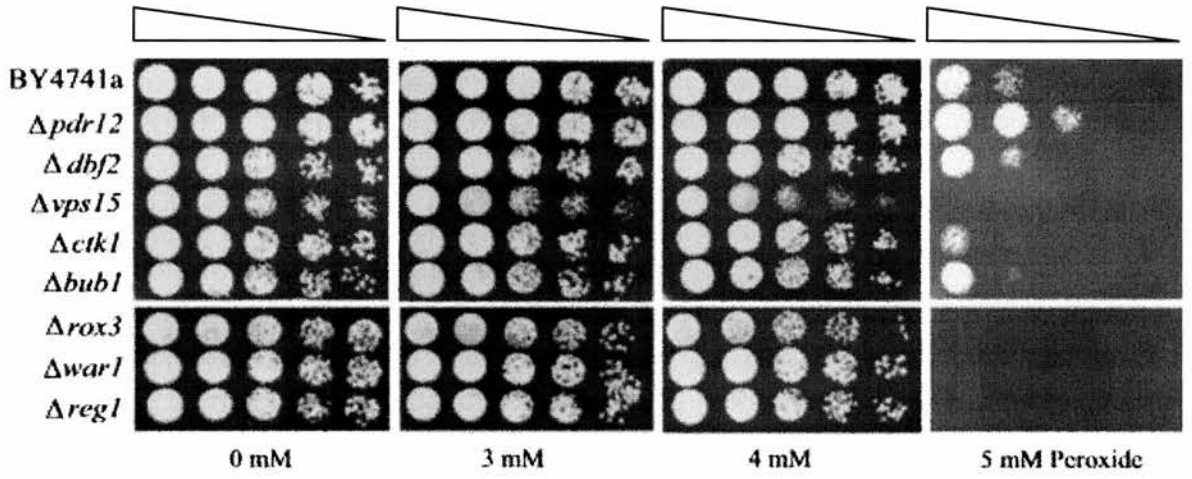


Figure 3.10 Growth under hydrogen peroxide. Wild type (BY4741a), deletion mutants (Δ*dbf2*, Δ*vps15*, Δ*ctk1*, Δ*bub1*, Δrox3, Δ*war1* and Δ*reg1*) and Δ*pdr12* (positive control) were subjected to five-fold serial dilutions (denoted by triangles) and spotted on YEPD, pH 4.5 plates containing 3, 4 and 5 mM peroxide. Plates were imaged after two days incubation at 30 °C. A representative result is shown.

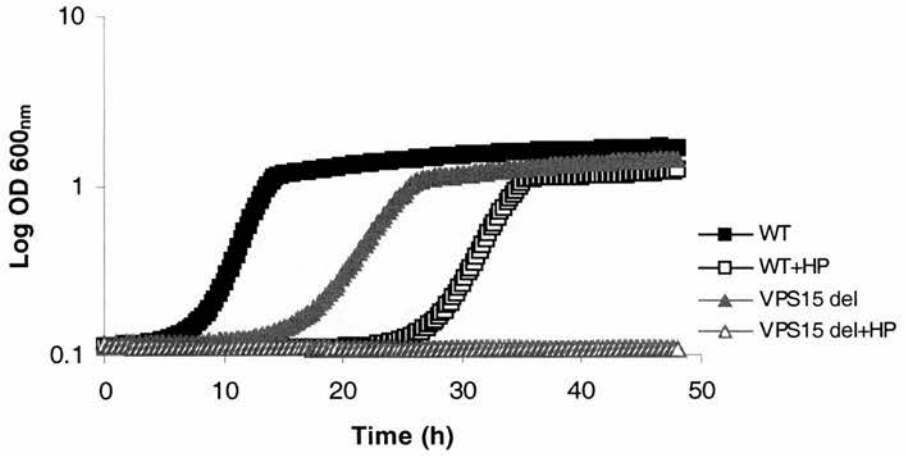


Figure 3.11 Growth under Hydrogen peroxide Wild type BY4741a (WT) and Δ*vps15* deletion mutant were grown in YEPD, pH 4.5 containing 4 mM hydrogen peroxide in a PowerWave ™ x S Universal Microplate Spectrophotometer (BIO-TEK® Instruments, GmbH). Readings were taken every 30 min. Plates were imaged after two days incubation at 30 °C. A representative result is shown

3.6.4 Ethanol stress

The effect of a range of ethanol concentrations on the seven deletion mutants was analyzed both by spotting on YEPD, pH 4.5 plates and by growth curves. Deletions of *DBF2* and *VPS15* genes exhibited notable growth inhibition at concentrations higher than 4 % ethanol. Especially loss of *VPS15* gene exhibited hypersensitivity to ethanol even at the lowest concentrations. Δ*bub1*, Δrox3 and Δ*reg1* showed also sensitivity to the stress factor (Figure 3.12).

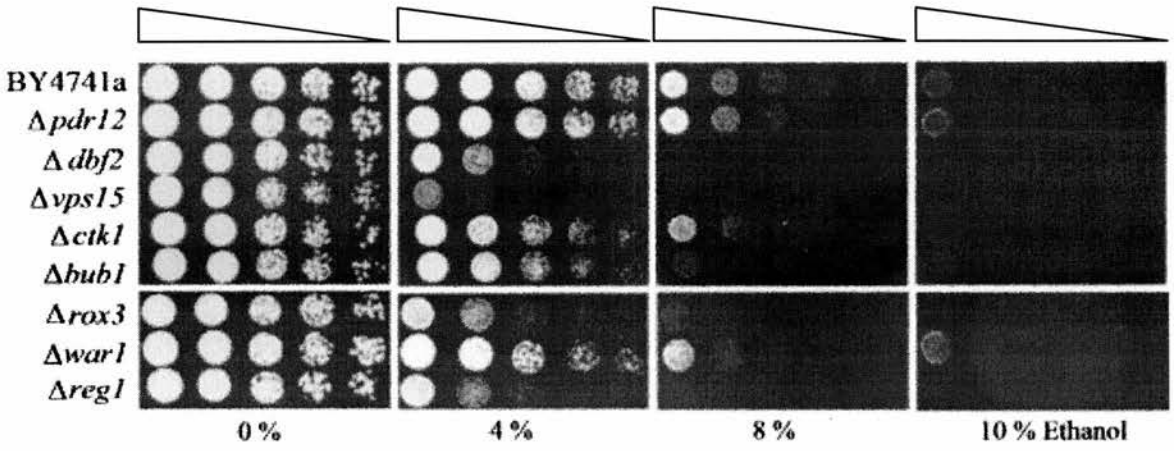


Figure 3.12 Growth under ethanol stress. Wild type (BY4741a), deletion mutants (Δ*dbf2*, Δ*vps15*, Δ*ctk1*, Δ*bub1*, Δrox3, Δ*war1* and Δ*reg1*) and Δ*pdr12* (positive control) were subjected to five-fold serial dilutions (denoted by triangles) and spotted on YEPD, pH 4.5 plates containing 4, 8 and 10 % ethanol. Plates were imaged after two days incubation at 30 °C. A representative result is shown.

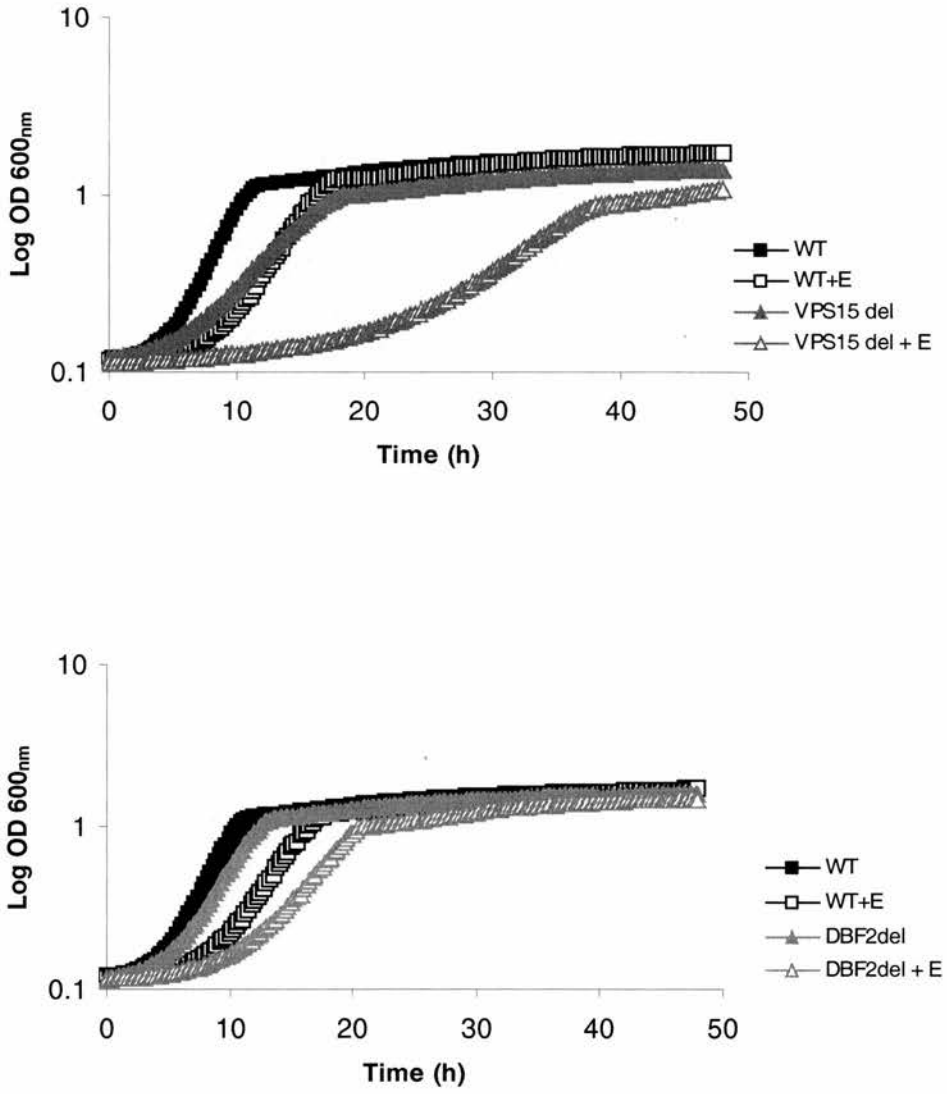


Figure 3.13 Growth under ethanol stress. Wild type BY4741a (WT) and deletion mutants (*Δdbf2*, *Δvps15*) were in YEPD, pH 4.5 containing 8 % ethanol (E) in a PowerWave ™ x S Universal Microplate Spectrophotometer (BIO-TEK® Instruments, GmbH). Readings were taken every 30 min. Plates were imaged after two days incubation at 30 °C. A representative result is shown.

3.6.5 Salt stress

Growth of the seven sorbic acid sensitive deletion mutants was also tested under osmotic stress conditions. The Δ*vps15* mutant failed to grow when challenged even with low concentrations of salt, whereas growth of Δ*dbf2* and Δ*bub1* mutants at salt concentrations higher than 0.5 M was severely inhibited (Figure 3.14). The Δ*rox3*, Δ*war1* and Δ*reg1* mutants did not show any growth inhibition under all concentrations of NaCl tested. (Figure 3.11A).

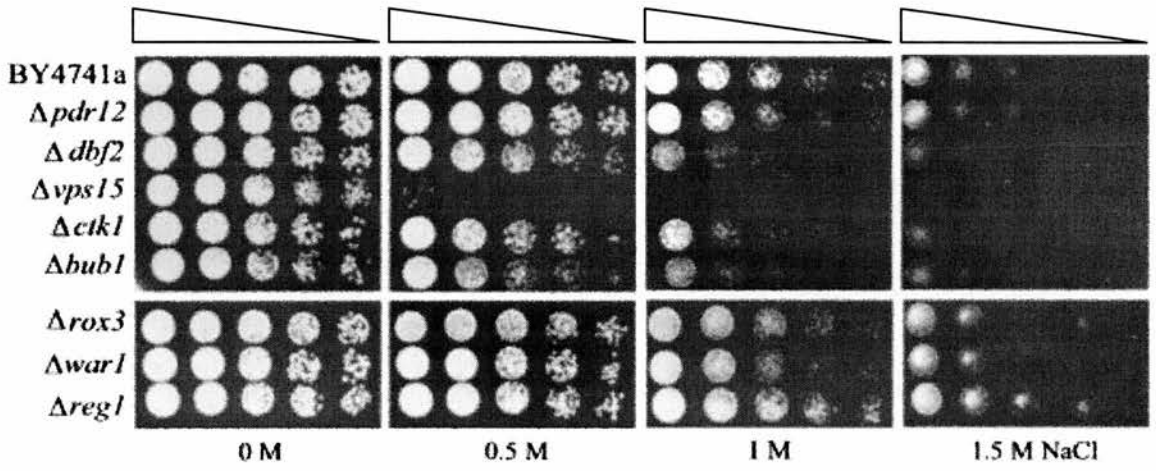


Figure 3.14 Growth under osmotic stress. Wild type (BY4741a), deletion mutants (Δ*dbf2*, Δ*vps15*, Δ*ctk1*, Δ*bub1*, Δrox3, Δ*war1* and Δ*reg1*) and Δ*pdr12* (positive control) were subjected to 5-fold serial dilutions (denoted by triangles) and spotted on YEPD, pH 4.5 plates containing 0.5, 1 and 1.5 M NaCl. Plates were imaged after 2 days incubation at 30 °C. A representative result is shown.

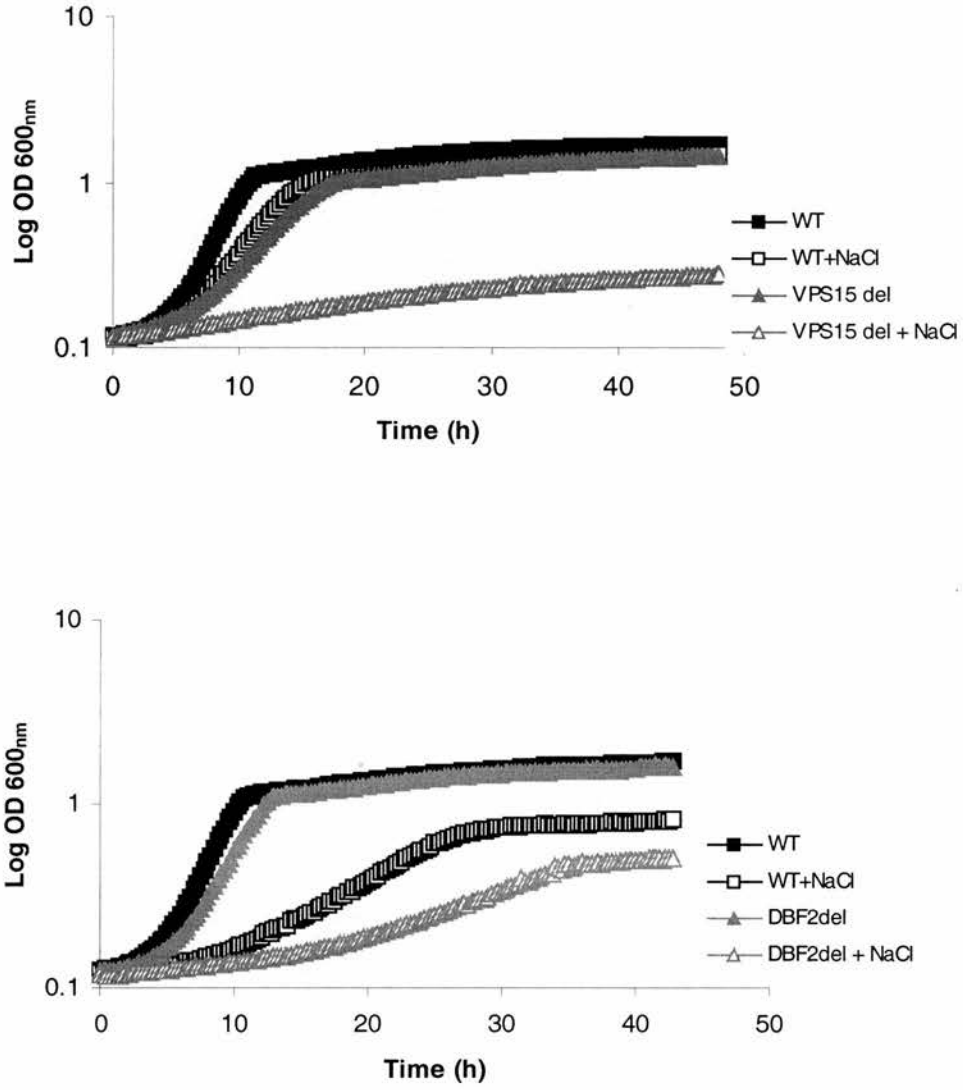


Figure 3.15 Growth under osmotic stress. Wild type BY4741a (WT) and deletion mutants (*Δdbf2*, *Δvps15)* were grown in YEPD, pH 4.5 containing 1 M NaCl in a PowerWave ™ x S Universal Microplate Spectrophotometer (BIO-TEK® Instruments, GmbH). Readings were taken every 30 min. A representative result is shown.

3.6.6 High temperature growth

Finally all deletion mutants were exposed to heat shock by shifting incubation temperatures from 30 °C to 37 °C or 40 °C. At 37°C growth of *Δvps15* was markedly inhibited. Growth at 40°C was totally impaired in *Δvps15*, Δ*dbf2* and Δ*rox3* mutants (Figure 3.16). The growth of Δ*ctk1* and Δ*war1* mutants was not inhibited upon exposure to heat stress. Whereas Δ*bub1* and Δ*reg1* displayed slightly slower growth at 40 °C compared to the parent strain. These data are consistent with those obtained from growth assays in liquid cultures at 37 °C (Figure 3.17). No growth was observed in liquid cultures at 40 °C.

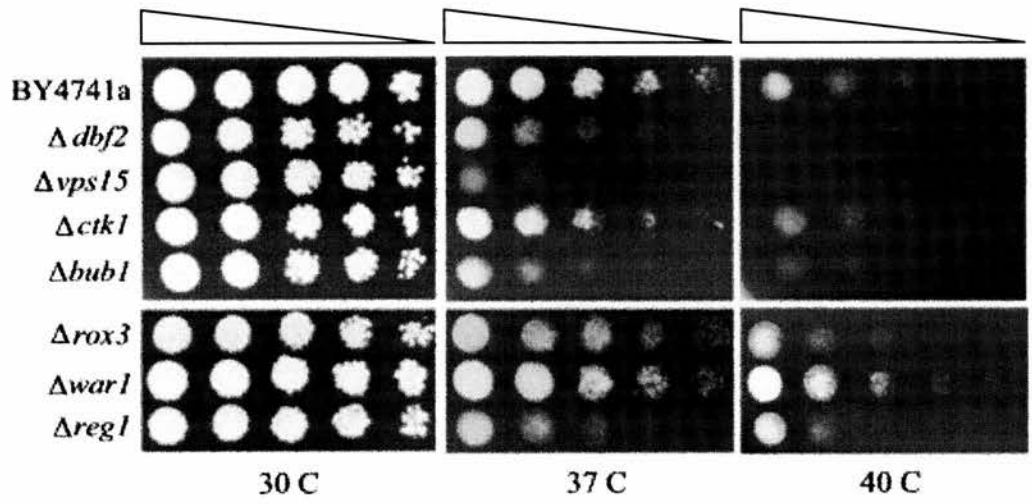


Figure 3.16 Growth under heat shock stress. Wild type (BY4741a) and deletion mutants (Δ*dbf2*, Δ*vps15*, Δ*ctk1*, Δ*bub1*, Δrox3, Δ*war1* and Δ*reg1*) were subjected to five-fold serial dilutions (denoted by triangles) and spotted on YEPD, pH 4.5 agar. Following incubation at 30 °C, 37 °C, and 40 °C for one day, plates were imaged. A representative result is shown.

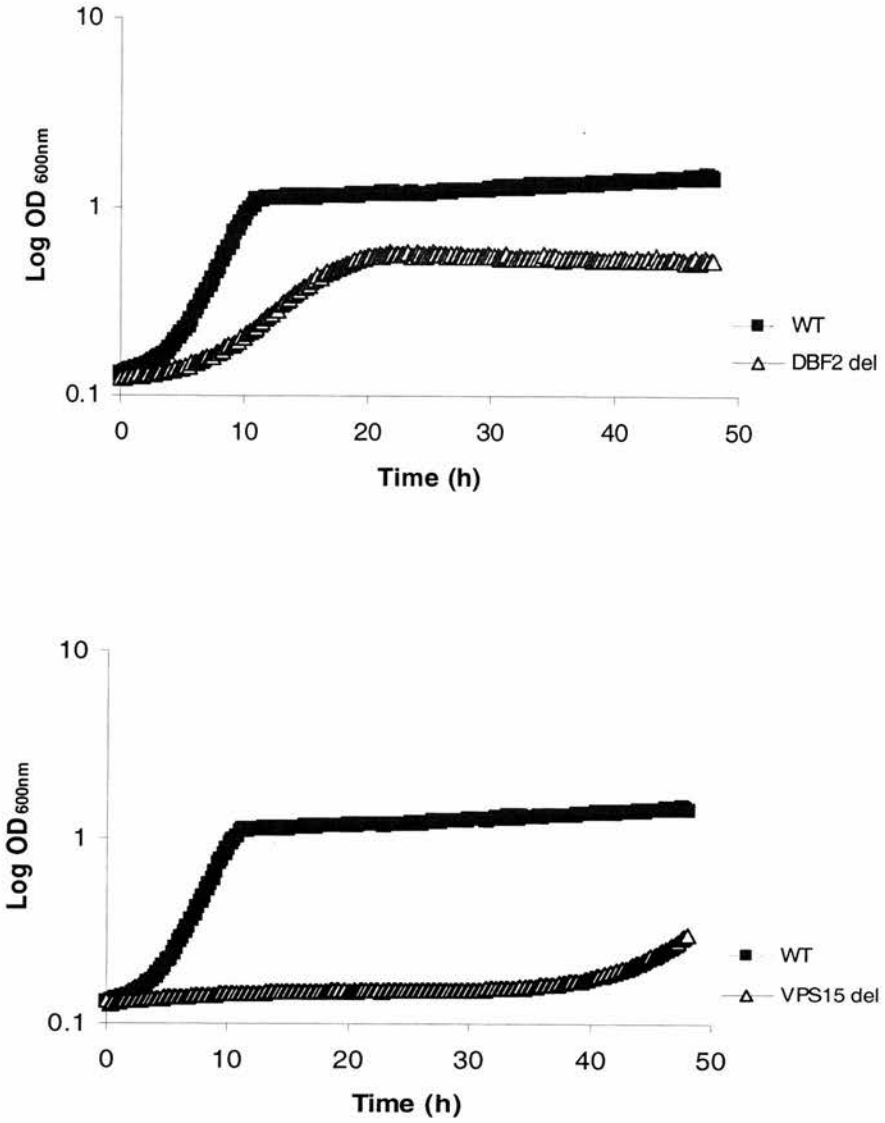


Figure 3.17 Growth under heat stress. Wild type BY4741a (WT) and deletion mutants (*Δdbf2, Δvps15)* were grown in YEPD, pH 4.5 at 37 °C in a PowerWave ™ x S Universal Microplate Spectrophotometer (BIO-TEK® Instruments, GmbH). Readings were taken every 30 min. A representative result is shown.

The overall results representing the effect of different stress factors on the aforementioned genes are listed in the following table (Table 3.2).

Table 3.2 Effect of different stress factors on the sorbic acid sensitive genes encoding regulatory proteins

	Sorbic	Benzoic	Acetic	Peroxide	Ethanol	Salt	Heat
DBF2	+	-	+	-	-	+	+
VPS15 *	+	+	+	+	+	+	+
CTK1	+	-	-	-	-	+	-
BUB1	+	-	-	-	+	+	+
ROX3	+	-	-	+	+	-	+
WAR1	+	+	-	-	-	-	-
REG1	+	+	+	+	+	-	-

+ sensitive, - not sensitive
* slow growing phenotype

3.7 Bioinformatic analysis of sorbate-sensitive regulatory proteins

In order to determine whether the sorbate-sensitive regulatory proteins shared a common signaling pathway a bioinformatic analysis was performed. To investigate if the sorbate-sensitive regulatory proteins (Dbf2p, Vps15p, Ctk1p, Bub1p, Rox3p, War1p and Reg1p) were sharing common interacting partners, a database was created containing all the published interacting proteins obtained from the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/) and the GRID Database (http://biodata.mshri.on.ca/grid/servlet/Index). Interacting proteins were originally identified via Yeast Two-Hybrid (Hazbun et al., 2003; Ito et al., 2001; Uetz et al., 2000), affinity precipitation (Gavin et al., 2002; Ho et al., 2002) and genetic interactions (Tong et al., 2004). All gene names were standardized against the ORF names to avoid duplications. The new database was screened for all first level interacting partners between the aforementioned regulatory proteins (shown in green in Figure 3.18). In the case that no interacting partners were found, second level of interacting partners was screened (*ie*

War1p). The analysis showed that the proteins were not directly linked to one another, although all proteins could be linked via intermediary partners (Figure 3.18).

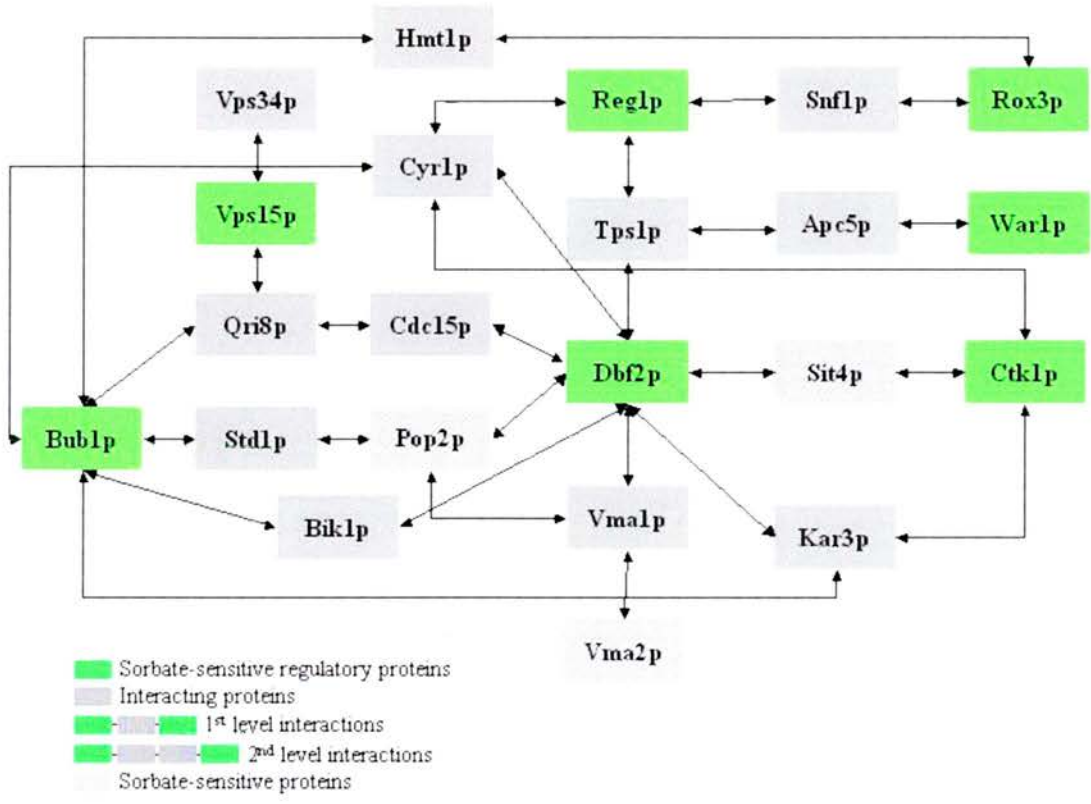


Figure 3.18 Interaction network of sorbate-sensitive regulatory proteins. The tested sorbate-sensitive regulatory proteins (shown in green boxes) and their first level interacting partners (shown in gray boxes) by bioinformatic analysis. Boxes shaded gray are the sorbate-sensitive proteins detected via the screen (Table 3.1).

3.8 Discussion

The genome-wide phenotypic screen of the deletion collection of *S. cerevisiae* BY4741a strain revealed many genes with different physiological roles and metabolic functions implicated in sorbic acid stress recovery. The number of sorbate-sensitive genes appeared to be proportional to the amount of sorbic acid used for the phenotypic selection. In this study, from the 4827 deletion mutants screened, 119 were identified as sorbate-sensitive upon exposure to 1.5 mM sorbic acid at pH 4.5. The screen of the viable deletion set did not result in sorbic acid resistant phenotypes. In contrast, in a concurrent study, where 1 mM sorbic acid was used, 86 out of the 4827 strains tested, reproducibly showed sorbate sensitivity (Schuller et al., 2004). Meanwhile, in a similar screen where 2 mM sorbate was used 237 strains were sorbate-sensitive (Mollapour et al., 2004). Only 15 mutants were found in all three independent screens, whereas there was a greater overlap between this study and the Mollapour study (48%) than between the Schuller study (18.5%) (Figure 3.19). However, Mollapour and coworkers identified 34 deletion strains that were more sorbate-resistant than the parent strain (Mollapour et al., 2004). Of these, some represented genes of unknown function while others were classified as genes that take part in the cell wall organization, ribosome biogenesis, vacuole function, transcription, cell cycle and response to endoplasmic reticulum (ER) stress.

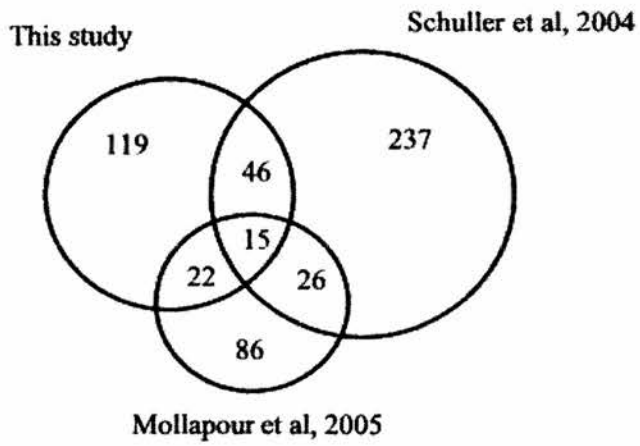


Figure 3.19 Overlap among the results of three independent sorbic acid sensitivity screens. The Venn diagram indicates the overlap among the data of this study and those obtained by the two independent screens (Schuller et al, 2004; Mollapour et al, 2005)

The ATP-binding cassette (ABC) transporter Pdr12p, which is known to confer the highest sorbic acid resistance, was also identified in this study. Previous studies have shown that Pdr12p is essential for growth in the presence of weak organic acids. Loss of this anion efflux pump results in hypersensitivity to water-soluble monocarboxylic acids with chain lengths from C_1 to C_7 (Holyoak et al., 1999; Piper et al., 1998). Concurrently with Kren and coworkers we also identified the *Δwar1* mutant as sorbate-hypersensitive and further studies have characterized War1p as a nuclear Zn_2Cys_6 zinc finger transcription factor and the only mediator of Pdr12p stress induction (Kren et al., 2003). Although further studies demonstrated that War1p is the major, if not the only stress regulator of Pdr12p, the actual signal transduction mechanism that activates War1p remains as yet unknown.

An unexpected finding from the screen was the identification of many deletion mutants from the aromatic amino acid synthesis pathway (e.g., *Δaro1*, *Δaro2*, *Δaro4*, *Δaro7*, *Δtrp1*, *Δtrp2* and *Δtrp3*). However, previous studies have shown that none of these were required for, or implicated in *PDR12* stress regulation (Schuller et al., 2004). Recently, Bauer and coworkers tested the aforementioned deletion mutants for acetate and sorbate sensitivity at pH 4.5 and discovered that defects in several of the enzymes of aromatic amino acid biosynthesis hypersensitized yeast to weak organic acid stress (Bauer et al., 2003). Further experiments showed that weak acid stress inhibited uptake of aromatic amino acids from the medium. This allowed the auxotrophic requirement for these amino acids to strongly influence the resistance phenotypes of mutant strains. Therefore, inability to synthesize tryptophan increased sorbic acid sensitivity (Schuller et al., 2004). Because these mutants show normal sorbate-sensing and signaling as judged by induction of Pdr12p, the effect is most likely caused by reduced tryptophan uptake (Bauer et al., 2003) through mechanisms that are unknown at present.

Strikingly, 29 of the 119 sorbate-sensitive mutants were defective in gene functions that are involved directly or indirectly with organelle protein sorting or processing (Table 3.1) These included genes important for Golgi complex or ER functions (e.g., *SSO2, VPS52, VPS53, RGP1, YPT6, GCS1*) as well as several *VPS* genes that are involved specifically

with vacuolar protein sorting or biogenesis (*PEP12*, *VPS15*, *VPS3*, *VPS33*, *VPS34, VPS36*). The yeast vacuole, equivalent to the mammalian lysosome, is the major site of macromolecular turnover (Kihara et al., 2001) and although *vps* mutants are generally characterized by increased stress sensitivity (Shimoni, 2002), many of them confered sorbic acid resistance, thus suggesting a new mechanism for the acidified vacuole in sorbic acid stress regulation. In accordance with this finding, in a recent deletion screen, novel pathways of vacuole priming and docking were discovered, which include ergosterol biosynthesis and phosphatidylinositol (4,5)-biphosphate turnover (Mayer, 2000; Seeley et al., 2002). Sorbate sensitivity from the present screen was associated with defects in phosphoinositide metabolism (*Δarg82*, *Δsac1*, *Δvps34*). In *S. cerevisiae*, the synthesis of inositol pyrophosphates is essential for vacuole biogenesis and the cell's response to certain environmental stresses. The kinase activity of Arg82p is required for the production of soluble inositol phosphates (El Alami et al., 2003). The phosphatase SacIp is required for normal vacuole morphology (Audhya et al., 2000), while Vps34 PtdIns 3-kinase activity is required for CPY pathway trafficking between the Golgi apparatus and vacuole (Stack et al., 1995) as well as functions in macroautophagy (Kiel et al., 1999; Kihara et al., 2001). Therefore all these linked functions are disrupted under sorbic acid stress.

Many genes belonging to the ergosterol biosynthesis pathway (*ERG2, ERG3, ERG4, ERG6*) were also identified as sorbate sensitive. Suggesting that perturbation of membrane ergosterol can lead to dramatic sorbic acid sensitivity. Interestingly, lipid metabolism is also important in vacuole fusion (Kato and Wickner, 2001; Seeley et al., 2002). Striking vacuole fragmentation was observed in several *Δerg* strains, and this was confirmed biochemically by use of specific inhibitors (Kato and Wickner, 2001).

Subunits of the vacuolar-translocating H^+-ATPase resulted in sorbate-sensitive phenotypes in this screen, further suggesting a role of the acidified vacuole in sorbic acid stress regulation. The subunits belonged to both the catalytic V_1 subcomplex (Vma2p, Vma5p, Vma8p, Vma13p) and the proton-translocating V_o subcomplex (Cup5p, Ppa1p) or proteins involved in its assembly (Vma22p) (Graham et al., 2000). A functional vacuolar H^+-

ATPase could be essential to rapidly and efficiently counteract the acidification of the cytosol in a similar way that the PM-ATPase counteracts the acidification of the cytosol after induction by lipophilic acids (Piper et al., 2001; Viegas et al., 1998; Viegas and Sa-Correia, 1991). In previous studies the vacuolar H^+-ATPases have been described as nature's most versatile proton pumps (Nishi and Forgac, 2002). They function not only to acidify a wide array of intracellular compartments in eukaryotic cells, but also to pump protons across the cell membrane. Full integrity of vacuolar ATPase appears to be required for efficient carboxypeptidase pathway (CPY) sorting, the main pathway for the delivery of protein to the vacuole (Bonangelino et al., 2002; Burd et al., 1998; Conibear and Stevens, 1998). Thus, sorbic acid regulation could be achieved through concurring genes involved in vacuolar protein sorting and the vacuolar pump.

Mitochondrial components also gave a sorbate-sensitive phenotype, suggesting that the mitochondrial integrity is crucial for sorbic acid resistance in yeast. In aerobic *S. cerevisiae* a major cause of weak acid sensitivity is elevated superoxide production by the mitochondrial respiratory chain (Morradas Ferreira et al, 2000). So, even though the loss of an assembled respiratory chain will eliminate the major source of free radical production, respiratory-deficient mutants (petites) are hypersensitive to oxidative stress (Morradas Ferreira et al, 2000). Notably, in this study loss of a mitochondrial porin, Por1p, gave a clear sensitive phenotype. In accord with this finding, microarray studies have shown that *POR1* expression levels were induced upon exposure to sorbic acid (de Nobel et al., 2001). Another sensitive mutant was the mitochondrial glutaredoxin, Grx5p. This protein is known to exhibit high sensitivity to oxidative stress, increased sensitivity to osmotic stress, and increased oxidation levels of cell protein (Molina et al., 2004; Rodriguez-Manzaneque et al., 1999). Deletion of *SOD1*, responsible for ionic homeostasis, also produced sorbic acid sensitivity. Loss of Sod1p is known to exhibit increased copper sensitivity, methionine auxotrophy and oxygen sensitivity (Jensen et al., 2004; Wei et al., 2001).

The cell wall in *S cerevisiae* functions to preserve the osmotic integrity of the cell and to sustain morphology during budding, sporulation and formation of pseudohyphae (Horie and

Isono, 2001). The screen revealed sorbate sensitivity associated with a number of cell wall components that are highly mannosylated proteins (Och1p, Gon7p) and cell wall protein involved in biosynthesis and organization (Dcw1p, Smi1). Further more extensive studies by Mollapour and coworkers identified a number of *mnn* mutants that displayed either an increased or a decreased sorbate resistance depending on their altered mannan content (Mollapour et al, 2005).

A large set of sorbate-sensitive genes were involved in transcription. Subunits of the CTDK-I complex (Ctk1p and Ctk3p) resulted in sorbate sensitivity. The CTDK-I complex plays an essential role in the control of mRNA synthesis and processing and phosphorylation is key feature of its function (Bouchoux et al., 2004; Hautbergue and Goguel, 2001). The CCR4 complex (Ccr4p, Mot2p, Pop2p) is a general transcriptional regulator affecting the expression of a number of genes in yeast (Liu et al., 1997). Sin4p and Rox3p, yeast global transcription factors were also important in sorbate resistance. Both proteins are implicated by genetic evidence in positive and negative transcriptional regulation (Gustafsson et al., 1997)

Identification of genes involved in the cell cycle, could be an indication that a large influx of weak organic acid into dividing cells might cause severe disruption of the events in spindle assembly, chromosome segregation and cytokinesis. Survival of the stress might require cells to undergo cell cycle arrest at the spindle checkpoint (Kai et al., 2005; Westmoreland et al., 2004).

Schuller and coworkers used microarrays to analyze the genome-wide response to sorbic acid stress, allowing for the identification of more than one hundred genes rapidly induced by weak acid stress (Schuller et al., 2004). A large set of sorbate-induced genes (35 %) were dependent on Msn2p/Msn4p, which govern the general stress response (Estruch, 2000). Only 4 genes, including *PDR12,* were induced in a *WAR1*-dependent manner. Thirteen genes were coregulated by War1p and Msn2p/Msn4p. Twenty-one out of the remaining genes, were assigned to a group that was neither regulated by War1p or

Msn2p/Msn4p, indicating the existence of a third sensing system. In a previous microarray analysis (de Nobel et al., 2001), following long term exposure to sorbic acid, 1.53 % of the genome showed increased transcription levels following adaptation to sorbate. Thus, a combination of phenotypic screening and transcriptional profiling is an efficient way of identifying key proteins/genes operating in different stress response pathways.

Seven sorbate-sensitive regulatory proteins (Dbf2p, Vps15p, Ctk1p, Bub1p, Rox3p, War1p and Reg1p) identified by screening and verified via complementation studies were further analyzed. When these deletion strains were subject to different stresses (section 3.6) the sorbate-sensitivity was not unique. In a recent screen for sorbate sensitivity these seven proteins were identified as sorbic acid sensitive (Mollapour et al., 2004), whereas in a previous screen only War1p was identified to have a role in sorbic acid stress (Schuller et al., 2004). Differences among the results of large scale screen projects could be explained due to different strategy and stringency between them.

Bioinformatic analysis of interacting partners between the regulatory proteins revealed a complex network of interacting partners. Interestingly, this network of proteins revealed a number of associations with sorbate sensitivity identified from the 'disruptome' screen (Pop2p, Vma2p, Vps34p). Consequently, a large-scale mapping of interactions would generate a much more complex network of proteins and would be likely to reveal more proteins with a sorbate sensitivite phenotype. However, from the bioinformatic data, none of the regulatory proteins appeared to interact directly with each other and no known signaling pathway was evident.

To investigate whether the sorbate sensitivity of Δ*dbf2*, Δ*vps15*, Δ*ctk1*, Δ*bub1*, Δrox3, Δ*war1* and Δ*reg1* mutants was specific, other stresses were tested (Table 3.2). Interestingly, loss of the *VPS15* gene resulted in a sensitive phenotype under all stress conditions used in this study. Strains in which the *VPS15* gene has been deleted are temperature sensitive for growth at 37 °C according to previous studies (Stack et al., 1995). However, the Vps15p general stress sensitivity is a characteristic of all *vps* mutants (Shimoni, 2002). In addition, Δ*vps15* has a slow growing phenotype that can account for

the general sensitive phenotype the mutant is exhibiting. The transcription factor Rox3p was found in addition to sorbic acid, to be sensitive to hydrogen peroxide, ethanol and heat stress conditions. Previous studies have shown that Rox3p functions in the global stress response pathway by preventing *CYC7* (iso-2-cytochrome c) RNA accumulation during heat shock and osmotic stress (Evangelista, 1996).

Deletion of *REG1*, a protein phosphatase results in defective growth under weak acids stress, acetic, peroxide and ethanol stress conditions. This protein shows slow growth and morphological defects and is involved in glusose metabolism (Dombek, 1999; Sanz, 2000). Deletion of both *CTK1* and *BUB1* caused sensitivity to salt and ethanol, salt and heat respectively. Ctk1p protein kinase has published evidence of slow growth, cold sensitivity and a moderate growth defect on NaCl (Gavin et al., 2002). Bub1p on the other hand, is a protein kinase involved in cell cycle regulation with no prior evidence of being involved in stress responses (Kitagawa, 2003). Deletions of *DBF2* and *WAR1* gave the most clear sorbate-sensitive phenotypes (Figure 3.4B). Both deletion mutants showed no growth defect in comparison to the parent, suggesting that growth inhibition following sorbic acid treatment was not a pleiotrophic effect. Δ*dbf2* sensitivity was further verified via complementation studies (section 3.5). As (Kren et al., 2003) concurrently with this study, identified but further characterized War1p as the main regulator of Pdr12p transporter, no further studies were pursued on this protein.

Dbf2p is a well-studied multifunctional protein kinase that acts as part of a network of genes in exit from mitosis, transcription and stress response (Lee et al., 1999; Liu et al., 1997; Toyn and Johnston, 1994). Interestingly, Δ*dbf2* mutants showed sensitivities to staurosporine and caffeine and were hypersensitive to elevated levels of heavy metals and divalent ions (Liu et al., 1997). In this study deletion of *DBF2* resulted in acetic acid and ethanol sensitivity whereas, osmotic stress also gave a strong sensitive phenotype, which was consistent with similar sensitivity reported in other Δ*dbf2* mutants in different strain backgrounds (Lee et al., 1999; Warringer et al., 2003). We also verified the effect of heat shock of 37°C (Liu et al., 1997). Prior published evidence linking Dbf2p to osmotic stress response (Lee et al., 1999) made it an excellent candidate for further studies. Thus, Dbf2p

was chosen as a target for further study to characterize its function in relation to sorbic acid resistance.

This study has postulated a novel role for Dbf2p in sorbic acid regulation via an as yet unknown mechanism. The further aims of this work are to identify key components that are controlled by the Dbf2p protein kinase, putative phosphorylation substrates of Dbf2p and elucidate the signaling pathway that mediates sorbic acid resistance.

CHAPTER 4

4. Sorbate-induced changes in the phosphoproteome and proteome of *S. cerevisiae* Δ*dbf2* mutant

4.1 Introduction

Dbf2p is a multifunctional protein kinase that acts as part of a network of genes involved in exit from mitosis, transcription and stress response (Lee et al., 1999; Liu et al., 1997; Toyn and Johnston, 1994). The *S. cerevisiae DBF2* gene was first characterized in 1991 (Johnston et al., 1990). Since then, it has been extensively studied over the past fifteen years. Homology studies have shown that all of the highly conserved residues that are characteristic of protein kinases occur in the *DBF2* protein (Figure 4.1) (Johnston et al., 1990). Dbf2p shares 97 % homology with Hemiascomycetes, 38.8% with fungi, 26.8% with plants and 25% with mammalia.

The Dbf2p kinase in *S. cerevisiae* is cell cycle regulated and loss of activity causes cell cycle arrest at the end of mitosis, where the cells have a fully extended spindle and divided chromatin, which is characteristic of telophase (Johnston et al., 1990). Consistent with a mitotic role *DBF2* is expressed under cell cycle control in the M phase. Similarly its kinase activity is also cell cycle regulated, with a peak in metaphase-anaphase B/telophase (Toyn and Johnston, 1994).

Dbf2p is part of the mitotic exit network (MEN) that regulates events in late mitosis (reviewed in Yeong et al., 2002). In budding yeast MEN consists of the protein phosphatase Cdc14p, the ras-like GTPase Tem1p, the Dbf2-binding protein Mob1p and the protein kinases Cdc15p, Cdc5p, Dbf2p (Yoshida and Toh-e, 2001). Localisation studies have shown that Dbf2p is found on the centrosomes/spindle pole bodies (SPBs) and at the bud neck, where it forms a double ring (Frenz et al., 2000). The localization of Dbf2p is cell cycle regulated. It is present on the SPBs during most of the cell cycle and migrates to the bud neck in late mitosis, consistent with a role in cytokinesis (Frenz et al., 2000).

The role of this kinase in the cell cycle is very important but not yet clear, and its physiological substrates remain undefined. Surprisingly, strains in which *DBF2* has

been deleted are viable (Toyn et al., 1991). This is due to the existence of a highly homologous protein, *DBF20* (Toyn et al., 1991). Deletion of both *DBF2* and *DBF20* is lethal, suggesting that they jointly perform an essential late mitotic function. However, *DBF2* accounts for 80 % of the activity, with *DBF20* playing only a minor role (Toyn et al., 1991). Recently, it has been found that both Dbf2p and Dbf20p functions are necessary for the execution of cytokinesis (Hwa Lim et al., 2003).

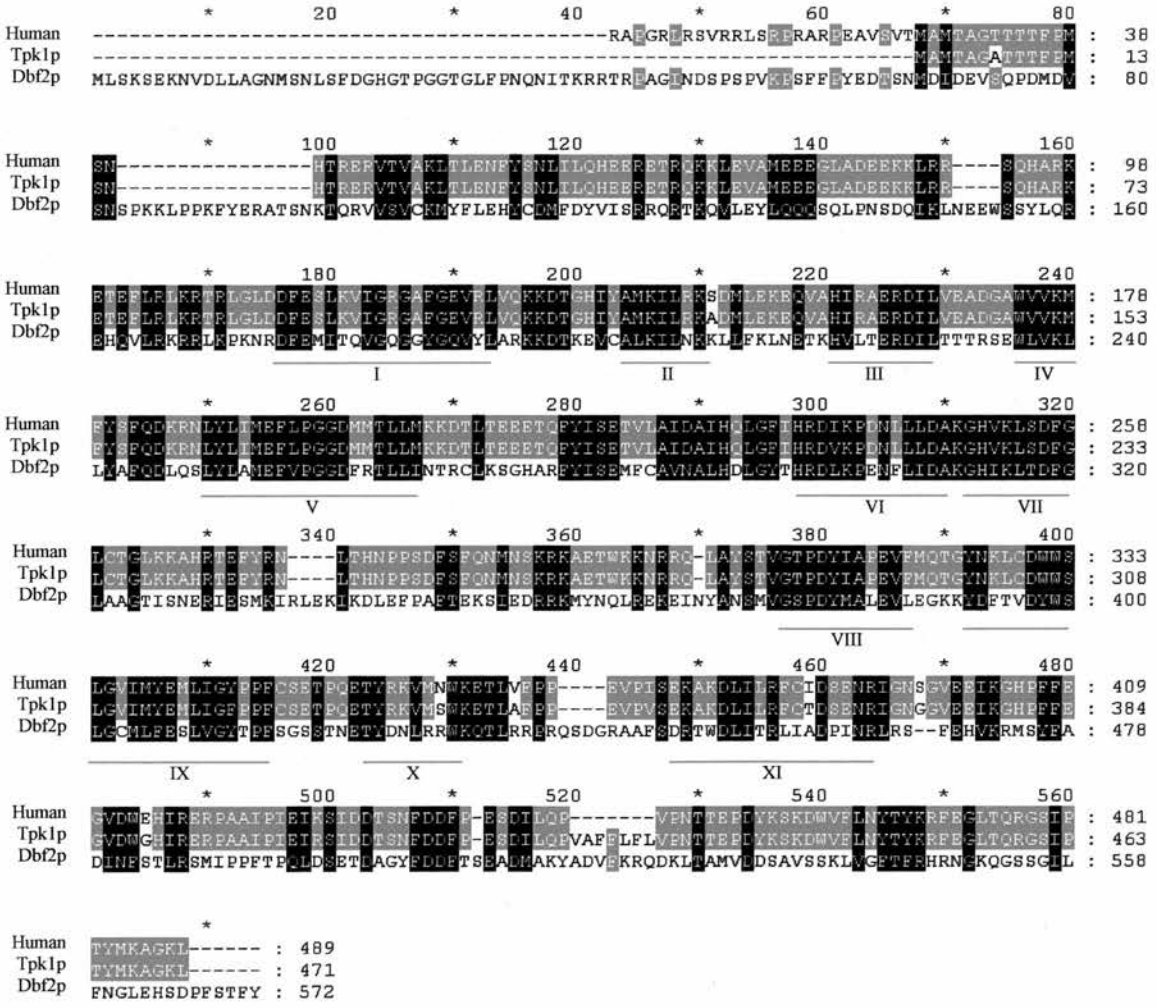


Figure 4.1 Sequence alignment of DBF2 with highly homologous proteins from other eukaryotes. Sequences identified using BLAST, and aligned using CLUSTA, conserved residues were coloured using BOXSHADE. Underlined domains (I toXI) represent the 11 highly conserved protein kinase domains. The sequences Asp-Leu-Lys-Pro-Clu-Asn in domain VI and Gly-Ser-X-X-Tyr-X-Ala-Leu-Glu (X being any amino acid) in domain VIII suggested that Dbf2p is a serine-threonine protein kinase (Johnston et al., 1990).

Dbf2p not only regulates cell cycle progression but also controls gene expression as a component of the CCR4 complex. The CCR4 protein complex is a general transcriptional regulator, which affects regulation of a number of genes (Draper et al., 1994; Liu et al., 1997). The complex contains a number of proteins in addition to Dbf2p. One of these is Caf1p/Pop2p that binds to both Ccr4p and Dbf2p. *CCR4* and *CAF1* defects also cause a partial block in late mitosis at a point similar to *DBF2* defects. These results suggested that one of the *DBF2* functions was to control gene expression through its association with the CCR4 complex. Further studies have shown that Δ*dbf2* resulted in similar transcriptional phenotypes to deletions of Δ*ccr4* and Δ*caf1*, further suggesting that *DBF2* functions as a transcriptional regulator (Liu et al., 1997). While previous studies indicate that Dbf2p plays an important role in the regulation of the cell cycle and gene expression, the mechanisms by which it is regulated and the identification of its cellular target proteins remain unclear. However, recently, it was found that Dbf2-Mob1 complex preferentially phosphorylated substrates that contain an RXXS motif. A subsequent proteome microarray screen revealed proteins that could be phosphorylated by Dbf2-Mob1 *in vitro*. These proteins were enriched for RXXS motifs (Mah et al., 2005).

Published evidence also has indicated a role for Dbf2p in stress response (Lee et al., 1999). In this study we report a new role for Dbf2p in sorbic acid resistance (Makrantoni, 2003). The aim of this work was to identify components of the signalling pathway through which Dbf2p mediates the sorbic acid stress response. To facilitate this we used a two-pronged approach: Firstly, we studied changes in protein phosphorylation in Δ*dbf2* mutant strains in the presence and absence of sorbic acid (phosphoproteome analysis) and compared these results with control wild type strain. Secondly changes in protein expression under the same conditions were analysed (proteome analysis).

We developed a new methodology in yeast in order to map and identify changes in phosphorylation due to the *DBF2* deletion, due to sorbic acid treatment or combination of both. For this a combination of affinity purification, 2D-PAGE and peptide mass fingerprinting was employed to study the phosphoprotein complement of *S. cerevisiae*. Protein extracts were first passed through a phosphoprotein affinity column and the

phosphoprotein-enriched eluate fractions were then separated by 2-D PAGE and proteins were visualised by staining with Silver or SYPRO Ruby. Proteins were excised from the gels and identified by peptide mass fingerprinting. A diagram illustrating this approach is shown in Figure 4.2. Additional experiments using a specific stain for phosphoproteins, prior incubation of the protein extract with alkaline phosphatase and blotting with monoclonal antibodies to phospho-threonine, -serine and –tyrosine demonstrated that the phosphoprotein affinity column was an effective way for enriching for phosphoproteins.

We measured changes in the proteome and phosphoproteome of *S. cerevisiae* control and Δ*dbf2* deletion mutants, occurring in response following growth in sorbic acid at pH 4.5. These proteins were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry of the peptides produced by in-gel digestion with trypsin, followed by database searching using the peptide masses derived from each trypsinized protein. Comparative analysis of the proteome and phosphoproteome of sorbate-stressed Δ*dbf2* mutants resulted in several significant changes.

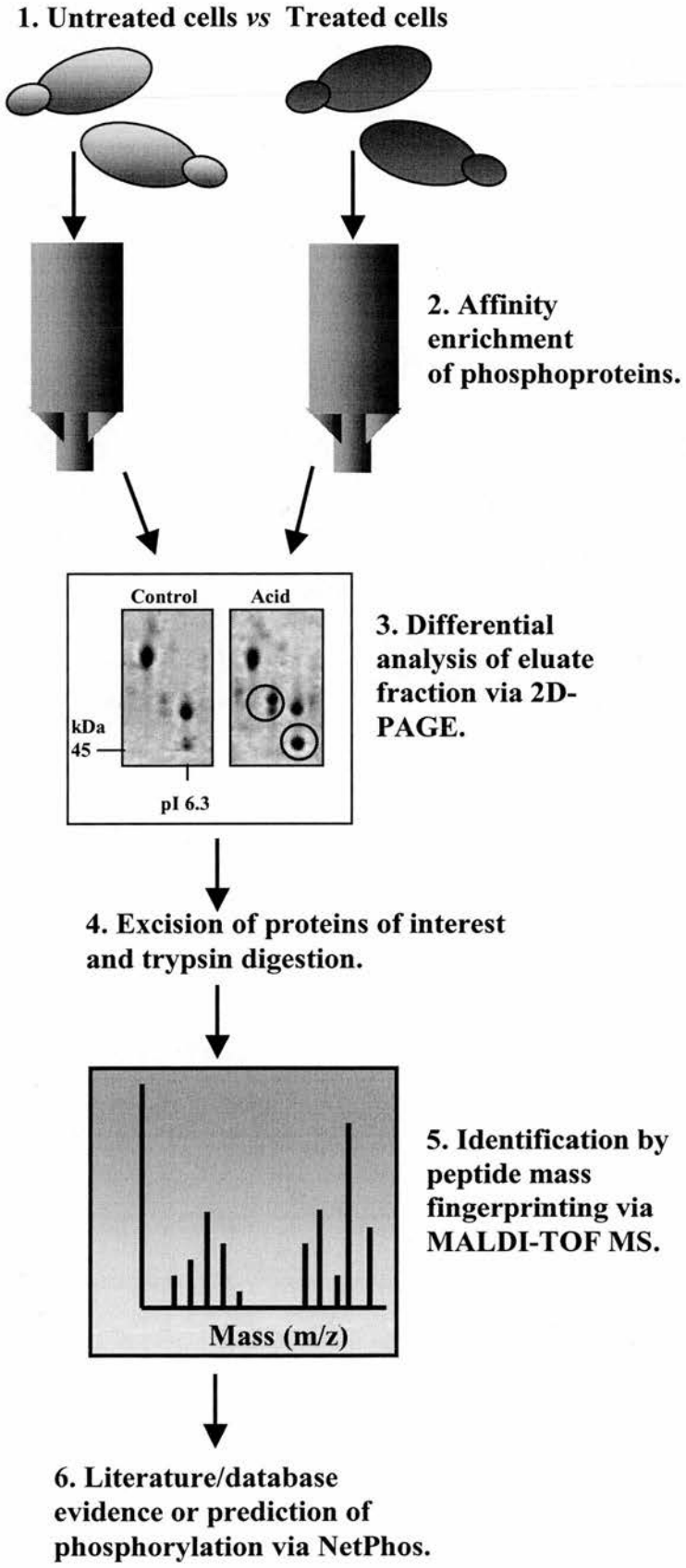


Figure 4.2 Schematic diagram illustrating the process for studying and identifying changes in the yeast phosphoproteome.

4.2 Weak acid induction of Pdr12p is not mediated by Dbf2p kinase

Previous studies have identified the Pdr12p ABC transporter as the major determinant of weak organic acid stress resistance in *S. cerevisiae* (Piper et al., 1998). Concurrent with this study, the transcriptional regulator War1p was identified to induce Pdr12p. Yet the upstream components transducing the signal to War1p remain elusive (Kren et al., 2003). In order to find out if Dbf2p kinase forms part of the Pdr12p induction pathway, western blot analysis of Pdr12p protein expression levels was performed using a monoclonal antibody against the protein (kind gift from Kuchler K.). Protein extracts from wild type (BY4741a) and Δ*dbf2* mutant strains protein extracts were grown in YEPD, pH 4.5 in the presence and absence of 1.5 mM sorbic acid. Loss of *DBF2* did not affect the basal or sorbate-induced levels of the Pdr12p transporter, as the protein levels remained consistent (Figure 4.3). Consistent with previous studies, the slowly migrating form represents the phosphorylated form of the protein upon induction with sorbate (Holyoak et al., 2000). Actin (Act1), a constitutively expressed protein was used as a loading control (Figure 4.3). These data suggested that the sorbic acid resistance mediated by Dbf2p occurs via a novel mechanism, independent of Pdr12p.

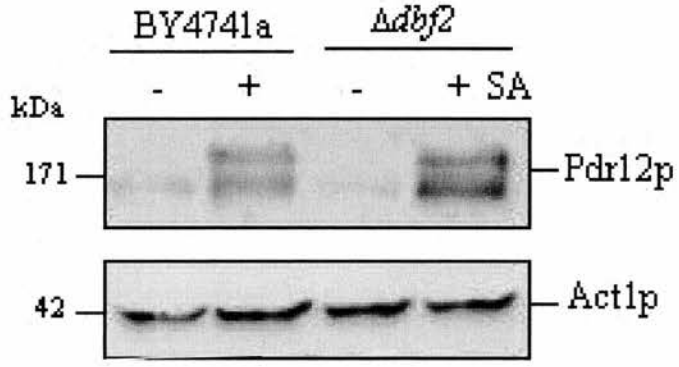


Figure 4.3 Immunoblot detection of Pdr12p levels. Wild type (BY4741a) and Δ*dbf2* mutant strains were grown in YEPD (pH 4.5) in the presence and absence (+/-) of sorbic acid (SA). Loss of *DBF2* displayed normal Pdr12p induction in the presence and absence of sorbic acid. Pdr12p is present in two isoforms seen as a doublet (Holyoak, 2000). Actin (Act1p) was used as a loading control. A representative result of three replicate experiments is shown.

4.3 Effect of sorbic acid stress on Dbf2p expression levels

To study changes in expression levels of Dbf2p under sorbic acid stress a TAP-tagged Dbf2p construct (Table 2.1) was used. Western blotting analysis to detect changes in Dbf2p expression levels was preformed using a commercially available antibody against the TAP-Tag (Table 2.4).

Western blotting analysis revealed that growth in the presence of 1.5 mM sorbic acid resulted in minor reduction in the expression of Dbf2p over a 5 h growth period (Figure 4.4). Actin (Act1p), a constitutively expressed protein, was used as a loading control (Figure 4.4).

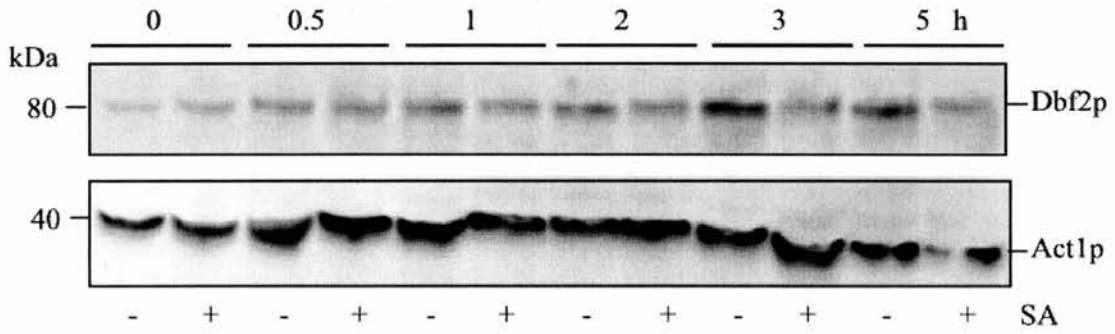


Figure 4.4 Time-course of expression levels of Dbf2p upon exposure to sorbic acid. Dbf2-TAP strain was grown in YEPD, pH 4.5 in the presence and absence (+/-) of sorbic acid (SA). Growth in the presence of 1.5 mM sorbic acid resulted in minor reduction in the expression of Dbf2p over a 5 h growth period. Actin (Act1p) was used as a loading control. A representative result of three replicate experiments is shown.

4.4 Development of a method to study changes in the yeast phosphoproteome

4.4 Validation of the phosphoprotein affinity column.

In this study an affinity purification column developed by Qiagen (Qiagen, UK) was optimised for yeast in order to analyse the yeast phosphoproteome. The ability of the column to specifically capture yeast phosphoproteins was studied and the following experiments were performed to validate the accuracy of the method.

4.4.1 Affinity purification produces a soluble protein fraction rich in phosphoproteins.

Study of the manufacturer's instructions on the affinity column, which had not previously been optimised for use with *S. cerevisiae*, revealed that loading 2.5 mg of a protein lysate from a range of mammalian cell types resulted in the capture of approximately 0.2-0.3 mg of phosphorylated proteins: indicating that in mammalian cells approximately 10 % of total soluble proteins are phosphorylated. However, when an equal quantity of a yeast lysate was loaded onto the column, only 0.02 mg of phosphorylated proteins was typically recovered. This low yield of phosphoproteins was not sufficient to achieve reliable identification by peptide mass fingerprinting after 2D-PAGE. To overcome this problem, the purification protocol was modified, by increasing the amount of lysate ten-fold (section 2.6.4). According to the manufacturer, this modification of the purification protocol would not compromise the efficacy of the affinity column.

After passage of the soluble yeast lysate through the affinity column and subsequent elution of phosphorylated proteins, both the flow-through and eluate fractions were collected and run on 2-D PAGE gels (Figure 4.5). Observation of the corresponding gels showed that the eluate fraction contained a different pattern of proteins compared to the flow-through fraction, indicative of enrichment for phosphoproteins. Many of the proteins, some of which were subsequently identified, appeared to have a multiple train of spots of different p*I*. This could signify post-translational modification, including phosphorylation (Guy et al., 1994). To determine whether the eluate fraction was

enriched for phosphoproteins, a random selection of 13 protein spots, representing a range of molecular weight, p*I* and staining intensities were excised from the gel. These were then identified by peptide mass fingerprinting (Table 4.1). Out of the 13 proteins identified, there was database (SwissProt:http://us.expasy.org/sprot/; MIPS:http://mips.gsf.de/genre/proj/yeast/index.jsp) or published literature evidence that 11 of these proteins were either phosphorylated or had ATP binding activity (Table 4.2).

Furthermore, the sequence of all 13 identified proteins were analysed using the publicly available NetPhos (v. 2.0 database; http://www.cbs.dtu.dk/services/NetPhos/). NetPhos is a neural network-based method for predicting potential phosphorylation sites at serine, threonine or tyrosine residues in protein sequences (Blom et al., 1999). NetPhos was 'trained' on a large data set of known phosphorylation sites and was reported to predict phosphorylation sites in independent sequences with reliability in the range of 69 to 96 %. Using this method we identified that there was evidence of phosphorylation sites on serine, threonine and tyrosine residues in all 13 of the proteins randomly selected from the eluate fraction (Table 4.2).

Figure 4.4 Comparative 2D-PAGE analysis of unphosphorylated and phosphorylated proteins from *S. cerevisiae* BY4741. Proteins present in the eluate and flow-through fractions from the phosphoprotein affinity column were separated in the first dimension by iso-electric focusing over the pH range 4.0-7.0, prior to second dimension separation via SDS-PAGE and subsequent silver staining. Map **A** Represents 150 μg of the flow-through fraction (unphosphorylated proteins), and Map **B** Represents 150 μg of the eluate fraction (phosphorylated proteins). As indicated, spots were excised from the gel of the eluate fraction and the corresponding protein identified by peptide mass fingerprinting. The identification of the numbered proteins is reported in Table 4.1. Molecular weight markers are indicated on the left and p*I* values at the top of the panels. A representative result of two replicate experiments is shown.

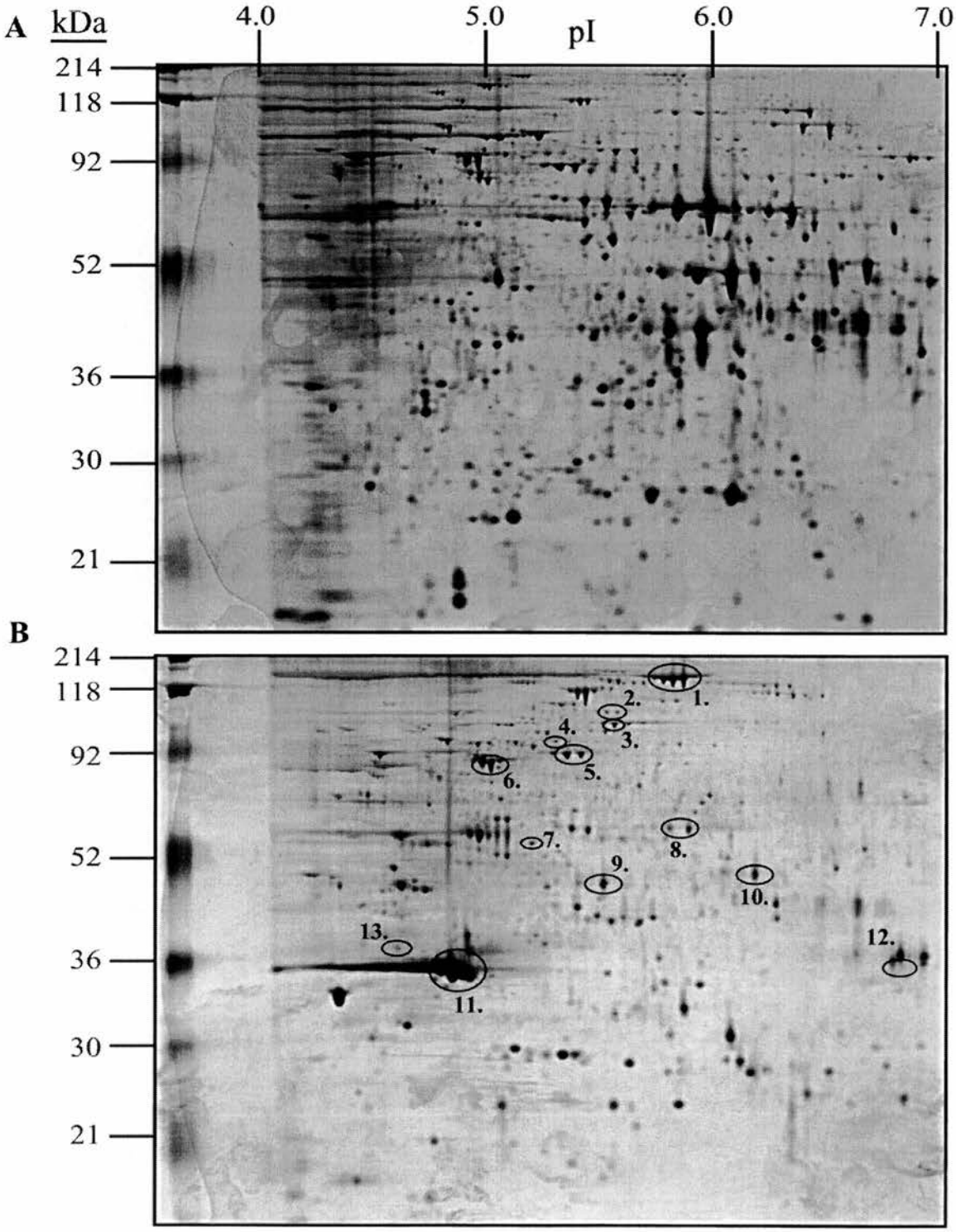

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Table 4.1 Identification of yeast phosphoproteins by peptide mass fingerprinting.

Spot No.	No. of matching peptides (%) [a]	Matched peptide coverage (%) [b]	Molecular mass (kDa) [c]		pI [c]		Top ranking protein in MS-Fit search [d]	CAI [e]	Swiss Prot Accession No.
			Exp	Pred	Exp	Pred			
1	30	14	120	116	5.90	5.95	Yef3p (translation elongation factor eEF3)	0.778	P16521
2	44	16	110	88	5.70	5.88	Prt1p (translation initiation factor eIF3 subunit)	0.304	P06103
3	28	29	110	77.3	5.20	4.97	Sse1p or Sse2p (heat shock protein 70)	0.521/0.192	P32589/90
4	60	16	90	70.6	5.30	5.48	Ssc1p (heat shock protein 70-related)	0.521	P12398
5	67	23	90	67	5.30	5.18	Ssb1 (cytoplasmic heat shock protein 70)	0.820	P11484
6	33	26	72	62.3	5.00	5.10	Ssz1 (Dnak homologue, heat shock protein 70)	0.455	P38788
7	60	13	60	55	5.30	5.52	Atp2 (beta subunit of F_1F_0 ATP synthase)	0.425	P00830
8	75	12	60	53.5	5.90	6.02	Srm1 (GDP/GTP exchange factor for Gsp1/Gsp2)	0.204	P21827
9	35	18	45	42	5.60	5.53	Act2p (cytoskeleton, actin binding)	0.209	P32381
10	33	21	53	47	6.10	5.67	Eno2p (EnolaseII; 2-phosphoglycerate dehydratase)	0.892	P00925
11	59	40	35	33.7	4.80	4.75	RppOp (conserved ribosomal protein PO)	0.794	P05317
12	50	12	34	36	7.00	8.29	Tdh1p (glyceraldehydes-3-phosphate dehydrogenase)	0.856	P00360
13	15	28	35	32.03	4.80	4.89	Pcl7p (cyclin like protein interacting with Pho85p)	0.131	P40186

[a] The number of tryptic peptides assigned to the protein divided by the total number of tryptic peptides predicted for the protein expressed as a percentage.
[b] The number of amino acids in the matched peptides divided by the total number of amino acids in the predicted protein sequence expressed as a percentage.
[c] Theoretical p*I* and molecular weights were obtained from the protein entry in the SGD database (URL:http://www.yeastgenome.org).
[d] The programme MS-Fit (URL: http://prospector.ucsf.edu/ucsfhtml4.0/msfithtm) was used to search the SwissProt database for proteins with calculated tryptic peptide masses that matched the measured experimentally derived masses (mass accuracy +/- 0.15Da).
[e] Codon Adaptation Index (CAI) from the SGD database.

Table 4.2 Evidence for identified proteins

Spot No.	Protein [a]	Net-Phos [b]	Functional evidence[c]	Database[d]	Reference[e]
1	Yef3p	+	ATP binding/phosphorylation	SwissProt	(Ficarro et al., 2002)
2	Prt1p	+	-	-	-
3	Sse1/2p	+	ATP binding	SwissProt	-
4	Ssc1p	+	ATP binding	SwissProt	-
5	Ssb1p	+	ATP binding	SwissProt	-
6	Ssz1p	+	ATP binding	SwissProt	-
7	Atp2p	+	ATP syntheisi/binding	SwissProt	(Ichikawa and Mizuno,
8	Srm1p	+	Phosphorylation	-	(Fleischmann et al., 1996)
9	Act2p	+	-	-	-
10	Eno2p	+	Phosphorylation	-	(Ficarro et al., 2002)
11	RppOp	+	Phosphorylation	-	(Grabowski et al., 1991)
12	Tdh1p	+	Phosphorylation	SwissProt	-
13	Pcl7p	+	Phosphorylation	MIPS	(Lee et al., 2000)

[a] Protein names according to the SGD database (URL: http://www.yeastgenome.org).
[b] A + indicates whether information concerning serine, threonine or tyrosine phosphorylated sites were predicted by NetPhos database (URL:http://www.cbs.dtu.dk/services/NetPhos/).
[c] Functional evidence was obtained from the protein entry in the SGD database (URL:http://www.yeastgenome.org) or the MIPS database (http://mips.gsf.de.genre/proj/yeast/index.jsp).
[d] Database entry of phosphorylation: SwissProt (URL: http://us.expasy.org/sprot/) and MIPS.
[e] References indicating previous studies on phosphorylation of the proteins listed.

4.4.2 Pro-Q Diamond phosphoprotein stain only identified proteins in the eluate fraction

To further validate the ability of the affinity column to specifically enrich for phosphoproteins, gels of the flow-through and eluate fraction were stained with Pro-Q Diamond phosphoprotein stain (Molecular Probes, Oregon, USA). This fluorescent stain detects phosphate groups attached to tyrosine, serine or threonine residues with a sensitivity limit between 1-16 ng per protein spot depending on the phosphorylation state of the protein (Martin et al., 2003; Schulenberg et al., 2003). No phosphoprotein staining was observed in the flow-through fraction. Therefore, no detectable phosphoproteins passed through the column. In the eluate fraction low levels of protein staining were observed and three proteins, Yef3p, Ssz1p and Rpp0p, were identified (Figure 4.6). These proteins had been previously identified in the phosphoprotein eluate fraction of SYPRO Ruby-stained 2-D gels (Figure 4.5). Comparison of eluate fraction gels stained with either Pro-Q Diamond or SYPRO® Ruby, revealed many more visible putative phosphoproteins on the SYPRO Ruby gel (Figure 4.6). Whilst the Pro-Q Diamond stain detected some phosphoproteins in the eluate fraction, the SYPRO® Ruby stain demonstrated greater sensitivity, as it has a higher detection capacity (sub-nanogram levels of protein).

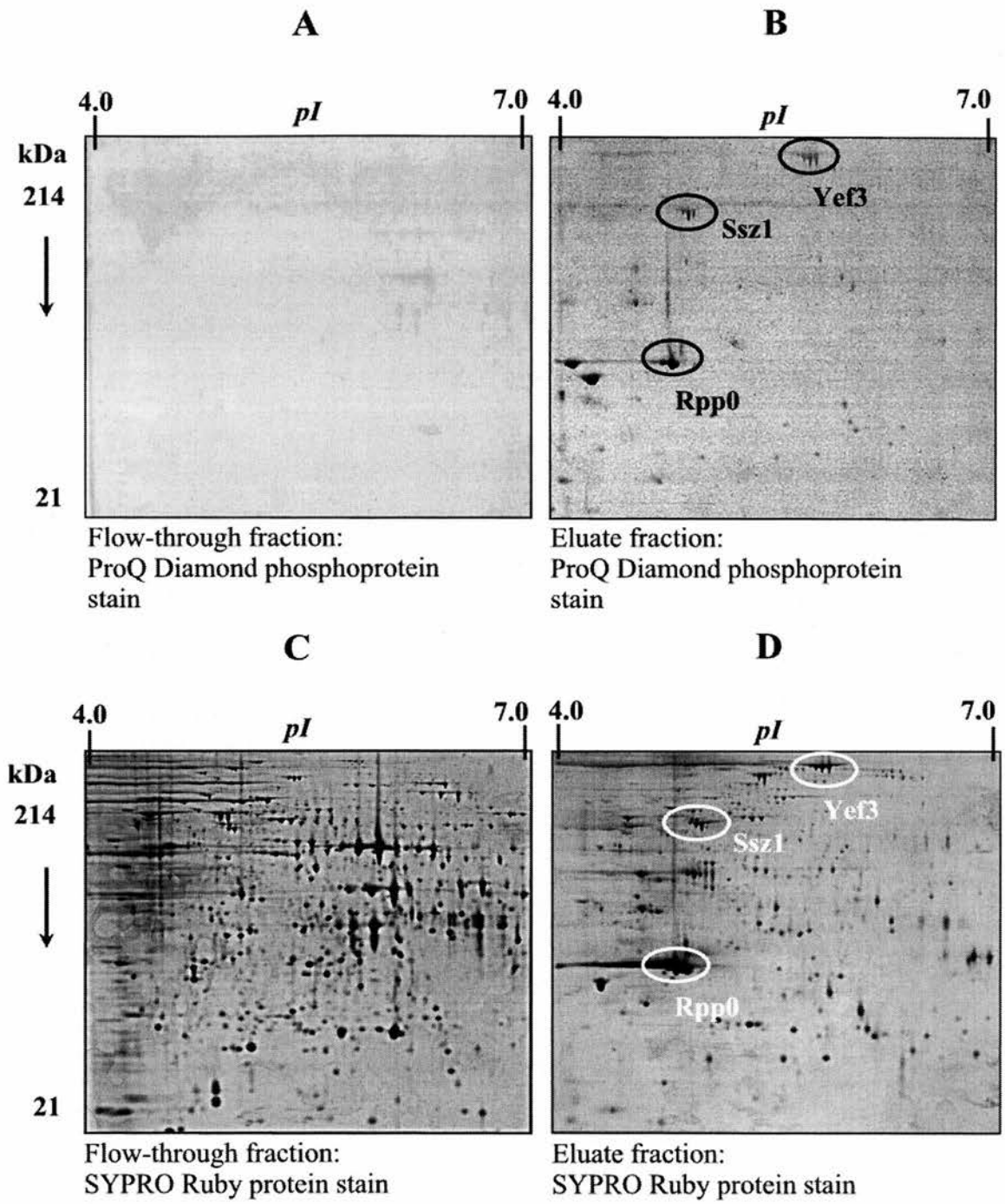


Figure 4.6 Comparative 2D-PAGE analysis of phosphocolumn purification. A. Unphosphorylated proteins (flow-through fraction) stained with ProQ-Diamond phosphoprotein stain. **B.** Phosphorylated proteins (eluate fraction) stained with ProQ-Diamond phosphoprotein stain, **C.** SYPRO® Ruby-stained map of unphosphorylated proteins (flow-through fraction), **D.** SYPRO® Ruby-stained map of phosphorylated proteins (eluate fraction). Molecular weight markers are indicated at the left and p*I* values at the top of the panels. Proteins identified by peptide mass fingerprinting are shown.

4.4.3 Prior alkaline phosphatase treatment reduced the protein content of the eluate fractions

A significant reduction in the number of phosphoproteins detected in the eluate fraction of the phosphocolumn was observed, when yeast soluble lysate was exposed to Calf Intestinal Phosphatase (CIP) prior to loading onto the column (Figure 4.7). This indicates less phosphorylated proteins were captured by the phosphocolumn. Pre-treatment with CIP completely abolished the presence of Yef3p from the eluate fraction (Figure 4.7A,C). CIP treatment had no significant effect on the protein map of the flow-through fraction (Figure 4.7B,D). This demonstrated that Yef3p was phosphorylated and specifically enriched by the phosphoprotein affinity column (Figure 4.7 and Figure 4.6 respectively).

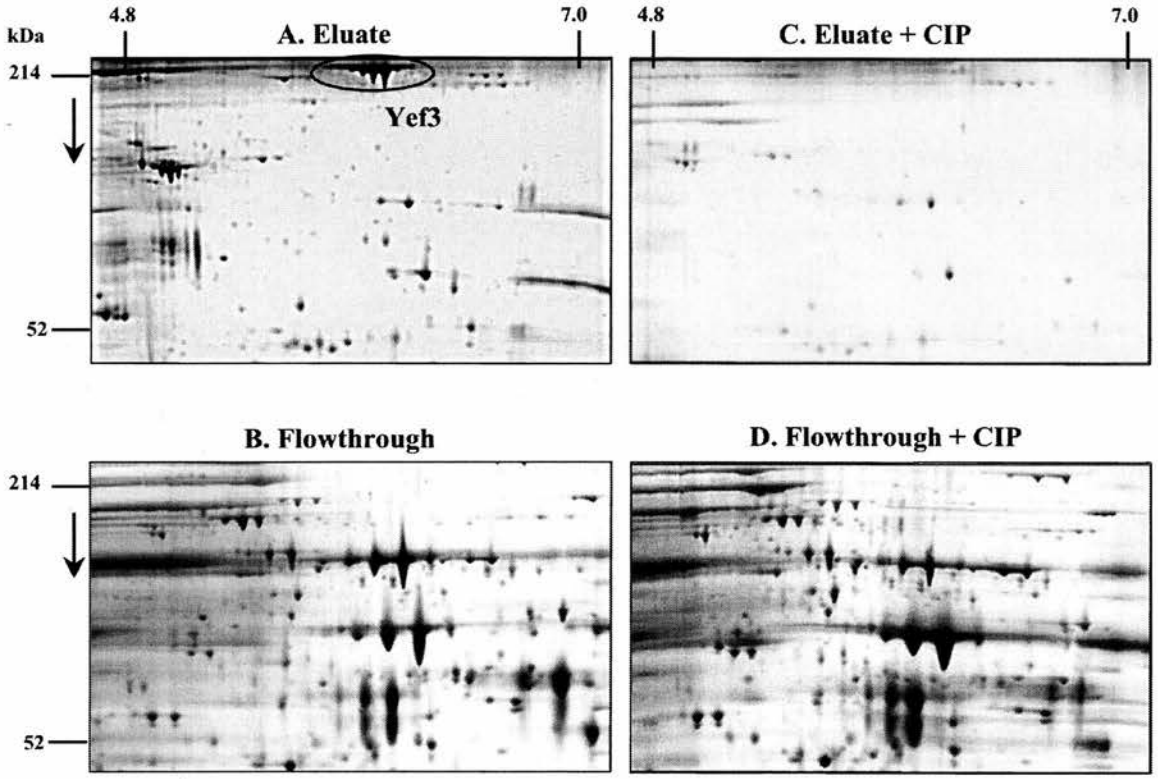


Figure 4.7 Comparative 2D-PAGE analysis of unphosphorylated and phosphorylated proteins after CIP treatment. SYPRO® Ruby-stained 2D-maps were prepared with either, unphosphorylated proteins (flow-through fraction), or phosphorylated proteins (eluate fraction) collected by affinity purification from phosphocolumn. **A.** Proteins of the eluate and **B.** the flow-through fractions were separated by IEF over the pH range of 4 to 7. **C.** Proteins of the eluate and the **D.** flow-through fractions were first treated with 50 mM Calf Intestinal Phosphatase (CIP) prior to loading onto the affinity column and then separated by IEF over the pH range of 4 to 7. Molecular weight markers are indicated on the left and p*I* values at the top of the panels. Yef3p identified by peptide mass fingerprinting is labelled.

4.4.4 Validation of phosphoprotein column specificity by using monoclonal antibodies

Monoclonal antibodies were used to verify the specificity of the phosphoprotein enrichment by the affinity column (Figure 4.8). A sample of phosphocolumn eluate and flow-through fractions were run on a 1D-SDS PAGE gel from cell extracts prepared in the presence and absence of 1.5 mM sorbic acid (Figure 4.8A). As reflected from 2D-PAGE maps (Figure 4.5), 1D gels also show that eluate (E) and flowthrough (F) lanes contain different proteins (Figure 4.8A). Following SDS-PAGE gels were blotted with antibodies that specifically recognise phospho-threonine, phospho-serine and phospho-tyrosine residues (Table 2.4, Figure 4.8B). The antibodies detected many proteins containing these phosphorylated residues in the phosphoprotein-enriched eluate fraction but only a few in the flow-through fraction (Figure 4.8B). The result further validates the phosphocolumn method to specifically enrich for phosphoproteins.

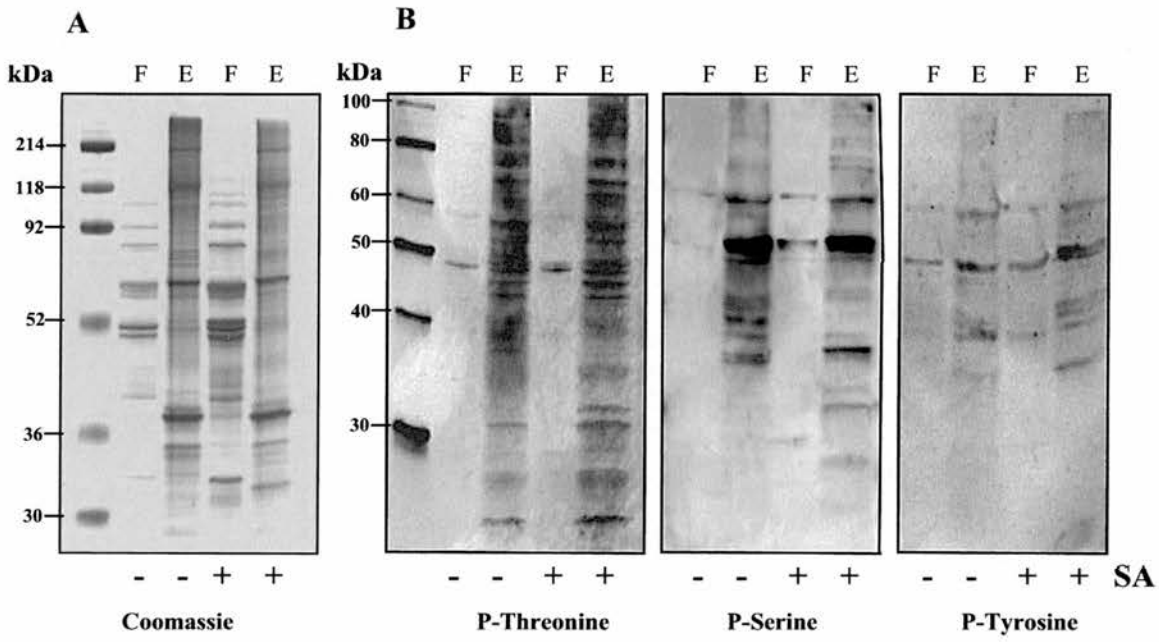


Figure 4.8 Western blot analysis of the phosphocolumn fractions. Flow-through (F: unphosphorylated proteins), and eluate (E: phosphorylated) fractions from the phosphoprotein affinity column are indicated. **A** Control (-) and sorbic acid-induced (+) cell extracts were stained with Coomassie. **B** Western blot detection of phospho threonine -serine, and -tyrosine containing proteins in the flow-through and eluate protein fractions from the phosphoprotein affinity column. Only a few phosphoproteins were detected in the eluate fractions. A representative result of three replicate experiments is shown.

4.5 Exposure to sorbic acid results in significant changes in the yeast phosphoprotcome.

Collectively, the validation results indicated that the phosphoprotein affinity column was an effective method for enriching yeast phosphoproteins. Thus, by coupling this affinity column with 2D-PAGE and peptide mass fingerprinting we have developed a relatively simple method that can be used to compare and measure changes occurring in the yeast phosphoproteome upon exposure to sorbic acid or due to *DBF2* deletion or to combination of both. Aim of this analysis is to identify components of the Dbf2p-mediated signalling pathway. A diagram illustrating this approach is shown in Figure 4.9.

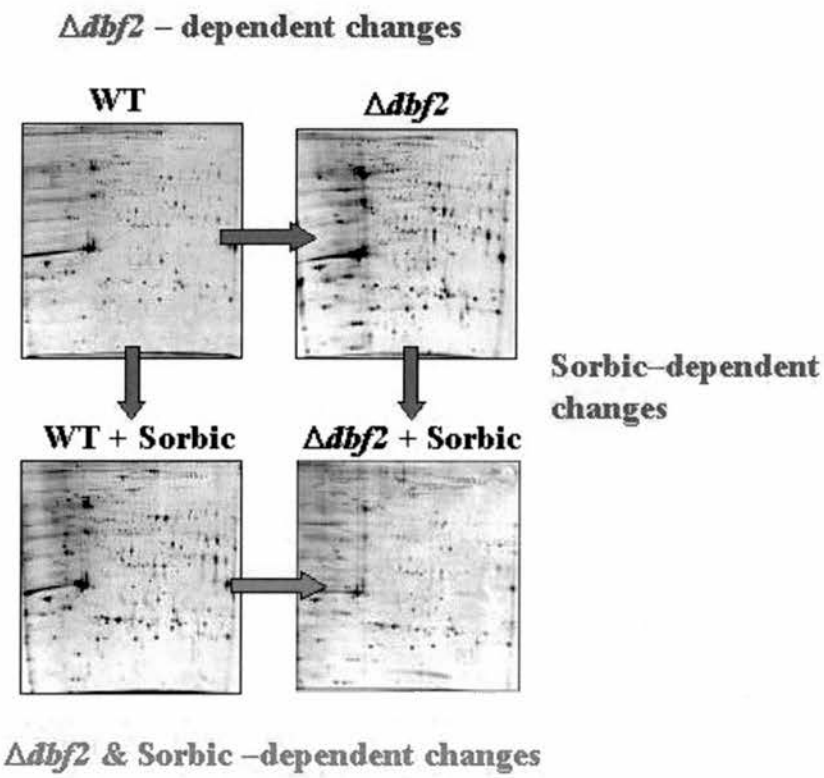
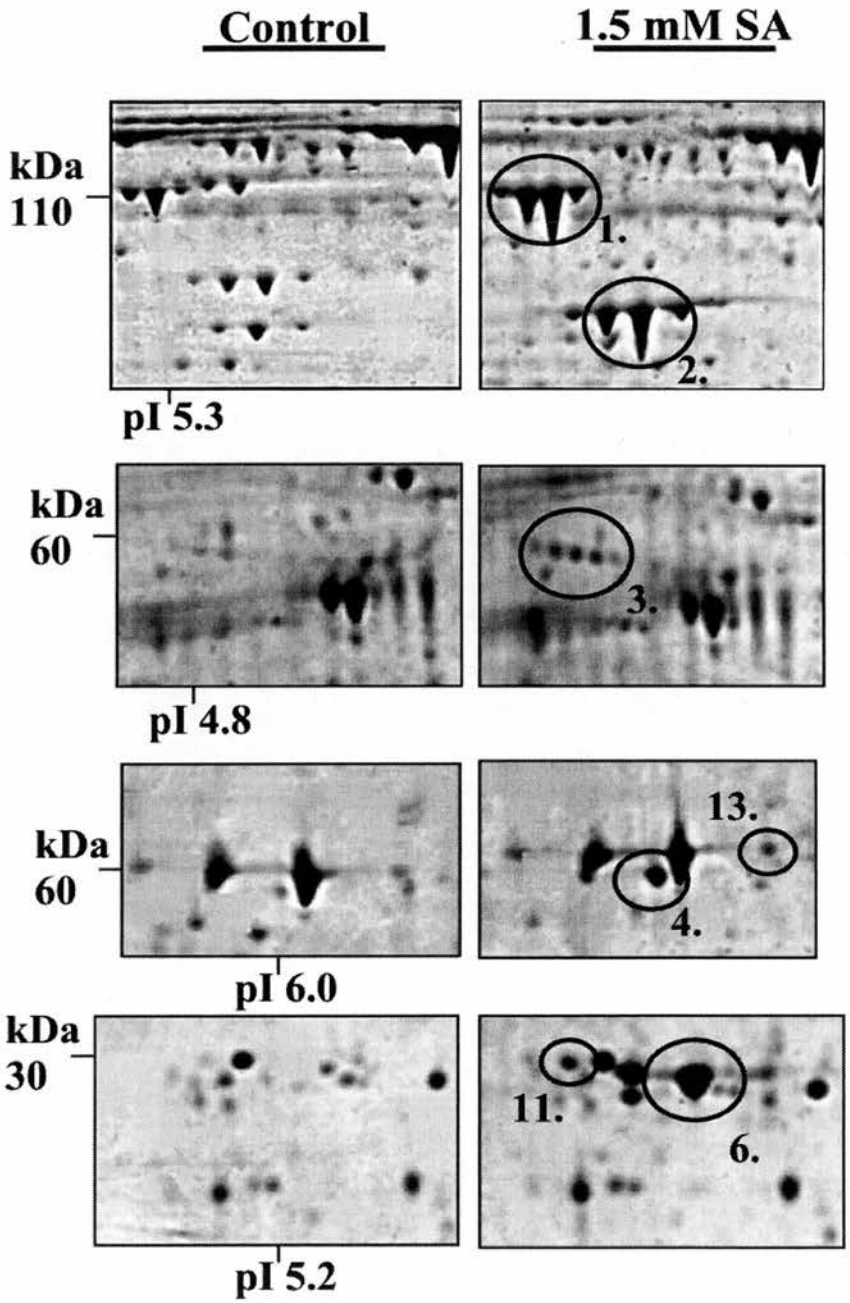


Figure 4.9 Schematic diagram illustrating the methodology followed for studying changes in the yeast phosphoproteome. Changes in the *Δdbf2* mutant strain phosphoproteome due to loss of *DBF2*, sorbic acid stress, and combination of both were identified. Wild type (WT) strain was used as a control.

4.5.1 Sorbic acid treatment results in significant changes in the yeast phosphoproteome of control and *Δdbf2* mutant strains

In this study the method discussed above has been used to characterize changes occurring in sorbic acid stressed control BY4741a and *Δdbf2* cells. Exposure of BY4741a cells to 1.5 mM sorbic acid resulted in the increased abundance, or unique expression, of 13 putative phosphoproteins that were subsequently identified by peptide mass fingerprinting (Figure 4.10, Table 4.3). Four putative phosphoproteins showed reduced phosphorylation due to sorbic acid (Figure 4.11, Table 4.5). Thirteen of these 17 identified proteins, had database (SwissProt: http://us.expasy.org/sprot/; MIPS: http://mips.gsf.de/genre/proj/yeast/index.jsp) or published literature evidence, indicating phosphorylation, or ATP binding activity (Table 4.4 and 4.6).

Exposure of *Δdbf2* cells to 1.5 mM sorbic acid resulted in the increased abundance of only two putative phosphoproteins (Glk1 and Pcl7) that were subsequently identified by peptide mass fingerprinting (Figure 4.12, Table 4.7). Five putative phosphoproteins showed reduced abundance, or unique expression due to sorbic acid (Figure 4.13 and Table 4.8). All of these identified proteins, had database (SwissProt: http://us.expasy.org/sprot/; MIPS: http://mips.gsf.de/genre/proj/yeast/index.jsp) or published literature evidence, indicating phosphorylation, or ATP binding activity (Table 4.9).


Control
1.5 mM SA
kDa
110
1.
2.
pI 5.3
kDa
60
3.
pI 4.8
kDa
60
13.
4.
pI 6.0
kDa
30
11.
6.
pI 5.2

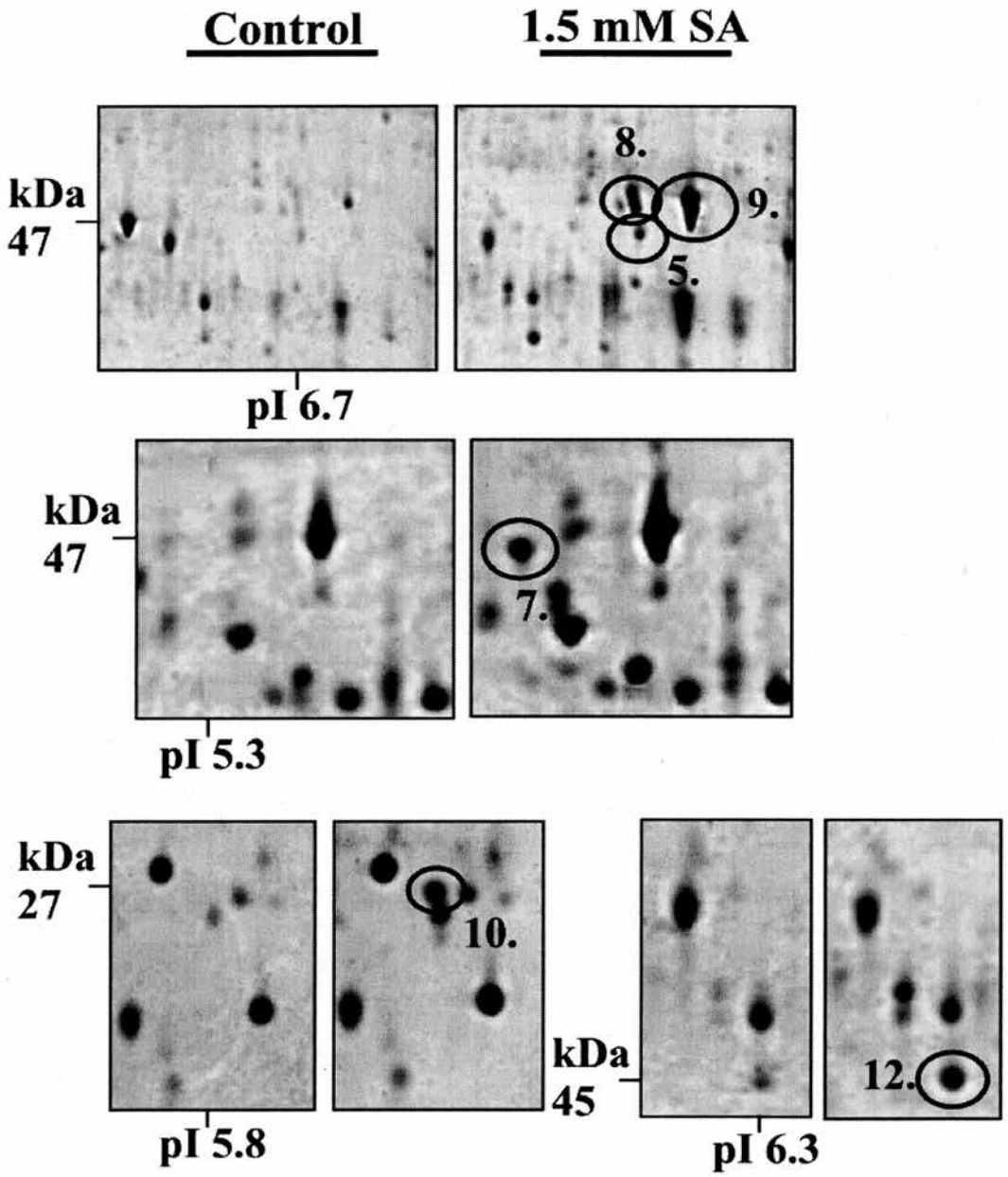


Figure 4.10 Proteins showing increased phosphorylation due to growth in the presence of sorbic acid. Proteins prepared from control cells (YEPD, pH 4.5) and cells grown in the presence of sorbic acid YEPD, pH 4.5 plus 1.5 mM sorbic acid) were passed through independent phosphoprotein affinity columns and the eluate fractions compared by 2D-PAGE in conjunction with SYPRO® Ruby staining. Protein spots showing obvious and reproducible changes in the level of protein phosphorylation are indicated and were identified by peptide mass fingerprinting (Table 4.3). A representative result of at least two replicate experiments is shown. Molecular weights are shown on the left, and p*I* values are indicated.

Table 4.3 Identification of yeast phosphoproteins by peptide mass fingerprinting: proteins showing enhanced phosphorylation due to sorbic acid.

Spot No.	No. of matching peptides (%) [a]	Matched peptide coverage (%) [b]	Molecular mass (kDa) [c]		*pI* [c]		Top ranking protein in MS-Fit search [d]	CAI [e]	Swiss Prot Accession No.
			Exp	Pred	Exp	Pred			
1	65	22	110	102	5.3	5.2	Hsp104 (heat shock protein)	0.305	P31539
2	34	16	95	77.6	5.4	5.38	Sse2 (heat shock protein 70)	0.192	P32590
3	56	16	60	46.9	4.89	4.72	Shp1 (potential regulatory subunit for Glc7p)	0.144	P34223
4	83	12	60	55.4	5.9	6.1	Glk1 (aldohexose specific glucokinase)	0.158	P17709
5	64	21	50	50.2	6.7	7.34	Cor1 (ubiquinol--cytochrome-c reductase protein)	0.293	P07256
6	14	37	29	23.8	5.31	5.22	Hsp26 (heat shock protein 26)	0.338	P15992
7	46	22	47 (frg) [f]	69.7	5.3	4.82	Ssa1 (heat shock protein 70)	0.709	P10591
8	60	35	60	46.9	5.8	5.67	Eno2 (enolase II (2-phosphoglycerate dehydratase)	0.892	P00925
9	67	29	50	46.8	6.7	6.6	Eno1 (enolase I (2-phosphoglycerate dehydratase)	0.871	P00924
10	27	8	27	26.8	5.74	5.86	Tpi1 (triose-phosphate isomerase)	0.817	P00942
11	24	12	31	60.9	5.1	6.31	Pck1 (phosphoenolpyruvate carboxykinase)	0.311	P10963
12	71	16	45	40.4	6.3	8.34	Qcr2 (ubiquinol--cytochrome-c reductase) chainII)	0.227	P07257
13	36	35	33	27.5	4.4	4.39	Grx4 (member of glutaredoxins Grx3, 4, 5)	0.144	P32642

[a - e] Refer to Table 4.1.

[f] The term 'frg' in brackets indicates that the protein detected was a proteolytic cleavage fragment.

Table 4.4 Evidence for identified proteins

Spot No.	Protein [a]	Net-Phos [b]	Functional evidence [c]	Database [d]	Reference [e]
1	Hsp104p	+	ATP binding	SwissProt	-
2	Sse2p	+	ATP binding	SwissProt	-
3	Shp1p	+	Regulator of phosphatase1	MIPS	Ficarro *et al.*, 2002
4	Glk1p	+	ATP binding/phosphorylation	SwissProt	-
5	Cor1p	+	-	-	-
6	Hsp26p	+	-	-	Ficarro *et al.*, 2002
7	Ssa1p	+	ATP binding	SwissProt	-
8	Eno2p	+	Converts 2-phospho-D-glycerate to phosphoenolpyruvate + H2O.	SwissProt	Ficarro *et al.*, 2002
9	Eno1p	+	Converts 2-phospho-D-glycerate to phosphoenolpyruvate + H2O	SwissProt	Ficarro *et al.*, 2002
10	Tpi1p	+	Catalyses the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate.	MIPS	-
11	Pck1p	+	ATP binding/phosphorylation	SwissProt	-
12	Qcr2p	+	-	-	-
13	Grx4p	+	Phosphorylation	-	Lopreiato *et al.*,

[a] - [e]Refer to Table 4.2.

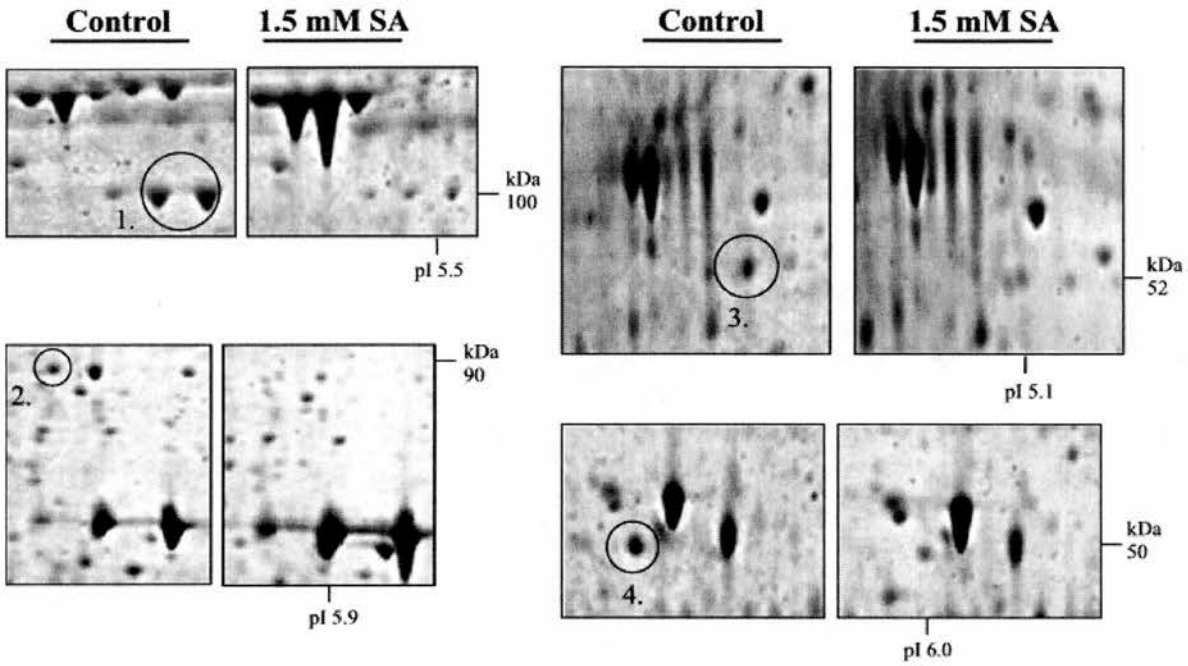


Figure 4.11 Proteins showing reduced protein phosphorylation due to growth in the presence of sorbic acid. Proteins prepared from control cells (YEPD, pH 4.5) and cells grown in the presence of sorbic acid YEPD, pH 4.5 plus 1.5 mM sorbic acid) were passed through independent phosphoprotein affinity columns and the eluate fractions compared by 2D-PAGE in conjunction with SYPRO® Ruby staining. Protein spots showing obvious and reproducible changes in the level of protein phosphorylation are indicated and were identified by peptide mass fingerprinting (Table 4.5). A representative result of at least two replicate experiments is shown. Molecular weights are shown on the left, and p*I* values are indicated.

Table 4.5 Identification of yeast phosphoproteins by peptide mass fingerprinting: proteins showing reduced phosphorylation due to sorbic acid.

Spot No.	No. of matching peptides (%) [a]	Matched peptide coverage (%) [b]	Molecular mass (kDa) [c]		pI [c]		Top ranking protein in MS-Fit search [d]	CAI [e]	Swiss Prot Accession No.
			Exp	Pred	Exp	Pred			
1	44	16	110	88.1	5.7	5.88	Prt1p (translation initiation factor eIF3)	0.304	P06103
2	61	19	92	75.4	5.9	5.88	Grs1p (glycine-tRNA ligase)	0.414	P38088
3	27	12	52	41.8	5.1	4.91	Sam1p (S-adenosylmethionine synthetase1)	0.494	P10659
4	23	22	50	36.4	6.0	8.34	Esf2p (Eighteen S processing Factor)	0.179	P53743

[a - e] Refer to TABLE 4.1.

Table 4.6 Evidence for identified proteins

Spot No.	Protein [a]	Net-Phos [b]	Functional evidence [c]	Database [d]	Reference [e]
1	Prt1p	+	-	-	-
2	Grs1p	+	ATP binding	SwissProt	-
3	Sam1p	+	ATP binding	SwissProt	-
4	Esf2p	+	-	-	-

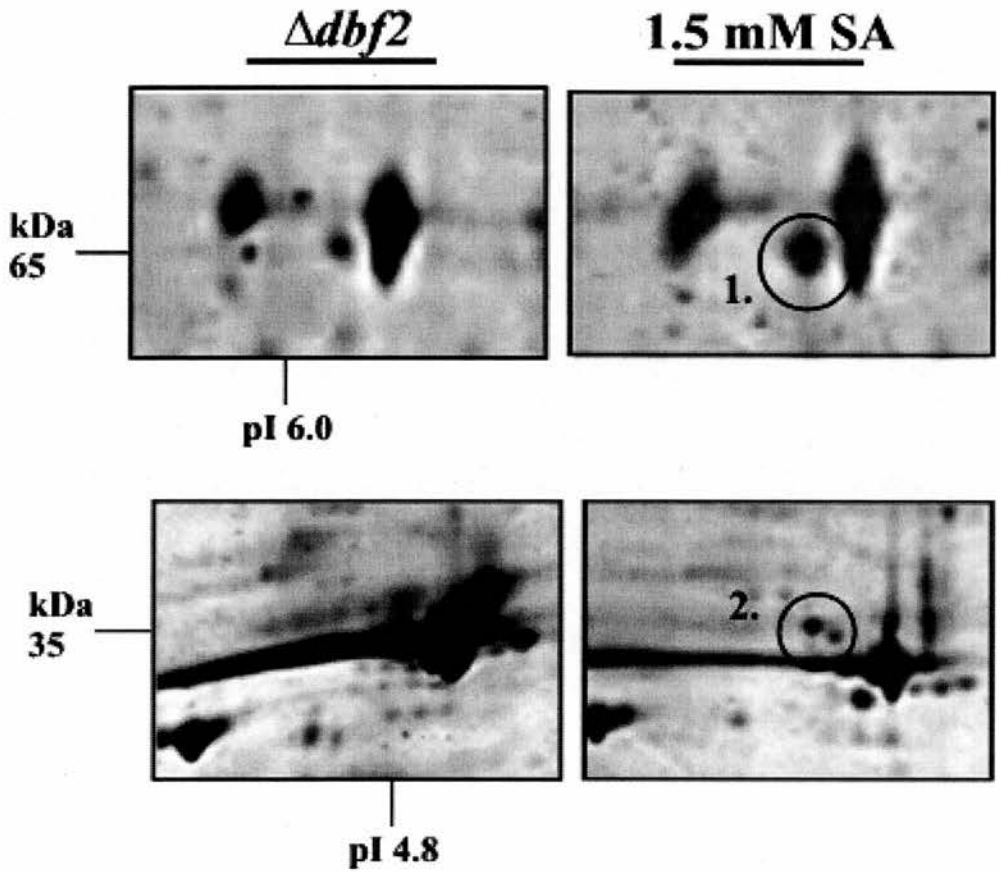


Figure 4.12 Proteins showing increased protein phosphorylation due to growth in sorbic acid in *Δdbf2* mutants. Proteins prepared from *Δdbf2* cells and cells grown in the presence of sorbic acid (YEPD, pH 4.5 plus 1.5 mM sorbic acid) were passed through independent phosphoprotein affinity columns and the eluate fractions compared by 2D-PAGE in conjunction with SYPRO® Ruby staining. Protein spots showing obvious and reproducible changes in the level of protein phosphorylation are indicated and were identified by peptide mass fingerprinting (Table 4.7). A representative result of at least two replicate experiments is shown. Molecular weights are shown on the left, and p*I* values are indicated.

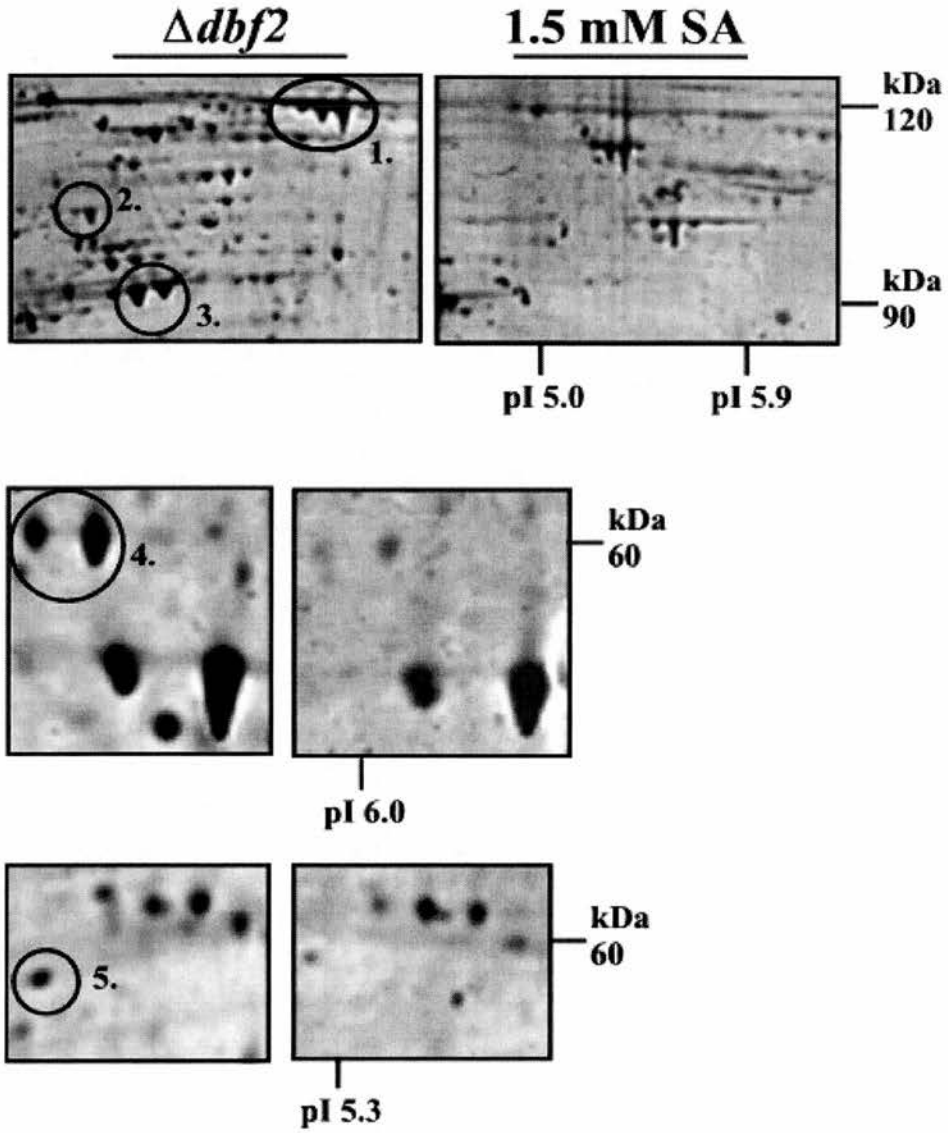


Figure 4.13 Proteins showing reduced phosphorylation due to growth in sorbic acid in the *Δdbf2* mutants. Proteins prepared from *Δdbf2* cells (YEPD, pH 4.5) and cells grown in the presence of sorbic acid YEPD, pH 4.5 plus 1.5 mM sorbic acid) were passed through independent phosphoprotein affinity columns and the eluate fractions compared by 2D-PAGE in conjunction with SYPRO® Ruby staining. Proteins showing obvious and reproducible changes in the level of protein phosphorylation are indicated and were identified by peptide mass fingerprinting (Table 4.8). A representative result of at least two replicate experiments is shown. Molecular weights are shown on the right, and p*I* values are indicated.

Table 4.7 Identification of yeast phosphoproteins by peptide mass fingerprinting: proteins showing increased phosphorylation due to sorbic acid in the Δ*dbf2* mutant strain.

Spot No.	No. of matching peptides (%) [a]	Matched peptide coverage (%) [b]	Molecular mass (kDa) [c]		pI [c]		Top ranking protein in MS-Fit search [d]	CAI [e]	Swiss Prot Accession No.
			Exp	Pred	Exp	Pred			
1	70	16	60	55.4	5.9	6.1	Glk1p (aldohexose specific glucokinase)	0.158	P17709
2	15	28	35	32.03	4.80	4.89	Pcl7p (cyclin like protein interacting with Pho85p)	0.131	P40186

Table 4.8 Identification of yeast phosphoproteins by peptide mass fingerprinting: proteins showing reduced phosphorylation due to sorbic acid in the Δ*dbf2* mutant strain.

Spot No.	No. of matching peptides (%) [a]	Matched peptide coverage (%) [b]	Molecular mass (kDa) [c]		pI [c]		Top ranking protein in MS-Fit search [d]	CAI [e]	Swiss Prot Accession No.
			Exp	Pred	Exp	Pred			
1	30	14	120	116	5.90	5.95	Yef3p (translation elongation factor eEF3)	0.778	P16521
2	28	29	110	77.3	5.20	4.97	Sse1p or Sse2p (heat shock protein 70)	0.521/0.192	P32589/90
3	67	23	90	67	5.30	5.18	Ssb1 (heat shock protein)	0.820	P11484
4	75	12	60	53.5	5.90	6.02	Srm1 (GDP/GTP exchange factor)	0.204	P21827
5	60	13	60	55	5.30	5.52	Atp2 (beta subunit of F_1F_0 ATP synthase)	0.425	P00830

Table 4.9 Evidence for identified proteins

Spot No.	Protein [a]	Net-Phos [b]	Functional evidence [c]	Database [d]	Reference [e]
1	Yef3p	+	ATP binding/phosphorylation	SwissProt	Ficarro *et al.*, 2002
2	Sse1/2p	+	ATP binding	SwissProt	-
3	Ssb1p	+	ATP binding	SwissProt	
4	Srm1p	+	Phosphorylation	-	Fleischmann *et al.*, 1996
5	Atp2p	+	ATP synthesis/binding	SwissProt	Ichikawa *et al.*, 2004

4.5.2 Deletion of *DBF2* results in significant changes in the yeast phosphoproteome

Comparison of the wild type phosphoprotein map with that of the Δ*dbf2* mutant strain resulted in the increased abundance, or unique expression, of four putative phosphoproteins, due to deletion of *DBF2*. These putative phosphoproteins were subsequently identified by peptide mass fingerprinting (Figure 4.14 and Table 4.10). Glk1p, Eno2p, Ssc1p and Egd2p were found to have enhanced phosphorylation in the Δ*dbf2* strain.

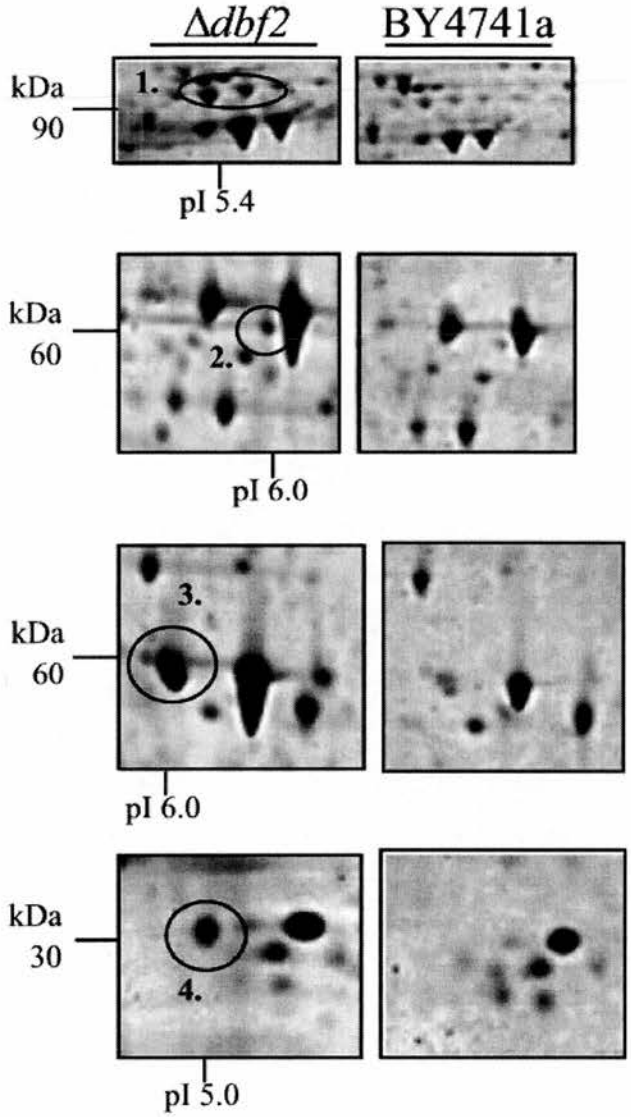


Figure 4.14 Proteins showing reduced phosphorylation due to loss of *DBF2*. Proteins prepared from Δ*dbf2* mutants (YEPD, pH 4.5) and wild type (WT) grown in the presence of sorbic acid YEPD, pH 4.5 plus 1.5 mM sorbic acid) were passed through independent phosphoprotein affinity columns and the eluate fractions compared by 2D-PAGE in conjunction with SYPRO® Ruby staining. Proteins showing obvious and reproducible changes in the level of protein phosphorylation are indicated and were identified by peptide mass fingerprinting (Table 4.10). A representative result of at least two replicate experiments is shown. Molecular weights are shown on the right, and p*I* values are indicated.

Table 4.10 Identification of yeast phosphoproteins by peptide mass fingerprinting: proteins showing induced phosphorylation due to loss of *DBF2* under acidic conditions

Spot No.	No. of matching peptides (%) [a]	Matched peptide coverage (%) [b]	Molecular mass (kDa) [c]		pI [c]		Top ranking protein in MS-Fit search [d]	CAI [e]	Swiss Prot Accession No.
			Exp	Pred	Exp	Pred			
1	60	16	90	70.5	5.3	5.48	Ssc1p (mitochondrial matrix protein involved in protein import)	0.521	P12398
2	33	18	60	55.7	6.0	5.8	Glk1p (aldohexose specific glucokinase)	0.158	P17709
3	60	35	60	47	6.0	5.6	Eno2p (enolase II (2-phosphoglyceratedehydratase)	0.892	P00925
4	34	45	30	18.2	5.0	4.84	Egd2p (subunit of the heteromeric nascent polypeptide)	0.617	P38879

4.5.3 Deletion of *DBF2* resulted in reduced abundance of the regulatory subunit of the vacuolar H^+-ATPase protein, Vma2p

Due to the low amounts of protein on the gels and to limitations of mass spectrometry facility at the time of the study, comparison of the *S. cerevisiae* BY4741a and *Δdbf2* phosphoproteome resulted in the identification of one protein (protein 1) that was reproducibly absent from the *Δdbf2* strain relative to the parent (Table 4.11; Figure 4.15). The protein was excised from the parent gel and identified by peptide mass fingerprinting to be Vma2p, the B subunit protein of the V-ATPase with 'non-catalytic' nucleotide binding sites (Liu et al., 1996; Vasilyeva and Forgac, 1996) (Table 4.11). In fact, two different isoforms of Vma2p were present on the gel from the parent strain. Protein (2) immediately adjacent to Protein (1) was also identified to be Vma2p, and this was the most abundant form with an approximate molecular weight of 58kDa and a *p*I of approximately 5.1. Only this isoform of Vma2p was present on the phosphoprotein gel from *Δdbf2*. Notably, the second iso-form of Vma2p (Protein 1) that was completely absent from the phosphoprotein gel of *Δdbf2* had a more acidic *p*I (approximately 4.9) and a slightly higher molecular weight, consistent with this isoform being phosphorylated.

The loss of the apparently phosphorylated isoform of Vma2p in *Δdbf2* was unaffected by the presence of sorbic acid and thus could be attributed solely to the deletion of *DBF2*.

Table 4.11 Vma2p is showing reduced abundance in the *Δdbf2* mutant strain under acidic conditions

Spot No.	No. of matching peptides (%) [a]	Matched peptide coverage (%) [b]	Molecular mass (kDa) [c]		*p*I [c]		Top ranking protein in MS-Fit search [d]	Swiss Prot Accession No.
			Exp	Pred	Exp	Pred		
1	70	12	60	57.7	5.0	5.1	Vma2p (H^+-ATPase V1 domain subunit, vacuolar)	P16140
2	62	13	60	57.07	5.1	5.1	Vma2p (H^+-ATPase V1 domain subunit, vacuolar)	P16140

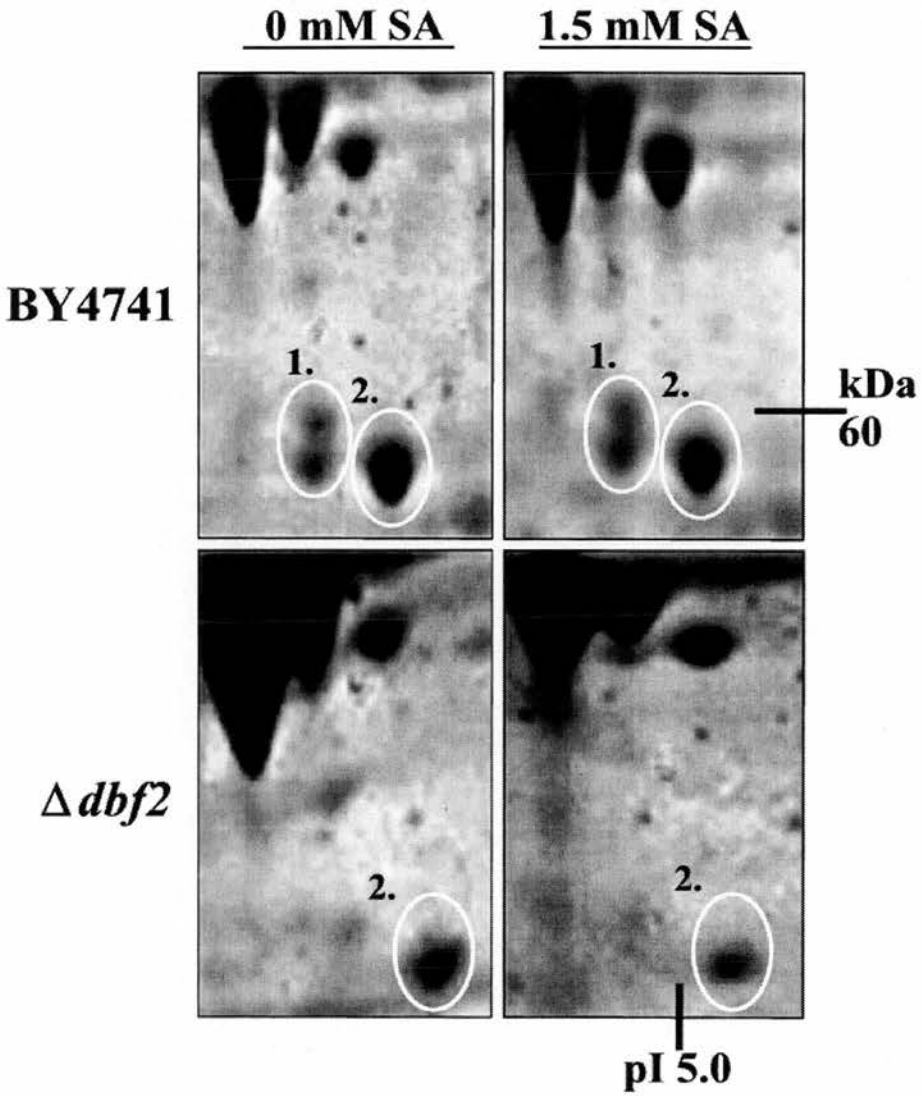


Figure 4.15 Comparison of protein phosphorylation in wild type and *Δdbf2* deletion strain in the presence and absence of sorbic acid. Total soluble proteins were separated by IEF over the pH range 4 - 7. Proteins were separated from untreated (control) cells (grown in YEPD medium, pH 4.5) and cells grown in the presence of sorbic acid (SA). We detected and subsequently identified a vacuolar protein, Vma2p (circled), whose phosphorylation was repressed in the *Δdbf2* mutant, both in the control and treated cells. Molecular mass (kDa) is shown on the right, and pI values are indicated. A representative result of two replicate experiments is shown.

4.5.4 Expression levels of Vma2p remain the same under sorbate stress

To determine whether the 2D-phosphoproteome analysis was indicating decreased expression of the putative Vma2p phosphoprotein the expression level of the protein was studied. Cell lysates of wild type (BY4741a) and Δ*dbf2* mutant strains stressed with the same amount of sorbic acid (1.5 mM) and harvested at the same OD as were in the phosphoproteome analysis. These samples were then run on 1D-PAGE and immunoblotted on nitrocellulose probing with commercially available monoclonal antibody against Vma2p.

Western blotting analysis showed that the proteins' basal and sorbate induced expression levels remained the same both in the presence and absence of *DBF2*. A constitutively expressed protein, Actin1 (Act1) was used as a loading control (Figure 4.16).

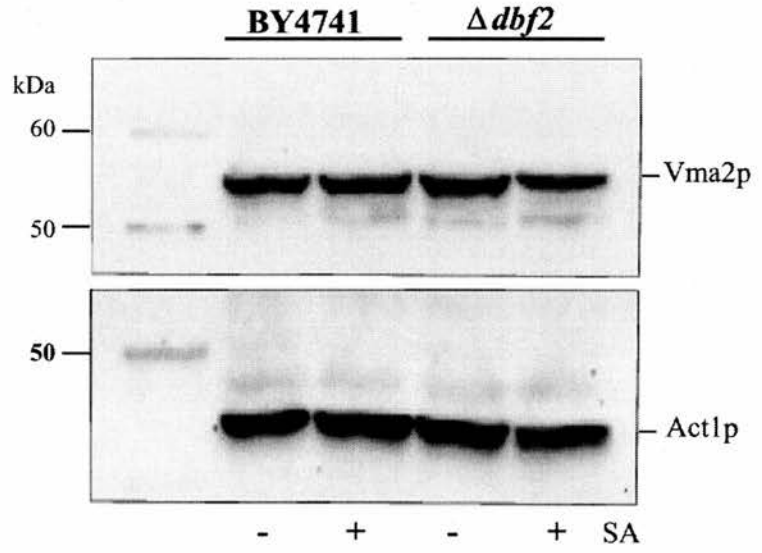


Figure 4.16 Loss of *DBF2* does not affect the basal or sorbate-induced levels of Vma2p. Wild-type (BY4741a) and Δ*dbf2* mutant cells were grown in YEPD, pH 4.5 medium at 30 °C. Cultures were divided and further grown with and without 1.5 mM sorbic acid (denoted by +/-) for 2 hours. Cell lysates were subjected to Western blotting with anti-H^+- ATPase 69 kDa subunit 8B1 monoclonal antibody (Molecular Probes). To check that equal amounts of protein were loaded the blot was stripped and reprobed for Act1p that is constitutively expressed. Vma2p expression levels remain consistent between BY4741a and Δ*dbf2* +/- sorbic acid. A representative result of three replicate experiments is shown.

4.5.5 Vma2p was only detected in the eluate fraction of the phosphocolumns

We have demonstrated that Vma2p is a putative phosphoprotein by 2-D PAGE analysis using affinity purification enriched for phosphoproteins. Furthermore, we have shown that reduced abundance of the protein was not a result of sorbic acid stress but due to the deletion of *DBF2*.

To provide further evidence that Vma2p is a putative phosphoprotein, total lysate treated and untreated with 1.5 mM sorbic acid was passaged through the affinity column and the subsequent flow-through (non-phosphorylated) and eluate (phosphorylated proteins) fractions were subjected to western blot analysis using monoclonal antibody against Vma2p (Figure 4.17). Vma2p was only detected in the eluate fraction as was expected, suggesting that Vma2p is a phopshoprotein (Figure 4.17B).

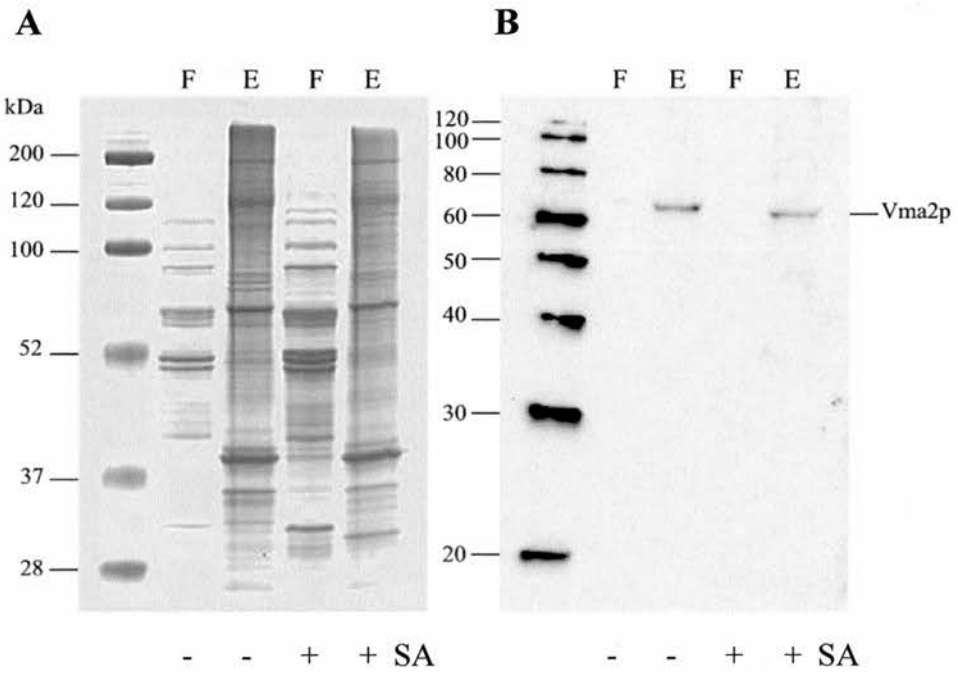


Figure 4.17 Phosphoprotein capture by affinity chromatography. Control and sorbate-induced BY4741a cells were applied to separate phosphocolumns and flow-through (F) and eluate (E) fractions were collected. Samples (20 μg) of these were run on 1D-PAGE and **A.** coomassie stained to show total protein in each fraction **B.** Western blotted to detect presence of Vma2p. Vma2p was detected only in the affinity-captured phosphoprotein fraction (eluate) in both control and sorbic acid treated cells.

4.6 Comparative analysis of the proteome of sorbic acid-stressed Δ*dbf2* mutant and control BY4741a strains

Proteomics was used to identify changes in protein expression between control BY4741a wild type and Δ*dbf2* mutant strains. Analysis of the *S. cerevisiae* proteome was performed using 2D-PAGE over three different pH ranges (pH 4.0-7.0, pH 4.5-5.5 and pH 6.0-11.0) in the presence and absence of 1.5 mM sorbic acid in both BY4741a and Δ*dbf2* strains. Proteins were only considered to have significant changes in levels of expression if the observed differences were repeated on a set of duplicate experiments. MALDI-TOF mass fingerprinting was used to determine the identities of these proteins.

4.6.1 Comparison of sorbate-induced changes in protein expression of control and Δ*dbf2* mutant strains

In the BY4741a wild type strain, over a pH range 4-7, four proteins were upregulated due to growth in the presence of 1.5 mM sorbic acid. The proteins were identified as: heat shock protein 26 (Hsp26p), two heat shock protein isoforms (Ssa1p and Ssb2p) and enolase I (Eno1p). The upregulated proteins on the pH 6-11 gels were identified as: alcohol dehydrogenase 3 (Adh3p), mitochondrial aconitase (Aco1p), a key enzyme in alcoholic fermentation, pyruvate decarboxylase (Pdc1p) and phosphoglycerate mutase of the glycolytic pathway (Gpm1p). Over the narrow range pH 4.5-5.5 following sorbic acid treatment the upregulated proteins identified were: Enolase 1 (Eno1p) and fructose 1,6-bisphosphate adolase (Fba1p). No proteins were found downregulated due to growth in the presence of sorbic acid.

The 2-D maps of the Δ*dbf2* deletion strain did not show significant differences following growth under sorbic acid stress. Therefore over a pH range of 4-7 the same proteins that were identified in the control gels were also upregulated under sorbic acid stress when *DBF2* was deleted (Figure 4.18 A, B). Over the pH range 6-11 only one protein, a translational elongation factor EF-1 alpha (Tef1p) was upregulated in regard

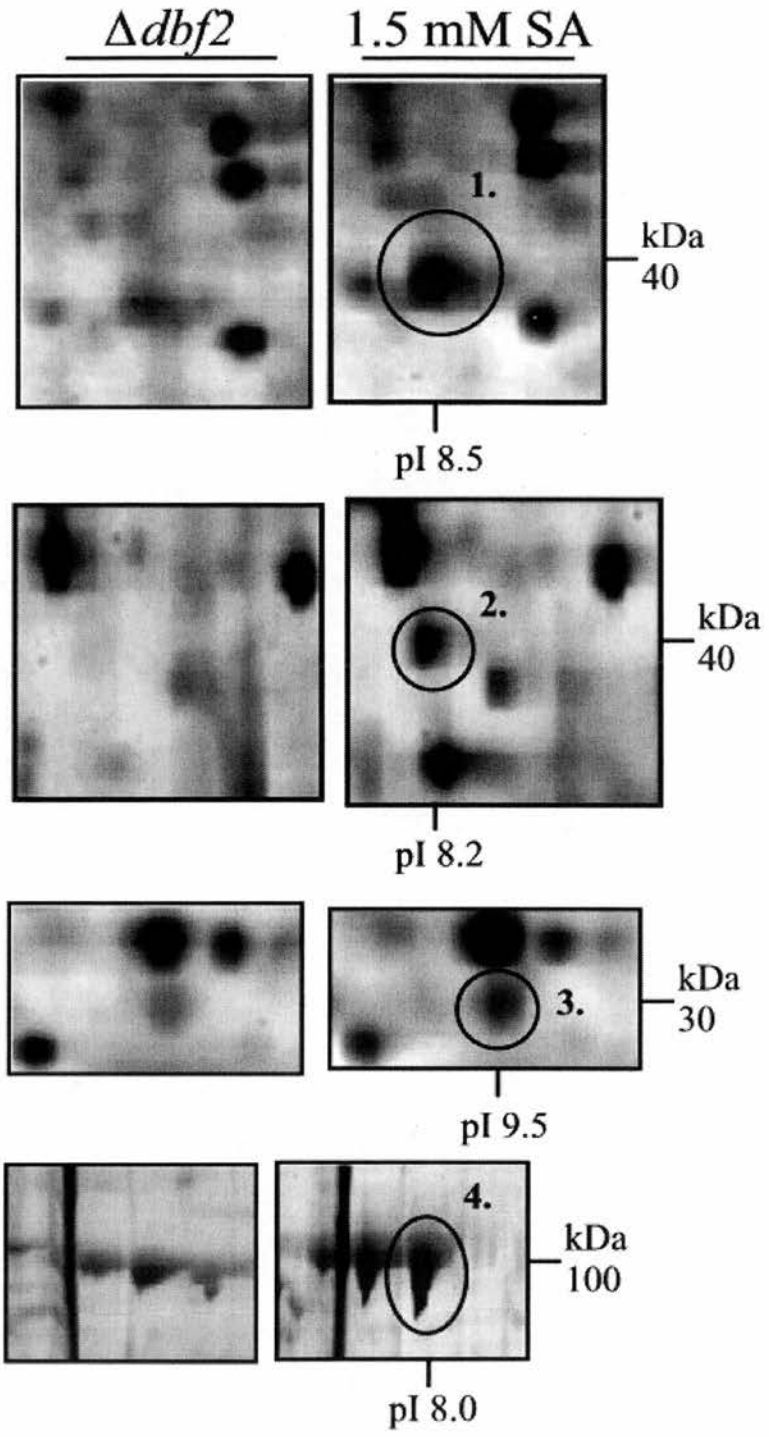
to sorbic acid stress (Figure 4.18A). No differences in protein expression were identified in the narrow range pH 4.5-5.5 gels.

Comparison of the experimental to predicted molecular weight, *p*I and per cent (%) of peptide coverage of the identified proteins are shown in Table 4.12. As the proteins upregulated in regard to sorbic acid were the same in both control and Δ*dbf2* deletion gels, only spots of the latter are shown (Figure 4.18; Table 4.12).

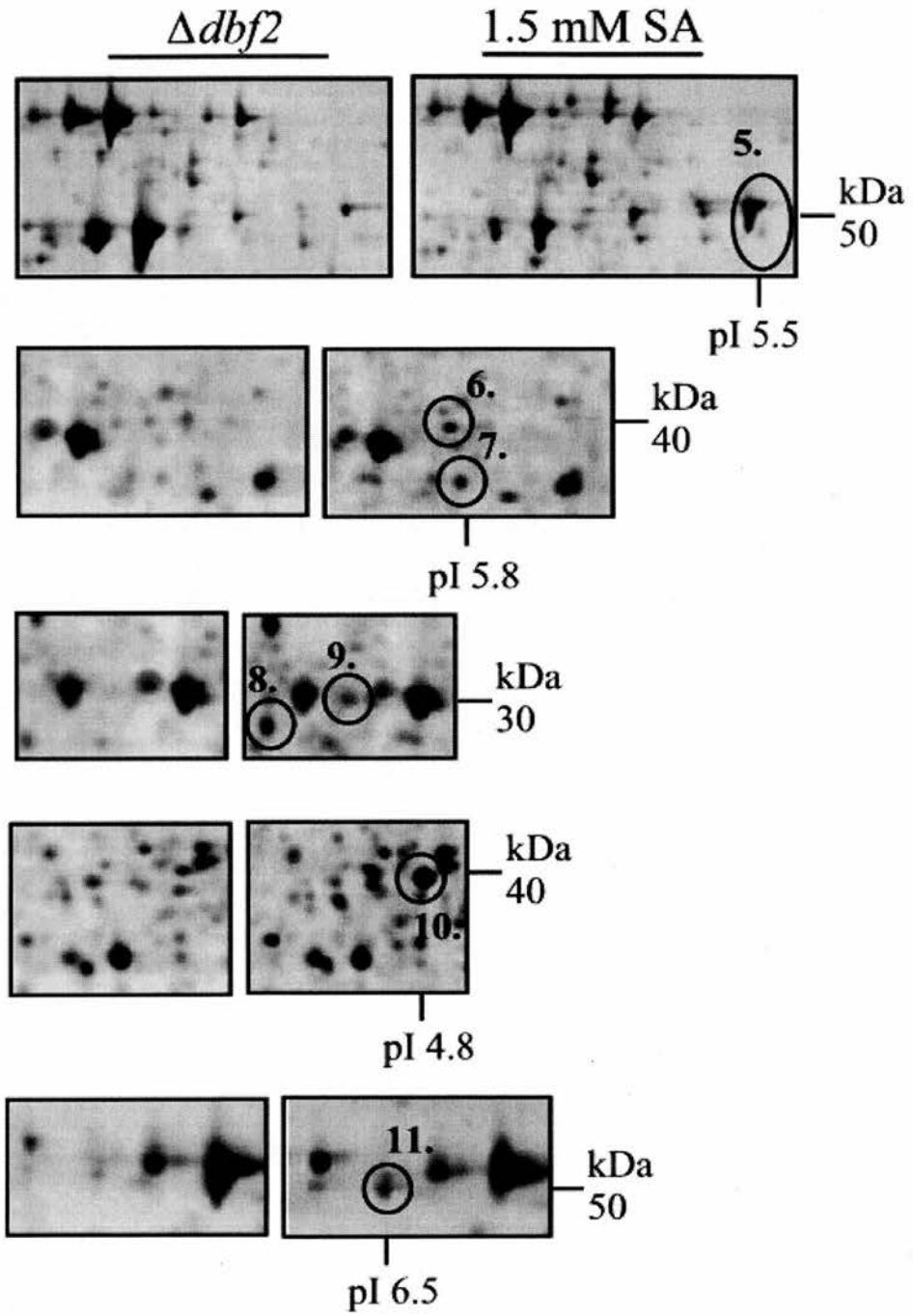
4.6.2 Comparison of sorbate-induced changes in protein expression due to *DBF2* deletion

Comparison between the control BY4741a and the Δ*dbf2* mutant 2-D gels following sorbic acid stress resulted in identification of a total of three proteins with altered expression. Two of them were upregulated due to loss of the *DBF2* gene. They were identified as a mitochondrial porin (Por1p) and the cytoplasmic malate dehydrogenase (Mdh3p) (Figure 4.13C; Table 4.12). Only one protein was identified as being downregulated, a protein component of the small ribosomal subunit (Rps0ap) (Figure 4.13C; Table 4.12).

A.


Δdbf2
1.5 mM SA
1.
kDa
40
pI 8.5
2.
kDa
40
pI 8.2
3.
kDa
30
pI 9.5
4.
kDa
100
pI 8.0

B.


Δdbf2
1.5 mM SA
5.
kDa
50
pI 5.5
6.
7.
kDa
40
pI 5.8
8.
9.
kDa
30
kDa
40
10.
pI 4.8
11.
kDa
50
pI 6.5

C.

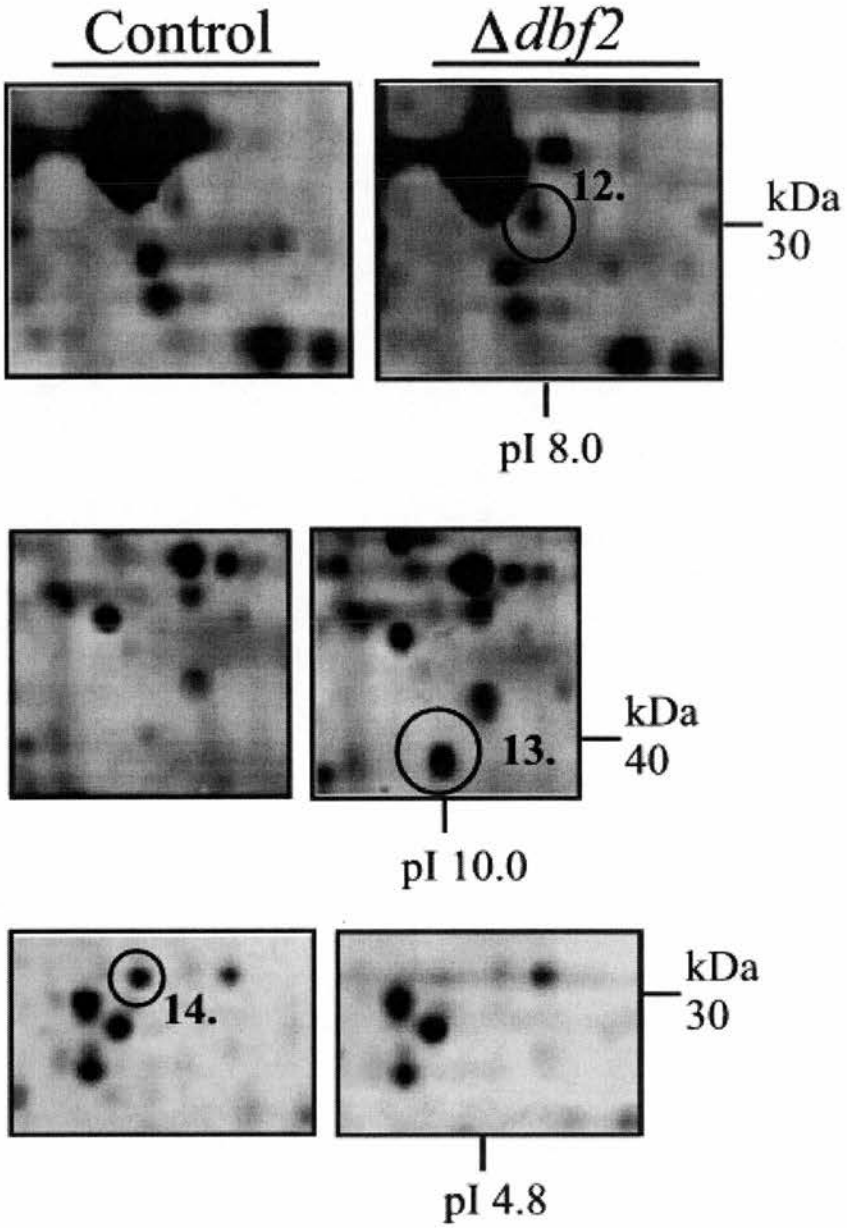


Figure 4.18 Proteins with altered expression levels. A, B. Proteins with altered expression due to sorbic acid stress in the Δ*dbf2* mutant strain and **C.** Proteins exhibiting changes in expression due to loss of *DBF2*. Comparison was achieved by 2D-PAGE in conjunction with SYPRO® Ruby staining. Proteins showing obvious and reproducible changes in the level of protein phosphorylation are indicated and were identified by peptide mass fingerprinting (Table 4.12). A representative result of at least two replicate experiments is shown. Molecular weights are shown on the right, and p*I* values are indicated

Table 4.12 Identification of yeast up- and down-regulated proteins by peptide mass fingerprinting

Spot No.	No. of matching peptides (%) [a]	Matched peptide coverage (%) [b]	Molecular mass (kDa) [c]		pI [c]		Top ranking protein in MS-Fit search [d]	Swiss Prot Accession No.
			Exp	Pred	Exp	Pred		
1	43	23	25 frg	40.36	8.4	8.3	Adh3p (Alcohol DeHydrogenase)	P07246
2	25	28	37	40.5	8.5	8.13	Tef1p (translation elongation factor 1-alpha)	P02994
3	43	35	22	27.6	9.2	9.6	Gpm1p (phosphoglycerate mutase)	P00950
4	50	35	100	85.7	7.6	8.17	Aco1p (aconitate hydratase)	P19414
5	64	40	50	46.9	6.0	5.67	Eno2p (enolase 2-phosphoglycerate dehydratase	P00925
6	42	23	40	39.6	5.8	5.65	Fba1p (belongs to class II fructose-bisphosphate	P14540
7	35	15	40 frg	61.6	5.8	5.8	Pdc1p (pyruvate decarboxylase)	P06169
8	70	8	30 frg	66.5	5.4	5.24	Ssb2p (heat shock protein of HSP70 family)	P40150
9	61	42	28	23.8	5.4	5.31	Hsp26p (heat shock protein 26)	P15992
10	50	15	29frg	69.7	4.7	4.82	Ssa1p (heat shock protein)	P10591
11	67	50	50	46.7	6.5	6.6	Eno1p (enolase I/(2-phosphoglycerate)dehydratase	P00924
12	14	26	26	30.4	9.0	8.1	Por1p (porin voltage-dependent anion channel)	P04840
13	44	29	37	37.1	10.0	10.0	Mdh3p (malate dehydrogenase)	P32419
14	27	26	30	28	4.7	4.47	RpsOap (Ribosomal Protein of the Small subunit)	P32905

4.7 Discussion

4.7.1 Phosphoproteome analysis.

Recently, global analysis of the yeast phosphoproteome was reported by (Ficarro et al., 2002). The authors detected more than 1000 phosphopeptides, resulting in the identification of 216 peptide sequences defining 383 phosphorylation sites; only 18 of which were previously identified. Whilst this work presented a major advance in global studies of the phosphoproteome, limitations include the expensive and complex technical nature of the approach and the subsequent claim that this method was biased to multiply phosphorylated peptides and peptides originating from abundant proteins (Metodiev et al., 2004).

In the work presented in this chapter, a novel proteomic approach to the enrichment, visualisation and identification of phosphoproteins in *S. cerevisiae* is presented. Key to the methodology is the use of the Qiagen PhosphoProtein enrichment kit, originally developed for use with mammalian cells. The kit consists of an affinity column that binds phosphoproteins thus permitting their enrichment and specific buffers for washing and elution. Unfortunately, due to the proprietary nature of the affinity matrix, critical evaluation of the efficacy of the method was difficult. Therefore, the first part of this work concerns an assessment of the validity of using this approach to study the yeast phosphoproteome. In this regard, the data generated by (Ficarro et al., 2002), discussed above, in conjunction with other available literature and database evidence of protein phosphorylation, provided a useful benchmark to judge the validity of this affinity-based approach.

Two-dimensional PAGE analysis of the phosphoproteins eluted from the affinity column displayed approximately 296 protein spots (Figure 4.4), which compares favourably with the yeast phosphoproteome published by (Futcher et al., 1999), who detected approximately 300 distinct spots' using a radiolabelling approach with ^{32}P. Visual comparison of the protein pattern obtained by (Futcher et al., 1999) with the phosphoprotein-enriched, eluate fraction gel revealed a close similarity in appearance. Further supporting the efficacy of the affinity-based approach, (Futcher et al., 1999)

identified 14 phosphoproteins by ^{32}P labelling. Six of these were identified on the gels of the phosphoprotein-enriched eluate fraction (Rpp0p, Yef3p, Ssa1p, Ssb1p, Tpi1p and Eno2p). Furthermore, 24 of the 30 putative phosphoproteins identified during this study had previous literature evidence of phosphorylation (Tables 4.2 and 4.4). Only five proteins had no previous evidence of phosphorylation (Cor1p, Qcr2p, Prt1p, Act2p and Esf2p). Thus, the presented evidence suggested that this method reliably detects phosphoproteins. The above results, together with the observations that: a) monoclonal antibodies to phospho-serine, -threonine and –tyrosine detected the majority of proteins in the eluate fraction; b) Pro-Q Diamond phosphoprotein stain only detected proteins in the eluate fraction from the affinity column; and finally, c) prior incubation of the cellular protein extract with CIP before loading onto the phosphoprotein affinity column resulted in a significant reduction in the number of proteins detected in the phosphoprotein-enriched eluate fraction; lead to the conclusion that this affinity purification-based protocol used in this study is an effective method to analyse the yeast phosphoproteome. Furthermore, entry of all proteins whose phosphorylation state or expression was enhanced by sorbic acid or by loss of *DBF2* gene into the NetPhos database revealed putative phosphorylation sites on serine, threonine and tyrosine residues.

To assess the ability of the affinity purification step to allow detection of low abundance proteins, the codon bias of identified phosphoproteins, in the form of the codon adaptation index (CAI) from the SGD database, was examined. There is good correlation between CAI and protein abundance, with the relationship being log linear between 1,000,000 to 10,000 molecules per cell (Futcher et al., 1999). Thus, CAI values of 0.2 and 1.0 represent approximately 10,000 and 1,000,000 molecules per cell respectively. The majority (12 from 17) of the phosphoproteins detected with altered abundance due to exposure to sorbic acid, had a CAI greater than 0.2, and would be classed as abundant (Tables 4.1 and 4.3). However, 5 phosphoproteins, out of a total of 17, were detected with CAI values of less than 0.2, suggesting that affinity purification coupled to 2D-PAGE can allow for the detection of some low abundance proteins that would not normally be detected by standard 2D-PAGE gels (Futcher et al., 1999).

4.7.2 Exposure to sorbic acid and loss of *DBF2* gene resulted in changes in the phosphoproteome

Growth in the presence of sorbic acid resulted in 12 putative phosphoproteins reproducibly showing induced phosphorylation relative to the untreated sample in the control BY4741a 2-D maps and in two proteins in the Δ*dbf2* 2-D maps. Furthermore, growth in sorbic acid also resulted in five proteins with reduced phosphorylation relative to the untreated sample in each set of gels. This result could mean that either the expression of a phosphorylated protein increases without any increase in phosphorylation of the protein or the phosphorylation state of a protein is increased without any increase in expression of the protein. The affinity column cannot discriminate between these two possible scenarios or indeed, the possible combination of the two. However, this does not invalidate the method because putative phosphoproteins that were responding to sorbic acid stress were still detected. Thus, a genomic screening tool such as the presented can identify important candidate phosphoproteins but further work is required for a more detailed study of these proteins to determine their precise role *in vivo*.

To address the above problem, the extensive published data on sorbic acid inducible gene/protein expression was compared with the altered phosphoproteins identified in this work. This allowed us to determine if there were any sorbic acid-inducible genes/proteins common to the different methods. In a previous study, the expression of Hsp26p, Eno2p, Ssa1p, and via microarrays *HSP26* was induced by sorbic acid (de Nobel et al., 2001). Similarly, in a later analysis of sorbic acid-inducible changes in the transcriptome, (Schuller et al., 2004) identified increased expression of *HSP26*, *SSE2* and *GLK1*. Both of these previous studies demonstrated that members of the stress-inducible Hsp70 family, as well as other smaller heat-shock proteins, were induced at both the transcript and protein level. Thus, it seems likely that apparent 'induced phosphorylation' of many of these general stress proteins was detected because the actual expression, and thus abundance, of these proteins was induced due to sorbic acid exposure. Yet, this may not necessarily be true. There may be a combination of both induced expression and induced phosphorylation of these proteins. Importantly, of all

the other phosphoproteins detected with sorbic acid-dependent altered phosphorylation state there was no prior evidence of sorbic acid-induced changes in the proteome and transcriptome (de Nobel et al., 2001; Schuller et al., 2004). Thus, the induced phosphorylation observed cannot be simply explained by increased expression of the protein. It seems that in the majority of cases real changes in levels of protein phosphorylation are being detected.

We have presented initial evidence that Shp1p is phosphorylated upon exposure to sorbic acid stress. Shp1p was previously identified as a phosphoprotein in the global study of Ficarro et al., (2002) but little is known about the function of the protein. Shp1p possesses a 'ubiquitin regulatory X' (UBX) domain which has structural similarity to ubiquitin. Shp1p is known to bind to Cdc48p, an abundant ATPase involved in the ATP-dependent disassembly of oligomeric substrate proteins and ubiquitin-mediated protein degradation by the proteasome. Thus, it has been proposed that Shp1p is an adaptor of degradative Cdc48p activities (Schuberth et al., 2004). Supported by an independent study (Mollapour et al., 2004), we have also shown that Shp1p must play a crucial role in mediating resistance to sorbic acid because deletion of SHP1 renders cells hypersensitive to sorbic acid stress. In fact, deletion of SHP1 results in defects in the degradation of a ubiquitylated model substrate, and sensitivity to various stress conditions known to generate elevated levels of aberrant proteins; including heat and heavy metal stress (Schuberth et al., 2004). Thus, on the basis of this evidence, the hypothesis that the sorbic acid-induced phosphorylation of Shp1p results in the activation of Shp1p and the promotion of aberrant protein removal via the proteasome could be investigated. The identification of any upstream components of a possible signalling pathway that results in the sorbic acid-dependent phosphorylation of Shp1p could prove very interesting.

Previous work has also demonstrated that sorbic acid acts on the mitochondrial inner membrane, enhancing superoxide free radical production by the electron transport chain and causing oxidative stress (Piper, 1999). Consistent with this observation, mutants lacking the *GRX5* mitochondrial glutaredoxin, a determinant of oxidative stress resistance, are sensitive to sorbic acid stress (Schuller et al., 2004). Furthermore, two independent transcriptome studies have shown that the genes for a number of glutaredoxin, and other oxidative stress defence enzymes, are induced by sorbic acid (de

Nobel et al., 2001; Schuller et al., 2004). Interestingly, we observed that Grx4p, another member of the glutaredoxin subfamily related to Grx3p and Grx5p, was phosphorylated in response to sorbic acid stress. The Grx family of proteins maintain the redox state of target proteins and protect cells from oxidative damage (Rodriguez-Manzaneque et al., 1999). Grx4p is known to be phosphorylated on Ser-134 by the Bud32p protein kinase. It has been proposed that this phosphorylation regulates the activity of Grx4p and thus controls redox state maintenance (Lopreiato et al., 2004). However, although our findings are consistent with previous work (Lopreiato et al., 2004), how this phosphorylation actually affects Grx4p, in terms of its activity and possible role in protecting cells from stress-induced oxidative damage, is unclear. Sorbic acid-dependent phosphorylation of Cor1p and Qcr2p, two components of the ubiquinol-cytochrome c reductase complex of the respiratory chain was also detected. Possibly related to the fact that sorbic acid induces oxidative stress. Future work will aim to explore the hypothesis that the stress-inducible phosphorylation of these enzymes regulates their activity perhaps to reduce the generation of harmful oxidative free radicals.

Another interesting finding was the identification of the regulatory subunit of the catalytic sector (V1) of the H^+-ATPase complex, Vma2p (Anraku, 1992; Forgac, 2000), whose phosphorylation level was reduced in the absence of *DBF2*. There is no published evidence suggesting that Vma2p is a phosphoprotein but in this experiment we have shown that Vma2p is a putative phosphoprotein and that its phosphorylation is related to the existence of the *DBF2* gene in the presence or absence of sorbic acid stress. Further experiments listed in Chapter 5 will provide more insight into the role of Vma2p in sorbic acid resistance mechanism and to the importance of Dbf2p for its regulation under stress conditions.

4.7.3 Proteomic analysis following exposure to sorbic acid and loss of *DBF2* gene resulted in changes in protein expression

In this study we have carried out 2D-PAGE analysis of changes in the proteome due to loss of the *DBF2* gene during growth in the presence of sorbic acid. Gels of the wild type BY4741a strain were used as a control for the analysis. In cells exposed to 1.5 mM sorbic acid many stress proteins and chaperones, such as Hsp26p and Hsp70p isoforms were upregulated in wild type cells. These data were consistent with a previous analysis (de Nobel et al., 2001). In the *Δdbf2* mutant cells we also detected upregulation of Ssa1p, Ssb1p and Hsp26p proteins. This suggested that the increased expression of these proteins in sorbic acid is not influenced by loss of the *DBF2* gene since they are also upregulated in the control cells. Their upregulation could be explained by either increased damage of intracellular proteins or due to an inhibitory effect of sorbate on protein synthesis and turnover leading to increased function (de Nobel et al., 2001).

One protein was identified to have downregulated expression following exposure to sorbic acid in the *Δdbf2* mutant cells namely the protein component of the small ribosomal subunit, Rps0Ap, required for maturation of 18S rRNA. It is noteworthy that many of the ribosomal proteins of the small and the large subunits of the ribosomes influence sorbic acid resistance. Loss of individual ribosomal genes had resulted in decreased (Table 3.1; (Mollapour et al., 2004)) or increased resistance (Mollapour et al., 2004). Thus, subtle alterations to the ribosomal protein composition might be important to sorbic acid adaptation. However, previous evidence of transcript analysis did not reveal changes in expression of the *RP* genes following sorbic acid stress (de Nobel et al., 2001).

Notably, loss of *DBF2* resulted in the upregulation of the mitochondrial oxidoreductase Adh3p and a mitochondrial porin; Por1p. The latter is required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability. Interestingly the *Δpor1* mutant was sensitive to sorbic acid (Table 3.1; (Mollapour et al., 2004)). In addition to the sorbate sensitivity previous transcription studies have shown increased expression of *POR1* following exposure to sorbic acid (de Nobel et al., 2001).

Parallel and comparative analysis of the phosphoproteome and proteome in *S. cerevisiae* control BY4741a and Δ*dbf2* mutant strains yielded distinct information. Notably, little correlation was found between the two methodologies (Table 4.13) since only a small number of proteins were found to have both altered expression and phosphorylation levels in this study in both strains (Hsp26p, Ssa1p and Eno1p) (Table 4.13).

Table 4.13 Comparative analysis of Phosphoproteome and Proteome

Changes due to Sorbate				**Changes due to Δ*dbf2***	
BY4741a		**Δ*dbf2***			
Phosphopr.	**Proteome**	**Phosphopr.**	**Proteome**	**Phosphopr.**	**Proteome**
Hsp104p ↑[a]	Ssb2p ↑[c]	Glk1p ↑	Ssb2p ↑	Ssc1p ↑	Por1p ↑
Sse2p ↑	Aco1p ↑	Pcl7p ↑	Aco1p ↑	Glk1p ↑	Mdh3p ↑
Shp1p ↑	Tef1p ↑		Tef1p ↑	Eno2p ↑	Rps0a ↓
Glk1p ↑	Gpm1p ↑		Gpm1p ↑	Egd2p ↑	
Cor1p ↑	Adh3p ↑		Adh3p ↑	Vma2p ↓	
Hsp26p ↑	Hsp26p ↑		Hsp26p ↑		
Ssa1p ↑	Ssa1p ↑		Ssa1p ↑	**Changes due to Δ*dbf2* and Sorbate**	
Eno1p ↑	Eno1p ↑		Eno1p ↑		
Eno2p ↑	Fba1p ↑		Fba1p ↑	Vma2p ↓	
Tpi1p ↑		Yef3 ↓[d]			
Pck1p ↑		Sse1 ↓			
Qcr2p ↑		Ssb1 ↓			
Grx4p ↑		Srm1 ↓			
Prt1p ↓[b]		Atp2 ↓			
Grs1p ↓					
Sam1p ↓					
Ynr054c ↓					

↑[a,c] Arrow indicates enhanced phosphorylation or upregulated expression
↓[b,d] Arrow indicates decreased phosphorylation or downregulated expression

In this work, we have combined affinity purification, 2D-PAGE and peptide mass fingerprinting to develop a fast, straight forward and sensitive method for studying changes in the yeast phosphoproteome (Makrantoni et al., 2005). The method provides enrichment with minimal sample handling and was ideally suited for phospho-proteomics. The approach provides a powerful genomic screening tool that can identify initial candidate phosphoproteins for further study. We performed a proteome analysis of wild type and Δ*dbf2* yeast strains and compared the results to the phosphoproteome

analysis. Little correlation was found between the two analyses of Δ*dbf2* mutants, further validating that the changes identified from the phosphoproteome analysis were not expression changes (Table 4.12).

From the phosphoprotein analysis we identified a vacuolar protein, Vma2p, whose phosphorylation level decreased when *DBF2* was deleted. Currently there is no published evidence of Vma2p modification by phosphorylation. Further experiments listed in the following chapter will provide more insight into the role of Vma2p in sorbic acid adaptation and the importance of the Dbf2p kinase for the regulation of Vma2p under acidic conditions.

CHAPTER 5

5. Investigating functional interactions between Dbf2p and Vma2p

5.1 Introduction

Vacuolar proton-translocating ATPases (V-ATPases) are large, complex enzymes responsible for the acidification of many internal compartments in eukaryotic cells (Nishi and Forgac, 2002; Stevens and Forgac, 1997) and are required for a range of cellular processes (Graham et al., 2000). In yeast, the V-ATPase acidifies the vacuole by driving the translocation of protons into the lumen of the vacuole (Graham et al., 2000). Vacuolar acidification is important for many cellular processes, including endocytosis, targeting of newly synthesized lysosomal enzymes and other molecular targeting processes. The V-ATPase is functionally and structurally related to the mitochondrial and the chloroplast F-ATPase. However, in contrast to the F-ATPase the V-ATPase is vital to almost every eukaryotic cell, excluding yeast, its destruction causes lethality (Nelson and Harvey, 1999).

The *S. cerevisiae* V-ATPase is composed of at least 13 subunits organized into two functional domains, V_1 and V_0 (Figure 5.1). The peripheral V_1 domain (subunits A-H) is responsible for ATP hydrolysis and is coupled to the mechanism of proton translocation. The integral V_0 domain is composed of five different subunits (subunits a, d, c, c′ and c″) and is responsible for the translocation of protons across the membrane (Figure 5.1, Table 5.1) (Graham et al., 2000).

Assembly of the yeast V-ATPase has been thoroughly studied. Deletion of any of the genes that encode V-ATPase subunits (except subunit H) leads to complete loss of assembly (Bowman and Bowman, 2000; Forgac, 1999; Stevens and Forgac, 1997). The nucleotide binding sites in the V-ATPase are located on the 70 kDa A and 60 kDa B subunits encoded by *VMA1* and *VMA2* respectively. Both subunits are present in three copies per complex (Forgac, 1999; Nishi and Forgac, 2002). The catalytic sites are located on the A subunits (Liu et al., 1996; Liu et al., 1997b; MacLeod et al., 1998). The B subunits also bind

nucleotides forming 'non-catalytic' nucleotide binding sites (Liu et al., 1996; Vasilyeva and Forgac, 1996). While the function of these sites is uncertain mutations suggest they may play a role in the regulation of activity (Forgac, 2000; MacLeod et al., 1998; Vasilyeva et al., 2000).

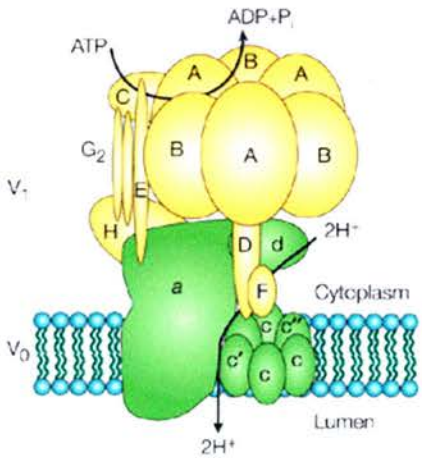


Table 5.1 Subunit composition of V-ATPases

Domain	Subunit	Gene	Function
V_1	A	*VMA1*	Catalytic site, regulation
	B	*VMA2*	Noncatalytic, targeting
	C	*VMA5*	Activity, assembly
	D	*VMA8*	Activity, assembly
	E	*VMA4*	Activity, assembly
	F	*VMA7*	Activity, assembly
	G	*VMA10*	Activity, assembly
	H	*VMA13*	Activity, not assembly
V_0	a	*VPH1*	H^+ transport, assembly, targeting
	d	*VMA6*	Activity, assembly
	c	*VMA3*	H^+ translocation, DCCD site
	c′	*VMA11*	H^+ translocation, DCCD site
	c″	*VMA16*	H^+ translocation

Figure 5.1 Structural model of the V-ATPase. The V_1 domain (shown in yellow) is a 570 kDa peripheral complex responsible for ATP hydrolysis, whereas the V_0 domain (shown in green) is a 260 kDa integral complex responsible for proton translocation. For details of subunits see Table 5.1 (Nishi and Forgac, 2002).

In this chapter we were interested in investigating the role the B subunit of the V-ATPase, encoded by *VMA2*, in sorbic acid resistance and the significance of its interaction with Dbf2p kinase. We have shown that loss of *VMA2* conferred marked sorbic acid sensitivity even at low concentrations (Table 3.1). This result is in agreement with published evidence from a concurrent study (Schuller et al., 2004). In the previous chapter, comparative phosphoproteome analysis using affinity phosphocolumns was performed between the sorbic acid sensitive Δ*dbf2* mutant and the wild type BY4741a strain. This analysis identified a reduced abundance of Vma2p in the Δ*dbf2* mutant strain, with only the less phosphorylated isoform of Vma2p present. To ensure that this result was not due to changes in expression levels of Vma2p, western blot analysis was performed using a commercially available monoclonal antibody against Vma2p. The Vma2p protein

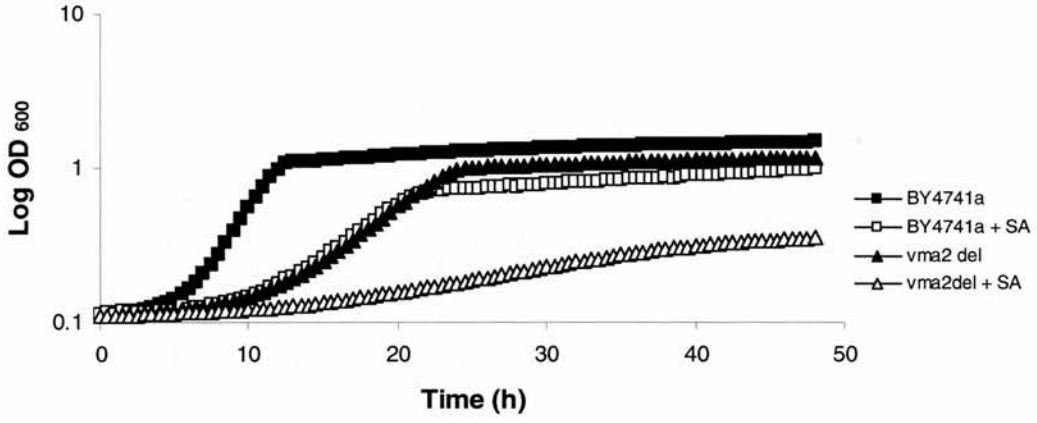
expression levels remained the same both in the presence and absence of *DBF2* under acidic conditions.

Following the previous experiments, in this chapter we have performed a series of biochemical experiments to investigate the possible link between the Dbf2p kinase and Vma2p subunit of the H^+-ATPase and their possible interaction in the same signaling pathway mediating sorbic acid resistance.

5.2 Deletion of *VMA2* results in significant growth inhibition upon exposure to sorbic acid

Growth of the *S. cerevisiae* Δ*vma2* mutant strain was almost completely inhibited in the presence of 1 mM sorbic acid both in YEPD, pH 4.5 medium and on agar (Figure 5.2A and 5.2B respectively). To confirm that the deletion of *VMA2* induced sorbate-sensitivity, complementation studies were performed as previously (section 2.5.1). Following the introduction of the *VMA2* into the Δ*vma2* deletion strain, subsequent testing on selective medium at pH 4.5 showed that sensitivity to sorbic acid was abolished (Figure 5.3). Notably, Δ*vma2* mutants are severely defective for growth in minimal medium (Figure 5.3).

A



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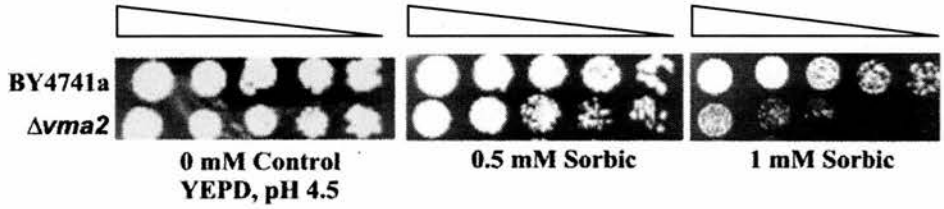


Figure 5.2 Comparison of growth in sorbic acid of wild type and Δ*vma2* strains showed severe Δ*vma2* growth inhibition. A. Cultures of control BY4741a strain and Δ*vma2* mutants were grown to late exponential phase in YEPD, pH 4.5 at 30°C with or without 1 mM sorbic acid. Growth was monitored for 48 hrs in a PowerWave ™ x S Universal Microplate Spectrophotometer (BIO-TEK® Instruments, GmbH). Readings were taken every 30 min **B.** Serial dilutions (denoted by triangles) of mid-exponential-phase cultures of BY4741a and Δ*vma2* strains were spotted onto YEPD [pH 4.5] agar with 0, 0.5 and 1.0 mM sorbic acid. Plates were incubated for 48h at 30°C before they were imaged. Data shown is representative of three independent experiments in each case.

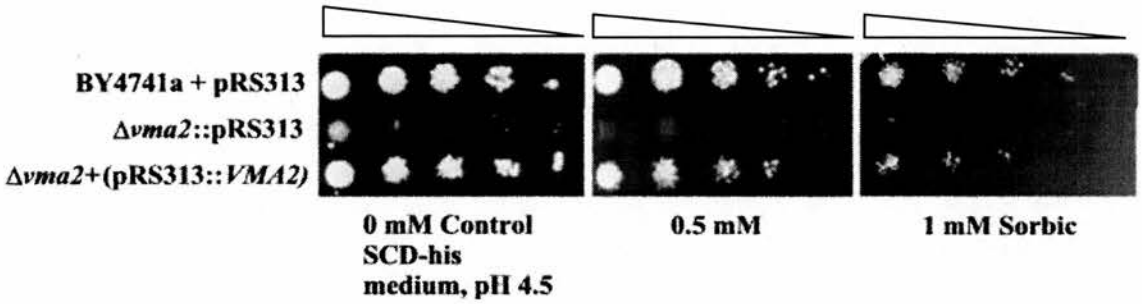


Figure 5.3 Complementation of the Δ*vma2* sorbic acid sensitive phenotype. Growth inhibition of *S. cerevisiae* BY4741, Δ*vma2* mutant, and Δ*vma2* mutant carrying *VMA2* on a shuttle plasmid (pRS313::*VMA2*) is shown on selective complete medium (SCD-his, pH 4.5) with 0, 0.5 and 1 mM sorbic acid. A representative result is shown.

5.3 Exposure to the vacuolar H^+-ATPase inhibitor bafilomycin mimics the effect of *VMA2* deletion following sorbic acid treatment

The vacuolar H^+-ATPase is inhibited with high specificity and potency by macrolide antibiotics such as bafilomycin both *in vitro* and *in vivo* (Figure 5.4) (Bowman et al., 2004; Drose and Altendorf, 1997; Drose et al., 1993). Despite the widespread use of this compound, understanding of the mode of action is rudimentary, though earlier reports indicate that bafilomycin acts on the Vo segment of the enzyme (Hanada et al., 1990; Zhang et al., 1994).

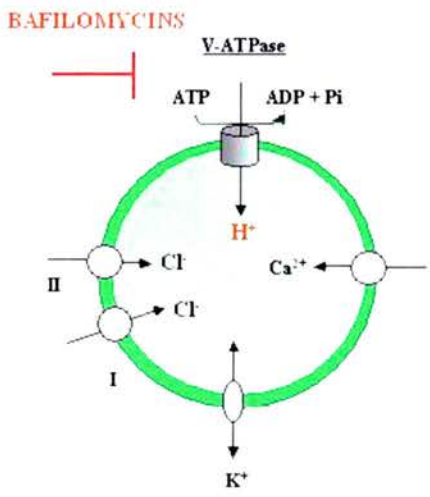


Figure 5.4 Schematic of inhibitory effect of bafilomycin. Bafilomycin is a highly potent inhibitor of V-ATPases, which is specifically inhibiting the proton pump possibly via acting on the Vo segment of the enzyme (Zhang et al., 1994).

In this study the effect of bafilomycin on BY4741a wild type strain was examined in the presence and absence of sorbic acid. As previously shown, treatment of wild type strains with 1 mM sorbic acid at pH 4.5 resulted in marked sensitivity (Piper et al., 1998). The same result was observed in the wild type strain (BY4741a) used in this study (Figure 5.2). Treatment of BY4741a cells with 10 μM bafilomycin on its own resulted in slight inhibition of growth (Figure 5.5). Notably, combination of sorbic acid and bafilomycin resulted in the same growth inhibition exhibited by deletion of *VMA2* gene as shown previously (Figure 5.2). Control experiments with 6% ethanol (solvent of bafilomycin) showed the same inhibition as bafilomycin on its own (Figure 5.5).

The above result indicates the importance of a fully functional vacuolar H^+-ATPase for optimal adaptation to sorbic acid stress. This observation is supported by previous published studies that showed that a functional V-ATPase is required for yeast adaptation

the organic herbicide 2,4-D (Fernandes et al., 2003) and that deletion of many of the components of the vacuolar H^+-ATPase results in sorbic acid sensitivity (Mollapour et al., 2004).

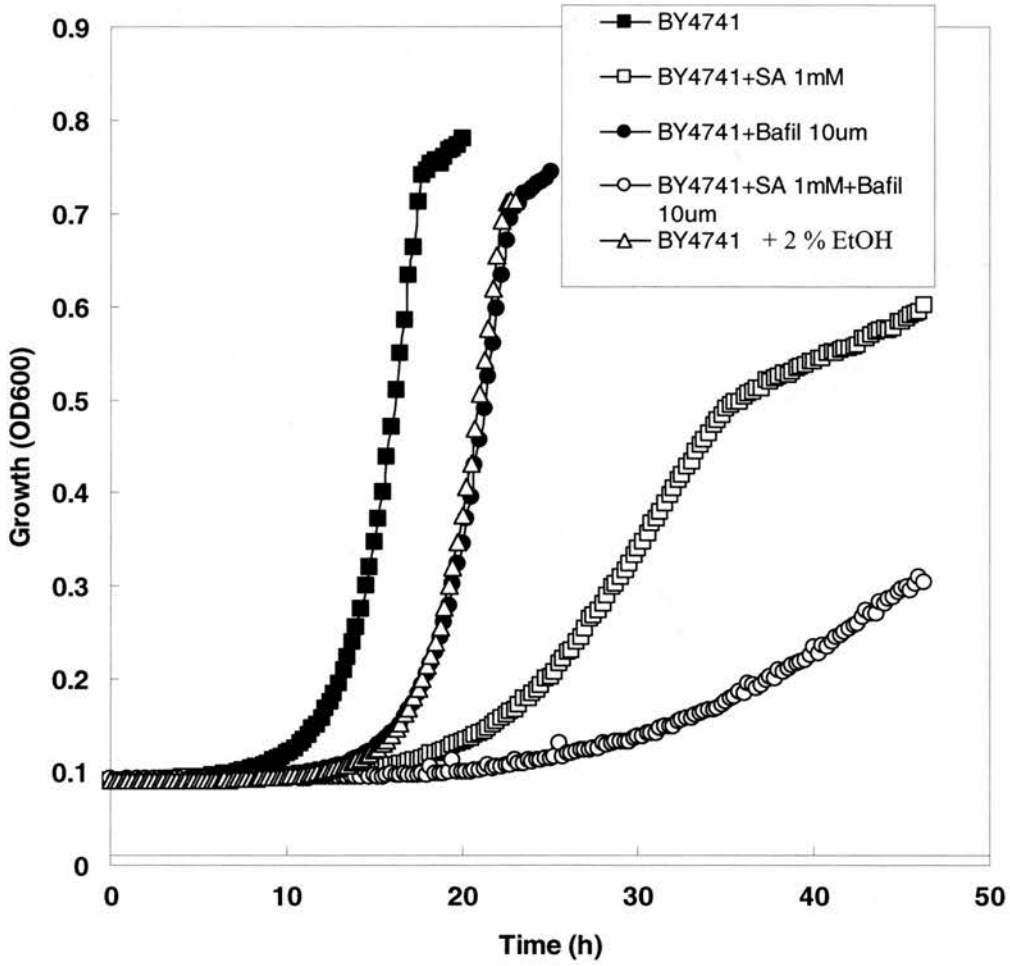


Figure 5.5 Effect of bafilomycin and sorbic acid on growth of *S. cerevisiae*. Wild-type (BY4741a) cells were grown in 48-well plates containing YEPD, pH 4.5 medium without sorbic acid (■), with 1 mM sorbic acid (□), with 10 μM bafilomycin (●), with combination of 1 mM sorbic acid and 10 μM bafilomycin A1 (O) and with 2 % ethanol (△) as a control (bafilomycin is disolved in ethanol). Growth was monitored for 48 hrs at 30 °C in a PowerWave ™ x S Universal Microplate Spectrophotometer (BIO-TEK® Instruments, GmbH). Readings were taken every 30 min. A representative result is shown.

5.4 Overexpression of Vma2p in the Δ*dbf2* mutant strain results in increased resistance to sorbic acid

To test whether overexpression of Dbf2p and Vma2p rescued the sorbate sensitivity, both *DBF2* and *VMA2* genes were cloned into the high copy vector pRS423 (Sikorski and Hieter, 1989) under the control of their own promoters (section 2.5.1). Overexpression of *DBF2* and *VMA2* rescued the sorbate-sensitive phenotype of the respective Δ*dbf2* and Δ*vma2* mutants but did not result in an enhanced resistance (Figures 5.6A and 5.6B respectively).

Additionally we investigated whether overexpression of *VMA2* in the absence of *DBF2* would make the cells more resistant to sorbate. The Δ*dbf2* mutant strain was transformed with the high copy vector pRS423 harboring *VMA2*. Overexpression of Vma2p in a Δ*dbf2* mutant background rescued the Δ*dbf2* phenotype when compared to a Δ*dbf2* mutant transformed with the empty plasmid (Figure 5.6C). This result provides additional compelling evidence that there is a functional link between Vma2p and Dbf2p proteins.

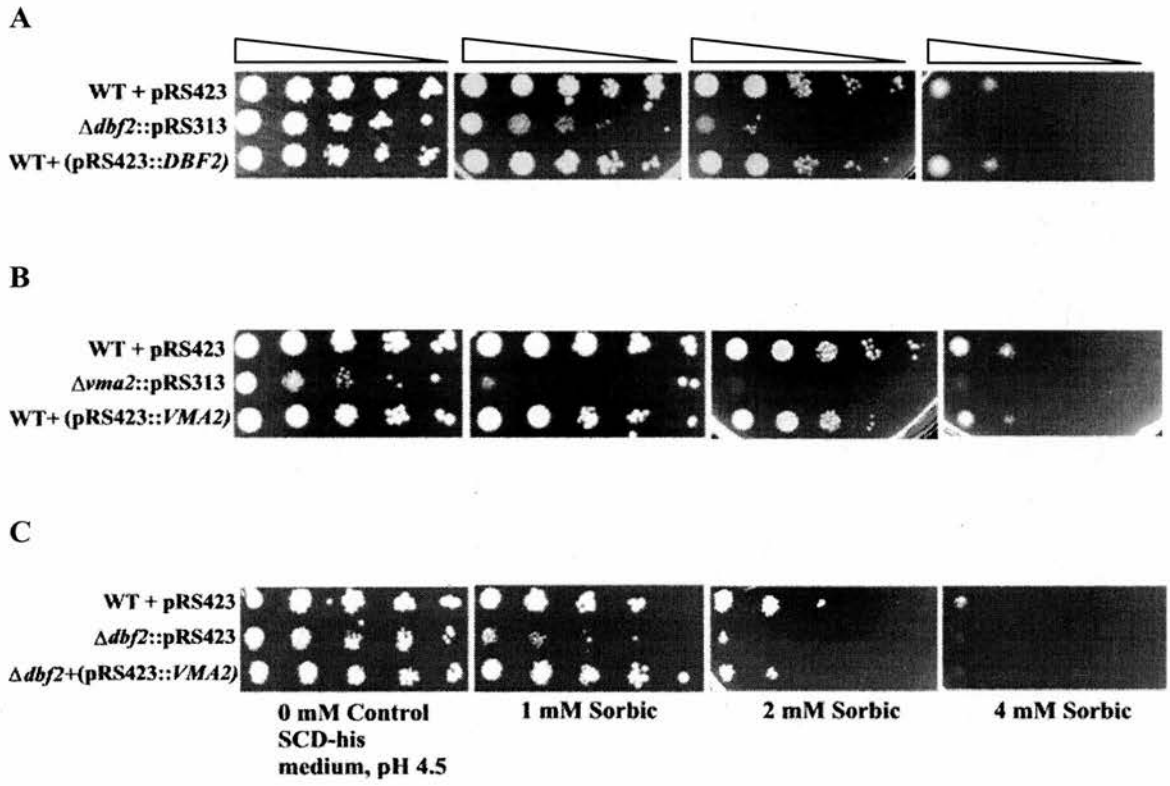


Figure 5.6 Overexpression studies of *DBF2* and *VMA2* under sorbic acid. Growth comparison on selective complete medium (SCD-His, pH 4.5) in 0, 1, 2 and 4 mM of sorbic acid in **A.** BY4741a (WT) transformed with an empty multicopy plasmid (pRS423), Δ*dbf2* transformed with empty vector (pRS313) and BY4741a transformed with (pRS423) carrying the *DBF2* gene and in **B.** Same as in **A** but for *VMA2* gene. Overexpression of Dbf2p and Vma2p in wild type strain does not alter sensitive phenotypes. **C.** WT and Δ*dbf2* strains transformed with empty pRS423 compared to Δ*dbf2* deletion strain transformed with *VMA2* on the multicopy vector. *VMA2* resques sorbic acid sensitivity in a Δ*dbf2* deletion strain. A representative result is shown.

5.5 Deletion of *DBF2* gene resulted in hypo-phosphorylation of Vma2p under acidic conditions

Phosphorylation of a protein causes an increase in negative charge and a subsequent shift in the overall charge making it more acidic. Using IEF and 2D-PAGE analysis resulted in the identification of a protein being shifted over a pH range of 4.0 to 7.0. In the previous chapters we have demonstrated through 2-D phoshoproteome analysis that Vma2p exists in two phosphorylated isoforms in the wild type BY4741a strain, whereas in a Δ*dbf2* mutant strain only the less phosphorylated isoform of Vma2p could be detected, judged by a shift in pH towards the acidic end of the gel (Figure 4.13). This suggested that the Dbf2p protein is important for subsequent Vma2p phosphorylation. To further validate the functional role of Dbf2p in Vma2p phosphorylation, 2-D immunoblots were performed. A 50 µg sample of total yeast cell lysate was separated by 2-D electrophoresis (mini 2D-gels) as previously described (section 2.6.6), electroblotted onto nitrocellulose and subsequently probed with a commercially available monoclonal antibody to Vma2p (Table 2.4).

When comparing BY4741a blots in the presence or absence of sorbic acid, a train of spots representing different isoforms was observed, corresponding to the theoretical isoelectric point of Vma2p (*p*I 4.79; Figures 5.7a,b). However, analysis of Vma2p from the Δ*dbf2* strain shows a movement of the predominant Vma2p isoforms on the blot toward the basic end, both in presence and absence of sorbic acid (Figures 5.7c,d). This shift of charged Vma2p isoforms to the basic direction indicates a loss of negatively charged phosphate groups. This result suggests that loss of *DBF2* results in the dephosphorylation of Vma2p or the presence of Vma2p in a less phosphorylated form. This finding is in accordance with the data obtained from the phosphoproteome analysis (Figure 4.13).

Notably, calf intestinal phosphatase (CIP) treatment of the BY4741a cells resulted in a rapid dephosphorylation of Vma2p and a concomitant basic shift in p*I* (shift to the right; Figure 5.7e). All the spots detected by the monoclonal antibody are isoforms of one distinct protein, Vma2p. Thus we can conclude that Vma2p is phosphorylated and that pre-

treatment with CIP reduces the phosphorylation state of the protein; a process that is exactly mimicked in cells lacking the protein kinase Dbf2p.

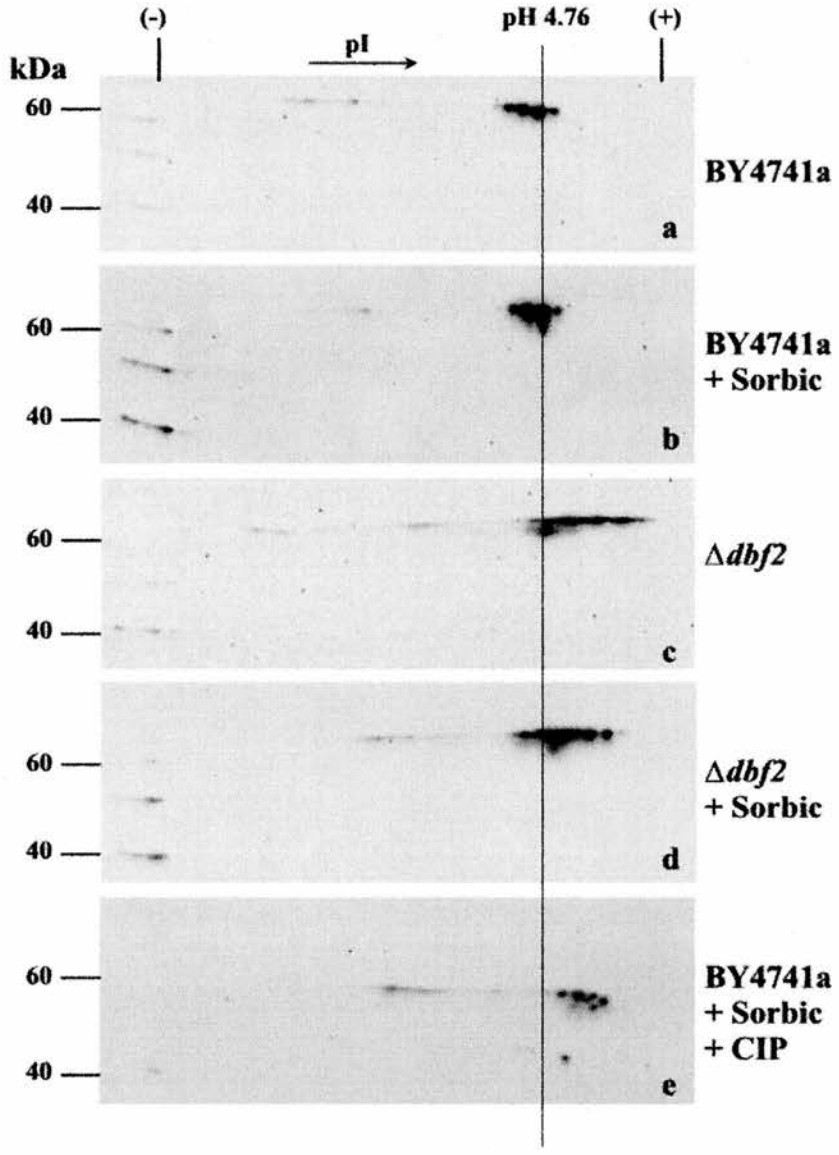


Figure 5.7 2-D Western Blot analysis of Vma2p phosphorylation states. Phosphorylation of Vma2p was assessed by a shift in p*I* toward basic pH values on 2D-PAGE gels. The acidic (-) and basic (+) directions are indicated. The IEF (p*I*) and PAGE (kDa) directions are marked. Total cell extracts (50 µg) from BY4741a wild type and Δ*dbf2* mutant strains were separated on 11 cm IPG strips (pH range 4.0-7.0) and 12.5% SDS-PAGE. Following electrotransfer to nitrocellulose membranes, Vma2p was visualized with anti-Vma2p monoclonal antibody. In the absence of *DBF2*, Vma2p was dephosphorylated both in the presence and absence of sorbic acid.

5.6 Loss of compartment acidification is concomitant with loss of V-ATPase activity and deletion of *DBF2* under acidic conditions

To study the effect of deletion of *VMA2* and *DBF2* on the activity of V-ATPase, vacuolar acidification was measured via quinacrine dye accumulation in both Δ*vma2* and Δ*dbf2* strains. Control BY4741a and Δ*vma1* strains were included. Quinacrine is a weakly basic dye that accumulates in acidic compartments in response to proton gradients and is frequently used to access the state of vacuolar acidification (Umemoto et al., 1990). In this study we further explored the functional relationship between Dbf2p and the regulatory subunit of the H^+-ATPase, Vma2p, by monitoring vacuolar acidification in wild type and mutant strains.

Cells grown in YEPD, pH 4.5 medium in the presence or absence of sorbic acid (1.5 mM) were collected and incubated with quinacrine (section 2.11). Control BY4741a cells, following treatment with quinacrine, displayed strong fluorescence that was localized to the vacuole (Figure 5.8a). Deletion of *VMA1*, the catalytic subunit of the H^+-ATPase, did not show vacuolar fluorescence (Figure 5.8b), indicating that the organelle was not acidified, as shown in previous studies (Morano and Klionsky, 1994). The Δ*vma1* cells were used as a negative control in this assay. The Δ*vma2* mutant cells also failed to accumulate quinacrine as no vacuolar fluorescence was evident (Figure 5.8c). Intriguingly, Δ*dbf2* cells also showed a pronounced loss of vacuole-localized fluorescence with consequent loss of compartment acidification (Figure 5.8d). These results are in agreement with the decreased cell growth in low pH medium following treatment with sorbic acid that these mutations exhibited (Figures 3.4B and 5.2A respectively). Addition of sorbic acid at the concentration used in the experiment did not affect the dye uptake (Figure 5.8e-h).

Together, these results suggest that Vma2p is necessary for vacuolar acidification and *DBF2* is important for compartment acidification, further indicating that the Dbf2p kinase may positively regulate V-ATPase activity *in vivo* via interactions with Vma2p.

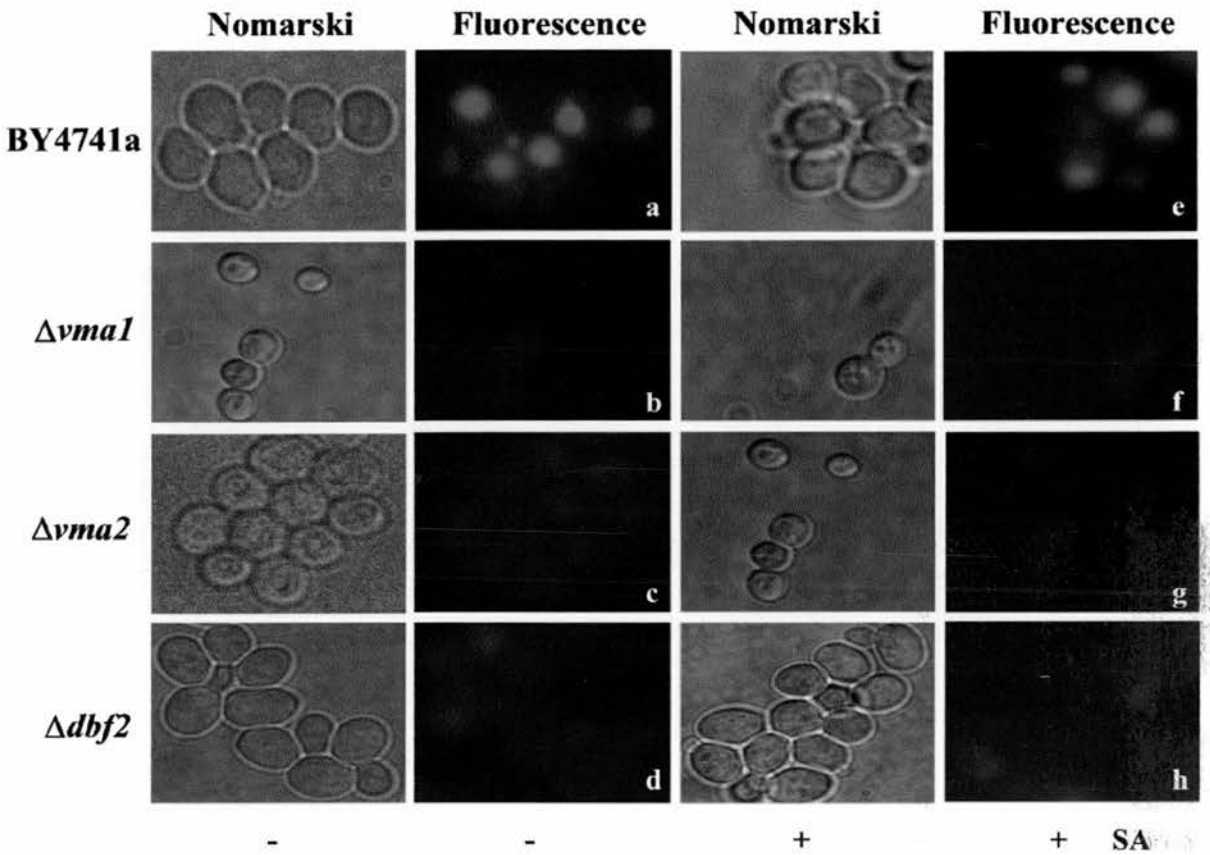


Figure 5.8 Vacuolar labeling with quinacrine. For each wild type and mutant strain a Nomarski image of whole cells (left panels) and corresponding quinacrine staining of the same cells (right panels) is shown. Vacuolar acidification was monitored by accumulation of the fluorescent quinacrine dye within the vacuole. Cells were grown in YEPD, pH 4.5 in the presence and absence of sorbic acid (+/- SA) at 30 °C to late exponential phase, then transferred to uptake buffer and stained with 200 μM quinacrine. Dye uptake was monitored by microscopic examination of single cells, using a Olympus IX70 Delta Vision microscopy.

5.7 Protein-protein interactions under sorbic acid treatment

As an alternative method to map out the network of protein interactions involved in signalling sorbic acid adaptation, via the Dbf2p kinase, we used a two-step affinity purification system followed by mass spectrometry. In this approach, TAP-tagged candidate genes (*DBF2* and *VMA2*) were purified by affinity chromatography from yeast whole-cell lysate (section 2.8.1). Proteins that interact with tagged proteins were separated by gel electrophoresis and then identified by mass spectrometry. Using this method, only strong interactions will be identified due to the nature of the two-step column purification.

5.7.1 Interactions of Vma2p after exposure to sorbic acid stress

To identify yeast proteins interacting with the target protein, Vma2p, the Tandem Affinity Purification (TAP tag) methodology was used (section 2.8.1) and pull down experiments were performed, whereby, interacting partners bound to Vma2p copurified with the TAP-tagged protein. The C-terminally TAP tagged Vma2p strain (Open Biosystems) was grown in YEPD, pH 4.5 in the presence and absence of 1.5 mM sorbic acid. Cells were lysed using glass beads as described in section 2.8.1 and yeast soluble lysate was clarified by centrifugation. The lysate was applied to an IgG column and eluted proteins were subsequently applied to a calmodulin column. TAP-Vma2p was released by high concentrations of EGTA. The tandem purification, with abundant washing steps, ensured a specific enrichment for Vma2p-TAP and its interactions. The concentrated eluate fraction was separated on SDS-PAGE and stained with SYPRO® Ruby. Control extracts (-SA) were compared to treated (+ SA) and different bands were identified by mass spectrometry. Mass spectrometry analysis verified the identity of Vma2p. Following sorbic acid treatment three proteins that were present in the untreated sample were reproducibly absent: alcohol dehydrogenase (Adh1p), glyceraldehyde-3-phosphate dehydrogenase (Tdh3p) and a component of the ribosomal subunit (Rps3p) (Figure 5.9; Table 5.2).

Vma2p in the presence and absence of sorbate was co-purified with Vma1p, Vma4p and Vma10p, suggesting that these proteins form a strong interaction and are most likely in a stable complex (Figure 5.9).

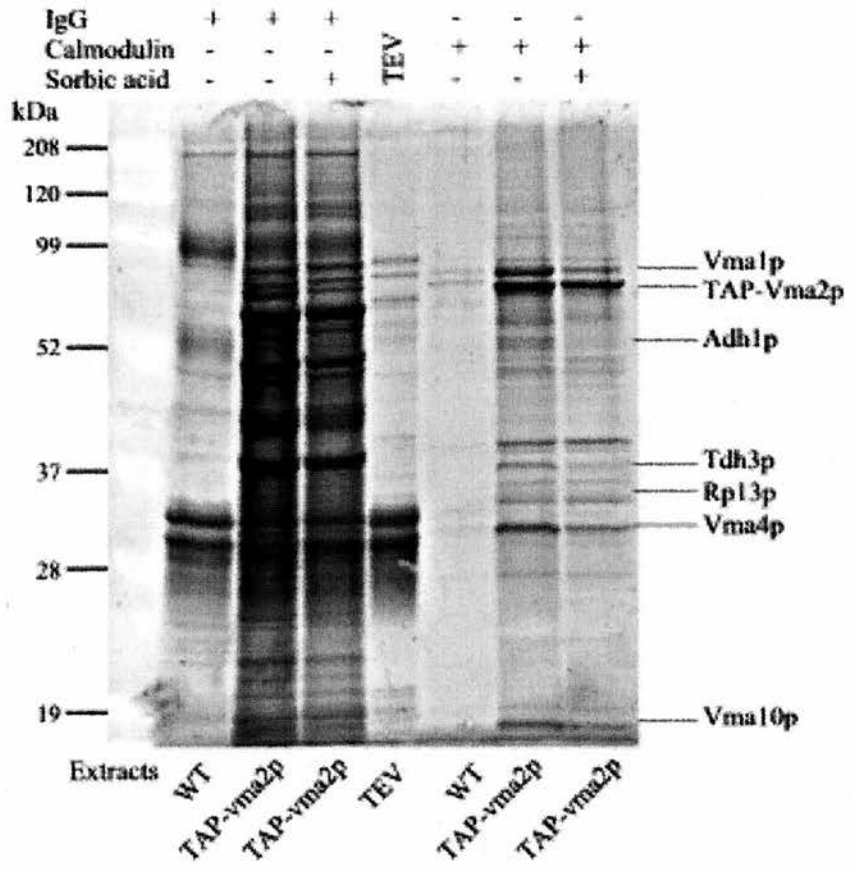


Figure 5.9 Interacting partners of Vma2p-TAP under sorbic acid conditions. SYPRO-Ruby stained protein gel depicting proteins recovered at the various stages of TAP-tagged Vma2p purification. The purification was performed from 2L of yeast cell culture, harvested at OD_{600} 0.2~0.3. Soluble yeast lysate was recovered and applied to first column (IgG). Bound proteins were eluted by TEV protease and applied to second column (Calmodulin). Wild type (WT) extracts were used as a control. Vma2p-TAP was released from calmodulin column by high concentration of EGTA. Eluted Vma2p and interacting proteins were subjected to SDS-PAGE and stained with SYPRO®-Ruby. Indicated protein bands were identified by mass spectrometry (Table 5.2).

Table 5.2 Mass Spectrometry data of identification of Vma2p interacting partners

Top ranking protein in MS-Fit search	Matched peptide coverage (%)	Molecular mass (kDa)	
		Exp	Pred
Vma1 (subunit A, vacuolar)	49	80	68
Vma2 (subunit B, vacuolar)	62	75	57.8
Adh1 (alcohol dehydrogenase)	31	50	37
Tdh1 (Glyceraldehyde-3-phosphate dehydrogenase)	54	37	35
Rp13 (component of the small (40S) ribosomal subunit)	43	33	26
Vma4 (subunit E, vacuolar)	30	30	26.5
Vma10 (subunit G, vacuolar)	42	15	12.7

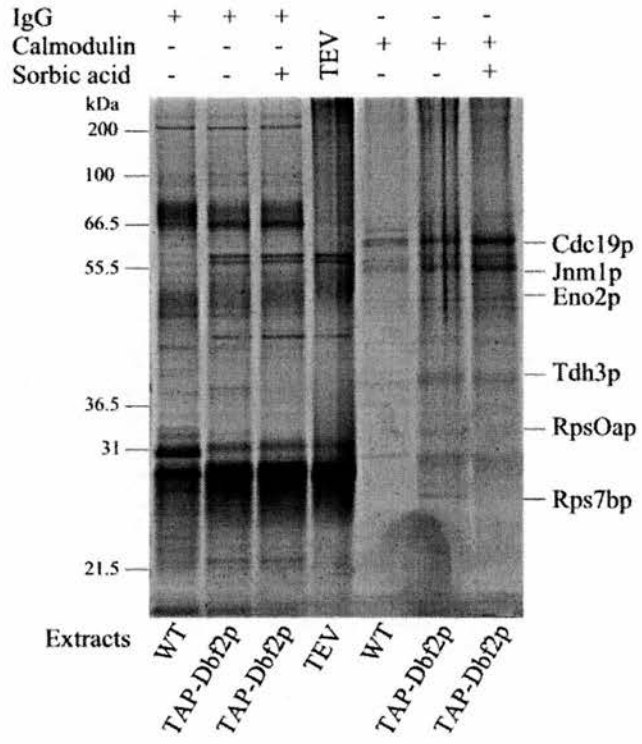
5.7.2 Interactions of Dbf2p following exposure to sorbic acid stress

The TAP tag methodology was also used to detect changes in the protein interactions of Dbf2p in the presence and absence of sorbic acid. The Dbf2p-TAP tagged strain (Open Biosystems) was grown to an OD_{600} of ~0.3. Soluble protein was TAP purified as described for Vma2-TAP. Strongly interacting partners will remain in complex with Dbf2p throughout the purification procedure and can be identified by mass spectrometry. Following sorbic acid treatment only two proteins were reproducibly absent: the protein components of the small ribosomal subunit; Rps0ap and Rps7bp (Figure 5.10A; Table 5.3). Tightly bound to Dbf2p in the presence and absence of sorbate stress were four proteins: a pyruvate kinase that functions in glycolysis (Cdc19p), a protein required for proper nuclear migration and spindle partitioning during mitotic anaphase (Jnm1p) enolase II (Eno2p) and a dehydrogenase, involved in glycolysis and gluconeogenesis (Tdh3p) (Figure 5.10A; Table 5.3).

Dbf2p could not be identified via mass spectrometry, presumably due to low expression levels, thus was visualized by immunobloting with a specific antibody against the TAP-tag (Figure 5.10B). The protein appeared as a doublet, probably due to a charge difference

between different isoforms, which migrate as two distinct bands. This is in accordance with previous evidence (Liu et al., 1997a).

A



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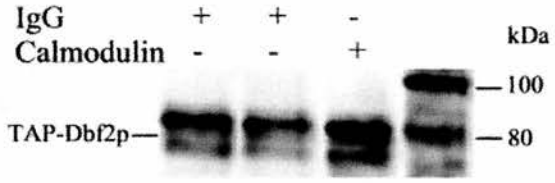

Figure 5.10 Protein-protein interactions of Dbf2p. A. Step-by-step analysis of Dbf2p-TAP purification. The purification was done from 2 L of yeast cell culture. Proteins identified by mass spectrometry to confirm changes due to sorbic acid treatment. Control wild type (WT) strain pull down was performed. Indicated protein bands were identified by mass spectrometry (Table 5.3). **B.** Immunoblot of TAP-Dbf2p, using commercially available antibody against the TAP-tag to detect the presence of the Dbf2p kinase after purification. It appears as a doublet at ~ 80 kDa. A representative result of both experiments is shown.

Table 5.3 Mass Spectrometry identification of Dbf2p interacting partners

Top ranking protein in MS-Fit search	Matched peptide coverage (%)	Molecular mass (kDa)	
		Exp	Pred
Cdc19p (pyruvate kinase)	13	60	54.5
Jnm1p (component of the yeast dynactin complex)	11	55	43.6
Eno2p (Enolase II)	19	50	46.9
Tdh3p (glyceraldehyde-3-phosphate dehydrogenase)	41	40	35.7
RpsOap (component of the small (40S) ribosomal subunit)	17	33	27.9
Rps7bp (component of the small (40S) ribosomal subunit)	24	26	21.6

5.8 Recombinant protein expression

To determine whether Dbf2p was directly phosphorylating Vma2p, both proteins were expressed and purified in order to perform kinase assays. Tagged versions of Dbf2p and Vma2p, incorporating a six-histidine tag at the C-terminus, were constructed for recombinant expression in *E. coli* (section 2.5.6). Upon expression both recombinant proteins formed insoluble inclusion bodies (Figure 5.11). Optimization of solubility was attempted by changing the induction temperature and the concentration of IPTG used to induce expression. However this proved unsuccessful. Solubilisation and refolding of the recombinant proteins forming inclusion bodies was also attempted, whereby the insoluble inclusion bodies were solubilised in appropriate buffer containing 8 M urea as a denaturant (section 2.8.3). A refolding protocol was then attempted by dialysis of the sample into a decreasing gradient of urea. Both proteins precipitated as concentrations of urea decreased, probably indicating unsuccessful refolding of these quite large proteins. Dbf2p was instead purified by the TAP methodology under native conditions in *S. cerevisiae* as seen previously (section 2.8.1) and as 13Myc-tagged protein in yeast (section 2.5.5).

Briefly, 13myc-Dbf2p was incorporated in the genome and purified via immunoprecipitation from yeast total lysate (section 2.5.5). Approximately 500 μg of total protein were incubated with anti-Myc antibody, washed and further incubated with protein G beads. Following washing steps, the beads with the bound Dbf2p on them were separated by 1D-PAGE. Due to the low expression of Dbf2p we were only able to detect it on a western blot (Figure 5.12). Vma2p was obtained by the TAP method in yeast for further biochemical assays using a commercial Vma2-TAP strain (Open Biosystems). Purified proteins were used in subsequent kinase assays.

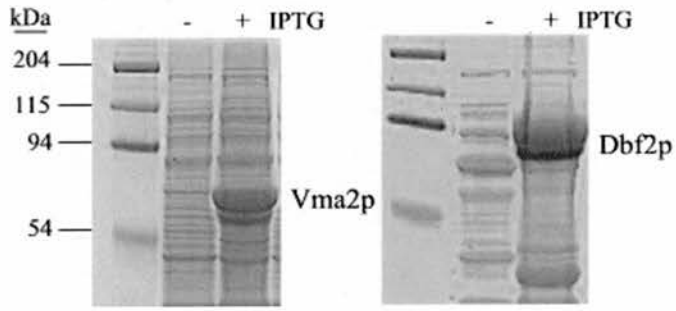

Figure 5.11 Overexpression of recombinant Vma2p and Dbf2p in *E. coli.* Vma2p and Dbf2p were c-terminally His-tagged and expressed in *E.coli.* Coomassie stained gels of pre- and post protein induction (-/+ IPTG) showed high levels of expression in both induced cultures. Proteins were expressed as insoluble inclusion bodies.

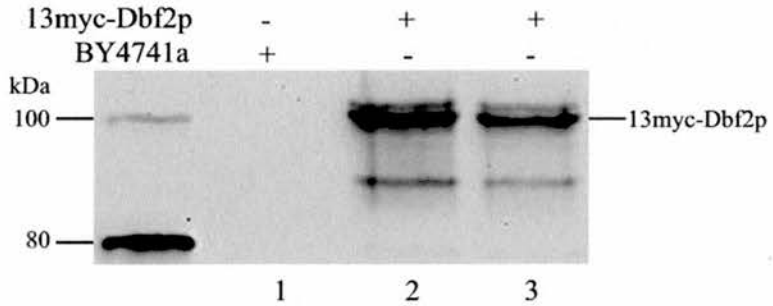

Figure 5.12 Expression of 13myc-Dbf2p. Strain BY4741a containing a 13myc-*DBF2* incorporated in the genome was immunoprecipitated and subjected to 1D-PAGE and subsequent blotting with an antibody against the Myc tag (lanes 2 and 3). BY4741a without carrying the 13myc-*DBF2* was used as a control (lane 1). Tagged Dbf2p appears as a doublet at ~ 100 kDa.

5.8.1 Vma2p is not directly phosphorylated by Dbf2p *in vitro*

In vitro kinase assays were performed (section 2.10.1) to determine whether Vma2p is a substrate for Dbf2p kinase. Increasing amounts (0-20 μg) of purified TAP-Vma2p were incubated with 500 μg of Dbf2p bound on IgG beads in the presence of radiolabelled ATP for 30 min at 30 °C. Samples were then subjected to SDS-PAGE and imaged via exposure to phosphoscreens. Both Dbf2p constructs were tested for kinase activity using histone (Figure 5.13), a protein known to be a substrate for Dbf2p (Toyn and Johnston, 1994);

The presence of increasing amounts of TAP-Vma2p to a large excess of 20 μg does not lead to Dbf2p directly phosphorylating a protein with a M_r of ~ 70 kDa that would correspond to TAP-Vma2p (Figure 5.14). For this experiment, two different constructs of Dbf2p were used: the C-terminal commercial TAP-tag and a C-terminal 13Myc-Dbf2p (section 2.5.5). Both constructs were originally tested for activity to histone, a positive substrate for Dbf2p kinase, and were both active (Figure 5.13). Using both versions of purified Dbf2p no direct phosphorylation of Vma2p, corresponding to a ~65 kDa, was observed (Figure 5.14), suggesting the existence of an intermediary protein *in vivo*.

However, under the experimental conditions used, Dbf2p was autophosphorylated both in the presence and absence of phosphatase inhibitors from the kinase reaction. This autophosphorylation was not Tag-specific (Figure 5.13, Figure 5.14). As the kinase reaction was performed in a whole-cell extract, which approximates the normal cellular environment better, Dbf2p was exposed to a wide variety of potential protein substrates, hence the lower molecular weight bands observed (Figure 5.14).

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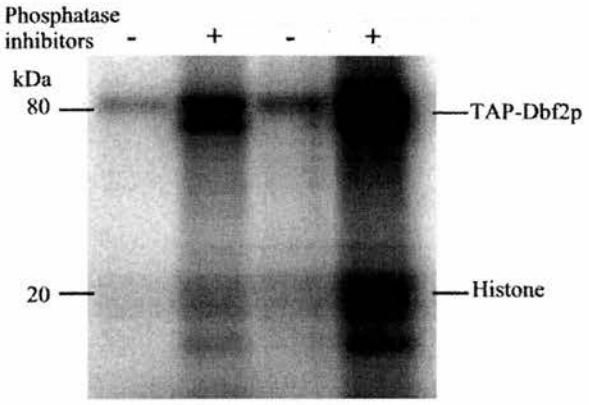

Figure 5.13 Activity of Dbf2p kinase against histone. Activity of Dbf2p kinase is detected both in the presence and absence of phosphatase inhibitors in the kinase assay buffer. The kinase is autophosphorylated and is phosporylating histone (positive substrate), proving its in an active state. Higher activity is detected when phosphatase inhibitors are not present in the assay.

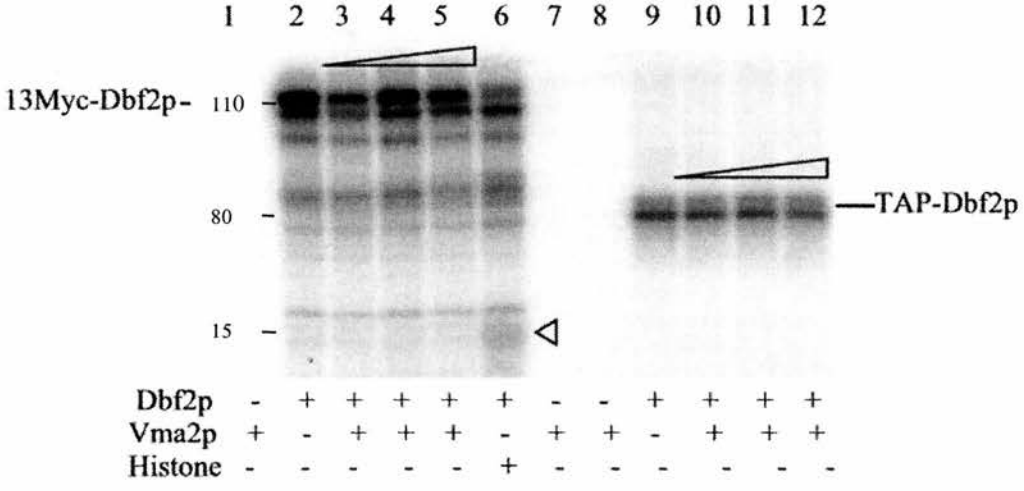

Figure 5.14 Activity of Dbf2p kinase against Vma2p. Activity of Dbf2p kinase is detected both in the culture derived from an integrated copy of c-13myc epitope-tagged *DBF2* (lanes1-7) and c-TAP tagged Dbf2p (lanes 8-12). Increasing concentrations of TAP-Vma2p (0, 5, 10 and 20 μg) (denoted by triangle) did not result in direct phosphorylation by Dbf2p kinase. Marked autophosphorylation of Dbf2p was observed under the experimental conditions used. A representative result is shown.

5.10 Discussion

In this work a variety of biochemical approaches were used to elucidate the interaction between Dbf2p and Vma2p and to test the hypothesis of a direct link between these two proteins or a possible involvement in the same signaling cascade that mediates sorbic acid resistance.

Deletion of the vacuolar H^+-ATPase subunit, Vma2p, resulted in marked growth inhibition when treated with sorbic acid. This result was consistent with data from concurrent studies for the identification of sorbate- sensitive genes (Schuller et al., 2004). Bafilomycin experiments performed in the wild type strain have shown that blocking the vacuolar H^+-ATPase pump results in significant growth inhibition under sorbic acid stress, thus, mimicking the effect of the *VMA2* deletion further supporting our finding. The sorbic acid sensitive phenotype in the Δ*vma2* mutant strain is specifically due to loss of *VMA2* gene, as we were able to rescue the sensitive phenotype by reinserting *VMA2* on a single-copy plasmid.

The yeast vacuole is an acidic compartment that plays essential roles in metabolic storage and in cytosolic ion and pH homeostasis. Storage of ions in the vacuole is necessary to maintain homeostasis, regulate intracellular pH (pH_i) and to serve as a mechanism of detoxification, *ie* for metal ions such as Cd^{2+} or Fe^{3+}. This acidification of the vacuole is achieved by the V-ATPase pumping ions into the vacuole via ATP hydrolysis to generate a proton gradient. Previous studies have identified that both War1p and Pdr12p dependent and independent processes confer weak acid tolerance (Kren et al., 2003; Piper et al., 1998). However the process of adaptation to weak acids must also involve the removal of accumulated weak acid protons to maintain pH_i homeostasis with the role of plasma membrane being H^+-ATPase crucial in this respect. There is no evidence that the activities associated with increased proton pumping and pH_i homeostasis during weak acid stress are dependent on War1p transcription factor. In this study we have shown that the vacuolar H^+-ATPase has a crucial role in allowing sorbic acid adaptation.

Consistent with a role for Vma2p in the cellular stress response, published evidence supports this hypothesis. *VMA2* was induced under citric acid stress (Lawrence et al., 2004), suggesting that a functional vacuolar membrane H^+-ATPase is also important for citric acid adaptation in yeast. Exposure to citric acid at low pH may have the effect of reducing pH_i, such that the activity of vacuolar enzyme is important in allowing the cell to maintain pH_i homeostasis (Lawrence et al., 2004). However, in the same study the authors proposed that citric acid adaptation may be exerted via disruption of calcium homeostasis, since in yeast cells the vacuole contains the major cytosolic Ca^{2+} pool (Eilam et al., 1985), sequestered via a low affinity Ca^{2+}/H^+ antiporter that utilizes the proton motive force generated by the vacuolar H^+-ATPase (Matsumoto et al., 2002). Tight control of cytosolic Ca^{2+} concentration in yeast is a pre-requisite for proper function of Ca^{2+}-dependent signal transduction pathways, which play a regulatory role in membrane biogenesis and maintenance, essential to protect cells from chemical stress (Forster and Kane, 2000; Tanida et al., 1995). In another study the inhibitory effect of citric acid on *Clostridium botulinum* has been reported to be due to citric acid chelating divalent metal ions, particularly calcium (Graham and Lund, 1986). Therefore, citric acid may be chelating Ca^{2+} from the medium, possibly resulting in Ca^{2+} ion depletion, implying that calcium homeostasis may be the principal inhibitory effect of citric acid stress. Preliminary data from the same study (personal communication) demonstrated that the inhibitory effect of citric acid was reduced in the presence of $CaCl_2$. Although the mechanism of citric acid adaptation in yeast is different to that of sorbic acid, a functional vacuolar membrane H^+-ATPase is crucial for adaptation to both acids.

Recently, activation of the vacuolar H^+-ATPase was seen in the *S. cerevisiae* adaptation and resistance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), a highly lipophilic weak acid (Fernandes et al., 2003). Studies on this herbicide are important to give insight to the number of toxicological problems and the emergence of resistant weeds due to the widespread and intensive use and misuse of herbicides (Bradberry et al., 2000). The main mechanism of adaptation and resistance to this herbicide involves the rapid and transient Pdr1/Pdr3-dependent transcription activation of PDR5 and TPO1 genes (Teixeira and Sa-Correia, 2002). These target genes encode multidrug resistance ABC transporters and major

facilitators superfamilies (MFS). Proteome analysis of the yeast response to the same herbicide found increased expression of Vma1p and Vma2p during yeast adaptation to 2,4-D (Teixeira et al., 2005). These findings suggest that the expression of the two vacuolar subunits is required for a more rapid and efficient yeast adaptation and resistance to the herbicide. This is possibly due to the role of the V-ATPase in counteracting the dissipation of the physiological H^+-gradient across the vacuolar membrane induced by the herbicide (Fernandes et al., 2003; Teixeira et al., 2005).

In another study, yeast mutants lacking a functional vacuolar H^+-ATPase show multidrug sensitivity {Parsons, 2004 #175;}. In this study ~4,700 viable yeast deletion mutants were screened for hypersensitivity to 12 diverse inhibitory compounds. Thus genes encoding subunits of the yeast vacuolar H^+-ATPase complex *(VMA2, VMA4, VMA5, VMA6, VMA7, VMA8, VMA10, VMA13, VMA22, VMA11, VPH2*) were sensitive to: wortmannin, tunicamycin, rapamycin, sulfometuron methyl, fluconazole, cycloheximide, caffeine, hydroxyurea and FK506. These findings are consistent with previous observations identifying some *vma* mutants as sensitive to staurosporine, vanadate and hydromycin B (Yoshida and Anraku, 2000). This multidrug resistance phenomenon may be conserved in other eukaryotes, notable evidence for this found in mammalian cells (Parsons et al., 2004). Three lines of evidence suggest that the role of the vacuolar H^+-ATPase in multidrug resistance may be conserved. First in mammalian cell culture, changes in pHi can alter drug accumulation (Simon et al., 1994). Second, the gene encoding vacuolar H^+-ATPase subunit C is overexpressed in multidrug-resistant HL60 cells (Ma and Center, 1992), and exposure to concanamycin, an inhibitor of vacuolar H^+-ATPase (Drose et al., 1993) can restore the sensitivity of drug-resistant cells to several anticancer drugs (Ouar et al., 2003). Third the effect of the actin depolymerizing agent latrunculin A on mammalian cells was enhanced by inhibition of the vacuolar H^+-ATPase with concanamycin A (Parsons et al., 2004).

In agreement with previous published evidence that Vma2p plays a role in stress response, we have shown that V-ATPase plays a crucial role in sorbic acid adaptation. The intriguing question remains: how is this response to weak acid adaptation activated, signaled and regulated? We have investigated the link between Vma2p and Dbf2p kinase via different

routes. Overexpression studies of both *VMA2* and *DBF2* rescued the sorbic acid sensitive phenotypes of both Δ*vma2* and Δ*dbf2* respectively but did not result in induction of enhanced resistance. However, when Vma2p was overexpressed in Δ*dbf2* background, the Δ*dbf2* sorbic acid sensitive phenotype was recovered. These results provide further convincing evidence of a functional interaction between Dbf2p and Vma2p in mediating sorbic acid resistance.

Protein interaction pull-down experiments were performed in order to identify interacting partners of Dbf2p and Vma2p in the presence and absence of sorbic acid. Due to the nature of the experiment including two separate purification steps only very stable complexes would remain intact and therefore could be identified by mass spectrometry. The tightly bound to Dbf2p in the presence and absence of sorbate stress interacting proteins Cdc19p and Tdh3p were involved in glycolysis, implying that Dbf2p could also function in another pathway. A few ribosomal proteins were reproducibly identified, which correlated with the results from the screen of the deletion library (Table 3.1), suggesting that ribosomal biogenesis is important in sorbic acid resistance. These interactions could simply be due to the fact that ribosomal proteins are highly abundant. However, highly non-specific hits are not likely due to the two-column purification. We cannot therefore rule out the existence of transient or less stable interactions that may be important between Vma2p and Dbf2p and their respective interacting partners that were not detected by this approach.

Shifts in *p*I from more acidic (phosphorylated) to more basic (less phosphorylated) clearly demonstrated that the phosphorylation state of Vma2p was decreased in the absence of the *DBF2* gene. Notably, pre-treatment of control cells with calf intestinal phosphatase (CIP) resulted in a marked, basic shift in the *p*I of the different Vma2p isoforms, further suggesting that Vma2p is phosphorylated in the presence of Dbf2p. Furthermore, experiments showing quinacrine staining of Δ*vma1* and Δ*vma2* mutants resulted in rapid loss of vacuolar acidification, which was in accordance with previous studies (Morano and Klionsky, 1994). Interestingly, quinacrine staining of Δ*dbf2* mutants also resulted in loss of vacuolar acidification, further suggesting a role for Dbf2p kinase in the V-ATPase regulation. Indirect evidence of a role for Dbf2p in vacuolar function comes from a recent

study that screened gene deletions affecting glycogen storage in yeast (Wilson et al., 2002). Gene deletions known to result in loss of V-ATPase function, for example *VMA1*, *VMA3*, *VMA4*, *VMA6* and *VMA10*, also resulted in hyperaccumulation of glycogen suggesting a significant role for the vacuole in the maintenance of yeast glycogen stores. Notably, it was also observed that deletion of *DBF2* also resulted in hyperaccumulation of glycogen supporting our observation that Dbf2p regulates the activity or assembly of the V-ATPase. Exposure to sorbic acid had no effect on the observed dephosphorylation of Vma2p, or the loss of V-ATPase activity/assembly, in the Δ*dbf2* strain. Thus, the sensitivity to sorbic acid observed in Δ*dbf2* cells is probably attributable to the loss of Vma2p phosphorylation and V-ATPase function. However, kinase assays showed that there is no direct phosphorylation of Vma2p by Dbf2 kinase *in vitro* but possibly there could be indirect phosphorylation *via* intermediary proteins of the signalling cascade mediating sorbic acid stress adaptation.

No known substrates for Dbf2p have been found so far and further work is required to elucidate if Dbf2p is an intermediary kinase in the signaling cascade that mediates sorbic acid adaptation. Interestingly, Snyder and co-workers developed protein chips to conduct high-throughput biochemical screens of protein kinases in *S. cerevisiae* in order to identify putative substrates (Zhu et al., 2000). The methodology comprises a high-density protein microarray containing >4000 purified yeast proteins from *S. cerevisiae*. Each ORF is expressed as a N-terminal GST (Glutathione-S-Transferase)-6xHis-fusion protein, purified and printed on glass slides. Using a protein kinase of interest in the presence of radiolabeled ATP it is feasible to screen against >4000 *S. cerevisiae* ORFs and identify potential kinase substrates (Zhu et al., 2000). Using the Phosphorylome Database that was constructed (http://networks.gersteinlab.org/phosphorylome/interface/index.cgi) it was interesting to find two vacuolar transporters, Avt3p and Avt4p to be phosphorylated by Dbf2p further suggesting a link between the kinase and the vacuolar function. Vma2p was absent from the chip.

Large-scale protein-protein interaction maps for *S. cerevisiae* demonstrate that the network of signaling proteins is intimately linked to the cytoskeleton, suggesting that this interconnected filamentous structure plays a crucial and distinct functional role in signal

transduction. Several recent studies have suggested an important link between V-ATPases and the cytoskeleton. A temperature-sensitive mutation in *VMA4* was reported to cause a defect in actin distribution and bud morphology (Zhang et al., 1998). In renal cells, the V-ATPase has been shown to interact with the PDZ protein NHE-RF, which in turn is able to bind actin-associated proteins such as ezrin (Breton et al., 2000). Direct binding of V-ATPase to actin filaments has been demonstrated in osteoclasts (Lee et al., 1999). These findings have led to investigation of a possible role of interaction between the V-ATPase and cytoskeletal elements in glucose-dependent dissociation of the V-ATPase in yeast (Xu and Forgac, 2001).

Notably, *VATB1* encoding the human kidney iso-form of the V-ATPase B subunit, shares 78% identity and 87% similarity with the yeast homologue Vma2p. Research has shown that mutations in *VATB1* cause distal renal tubular acidosis, a condition characterised by impaired renal acid secretion resulting in metabolic acidosis and sensorineural hearing loss (Karet, 1999). This was the first example of mutation in a member of the V-ATPase gene family causing human disease. Intriguingly, little is known about the function of the highly conserved human homologue of yeast Dbf2p (NDR1) and an interesting question arising from this work will be to ascertain whether NDR1 is involved in regulating the activity or assembly of human V-ATPase enzymes.

Modern functional genomic tools make global analyses of cellular responses to specific stress factors possible. However, deciphering the complexity of signaling cascades is still a challenging task. In this study we combined biochemical and proteomics techniques with the data from several recently established large-scale protein-protein interaction maps for *S. cerevisiae* to address the role of the Dbf2p kinase and Vma2p subunit of vacuolar H^+-ATPase in signaling sorbic acid stress. The results from this study demonstrate that the network of signaling proteins is more complex and involves more intermediary proteins than Dbf2p and Vma2p. Therefore, more approaches need to be considered in order to discover other intermediary proteins that may play a crucial and distinct functional role in the signaling cascade that allows sorbic acid tolerance in yeast.

CHAPTER 6

6. Discussion

6.1 Final Conclusions

Recent research has identified that *Saccharomyces cerevisiae* has evolved dedicated and sophisticated mechanisms that allow adaptation to different organic acids. Some examples include: the novel role of the yeast mitogen-activated protein kinase Hog1p in adaptation to citric acid stress (Lawrence et al., 2004); the requirement for the membrane-bound transporters Azr1p and Aqr1p for optimal adaptation to short chain monocarboxylic acids (Tenreiro et al., 2002; Tenreiro et al., 2000) and finally, the key role played by the vacuolar H^+-ATPase (V-ATPase) in resistance to the organic acid herbicide, 2,4-dichlorophenoxyacetic acid (Fernandes et al., 2003).

The best-characterised acid stress response to date in yeast is that induced by sorbic acid. Commonly used as preservatives in the food and beverage industries, sorbic acid and its Na^+ and K^+ salts mainly prevent the growth of spoilage yeasts and moulds. However, spoilage still occurs, mainly because the major spoilage organisms can adapt and grow even at low pH values in the presence of the maximum permitted levels of sorbic acid based preservatives (Loureiro, 1999). It is well known that some yeasts can grow at pH values as low as 2 and in the presence of >2.5% v/v acetic acid (Thomas, 1985). A major consequence of this ability of yeasts to adapt and grow at high acidity is huge economic losses of spoiled foods and beverages.

Upon exposure to sorbic acid, there are believed to be two detrimental effects that the yeast cell has to overcome: firstly, the intracellular accumulation of weak acid-anions and secondly, the intracellular accumulation of protons (reviewed in Piper et al., 2001). Recent research has revealed that yeasts are able to induce a specific adaptive response following exposure to sorbic acid that helps to overcome these inhibitory effects. Optimal adaptation to sorbic acid stress requires the induction of the ATP-binding cassette (ABC) efflux Pdr12p pump (Piper et al., 1998) (Figure 6.1). Pdr12p is present at low levels in unstressed cells but upon exposure to sorbic acid is highly induced and counteracts the intracellular accumulation of weak acid anions. Yeast cells lacking

Pdr12p become hypersensitive to water-soluble monocarboxylic acids with chain lengths from C3 to C8 (Hatzixanthis et al., 2003; Holyoak et al., 1999).

The rapid induction of *PDR12* by sorbic acid is regulated by the nuclear Zn_2Cys_6 zinc finger transcription factor, War1p (Kren et al., 2003). Δ*war1* mutants are hypersensitive to sorbic acid as they are unable to induce Pdr12p. To induce Pdr12p, War1p recognises and binds to a *cis*-acting weak acid response element (WARE) in the PDR12 promoter. War1p binds to the WARE constitutively, both in the presence and absence of sorbic acid stress, and it is believed that exposure to sorbic acid results in the phosphorylation, and thus activation, of War1p (Figure 6.1). Mutations that prevent phosphorylation result in a non-functional War1p (Schuller et al., 2004). Also, the appearance of phosphorylated War1p coincides with the transcriptional induction of *PDR12* (Kren et al., 2003). Notably the protein kinase, or signalling pathway, mediating the phosphorylation of War1p has not yet been identified.

One of the principal objectives of this study was to identify key regulatory proteins, such as protein kinases, involved in perception and signalling of weak acid stress. Screening of the yeast genome non-essential deletion library identified that the serine-threonine protein kinase Dbf2p was sensitive to sorbic acid. Further work showed that Dbf2p does not affect the expression of Pdr12p and thus mediates tolerance to sorbic acid via a novel mechanism. In this study, evidence is presented demonstrating that Dbf2p regulates the phosphorylation of the vacuolar H^+-ATPase (V-ATPase) non-catalytic subunit Vma2p. A fully functional Vma2p, and thus V-ATPase, is required for optimal adaptation to sorbic acid (Figure 6.1). Overexpression of *VMA2* rescued the sorbic acid-sensitivity of the Δ*dbf2* mutant, thus implying further functional interaction between Dbf2p and Vma2p. Notably, measurement of vacuolar acidification due to V-ATPase activity revealed that both Δ*vma2* and Δ*dbf2* strains were unable to acidify their vacuoles.

In this thesis, a novel, Pdr12p-independent mechanism of adaptation to sorbic acid stress mediated by a previously unknown role for the cell cycle-regulated protein kinase Dbf2p has been identified. A fully functional V-ATPase, regulated via the Dbf2p kinase is crucial for the adaptation to sorbic acid stress. Our results suggest that the vacuole contributes significantly to yeast survival under sorbic acid stress conditions. Most

likely by pumping up excess protons from the cytoplasm and storing them in the vacuole. It is already known that elimination of protons is one mechanism for sorbic acid tolerance as the plasma membrane H^+-ATPase is involved in this tolerance mechanism by excluding H^+ from the cytosol (Holyoak et al., 1996). We propose that the V-ATPase contributes to the sorbic acid tolerance mechanism in *S. cerevisiae* by accumulating excess H^+ from the cytoplasm in the vacuole (Figure 6.1).

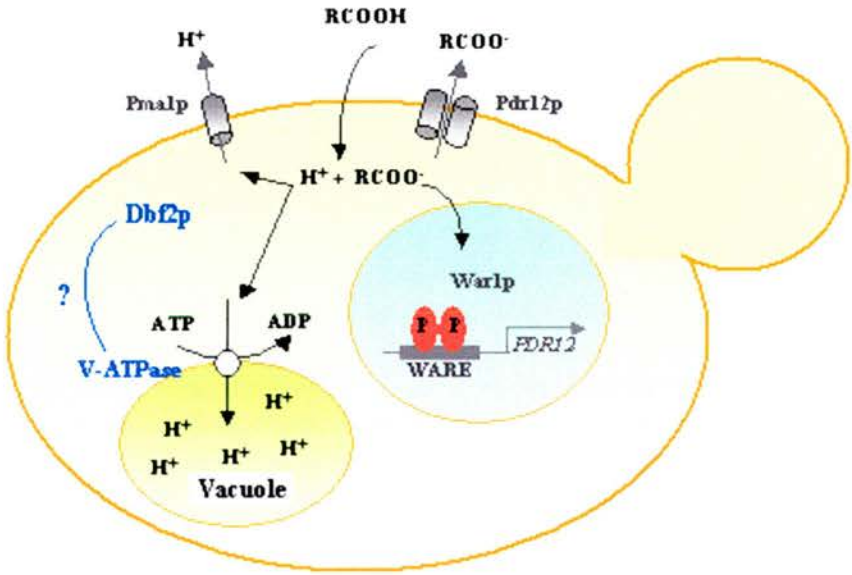

Figure 6.1. Schematic model of sorbic acid tolerance mechanisms in *S. cerevisiae*. Uncharged weak acids (RCOOH) enter the yeast cell and dissociate giving protons (H^+) and anions ($RCOO^-$). The acid anions activate the main mechanism of weak acid stress tolerance consisting of War1p and Pdr12p. We propose that the excess protons are extruded not only by the P-ATPase as described elsewhere (Holyoak et al., 1996) but also by a novel pathway via the vauole. A functional vacuolar H^+-ATPase is crucial for sorbic acid tolerance and is regulated via the Dbf2p kinase through an unknown signalling cascade.

In this study we propose that sorbic acid stress adaptation in *S. cerevisiae* is a multi-component stress response involving distinct mechanisms of tolerance. The main mechanism is controlled by the Pdr12p pump under its main stress regulator, War1p (Kren et al., 2003; Piper et al., 1998). The secondary mechanisms are that of the vacuolar H^+-ATPase regulated by the Dbf2p kinase and the plasma membrane P-ATPase. All three mechanisms are essential for sorbic acid adaptation in *S. cerevisiae*

(Figure 6.1). A reason as to why yeast cells require distinct and multiple mechanisms for weak organic acid resistance is possibly to avoid the unwanted effects of higher Pdr12p levels under general environmental stresses. This also highlights the importance of maintaining a neutral pH_i for optimal function of enzymes, the cell cycle and all the metabolic functions within the cell.

6.2 Future work

With the advent of genome/proteome wide approaches, insights into novel interactions between proteins involved in the same biological function are more easily gained. However, these interactions are often very complex. To appropriate a quote by John Donne "no protein is an island entire of itself"-or at least very few proteins are. Most seem to function within complicated cellular pathways, interacting with other proteins more commonly as components of larger complexes. Thus, to achieve a better understanding of the sorbic acid signalling cascade that mediates tolerance, many approaches could be further employed. Some of these are discussed below.

In order to identify possible up-stream components of the Dbf2p signalling pathway that allow sorbic acid tolerance, a multicopy suppressor screen of Δ*dbf2* sensitivity to sorbic acid could be performed. We could screen for multicopy suppressor gene(s) that rescue the sorbic acid sensitive phenotype of the Δ*dbf2* mutant strain using established methodologies (Rutter et al., 2002). This work has been initiated but due to time limitations it is still an ongoing process. Similarly, to identify whether there are other potential regulators of vacuolar H^+-ATPase activity we could also carry out a multicopy suppressor screen of Δ*vma2* in response to sorbic acid.

Recently, Snyder and colleagues employed protein chips to examine the substrate specificity of the majority of the protein kinases encoded by the yeast genome (Zhu et al., 2000). This approach could be useful in this work in order to identify kinase(s) that directly phosphorylate Vma2p under acidic conditions. In this way we could begin to build up the signalling cascade between Dbf2p and Vma2p. Phosphoproteome analysis

of a *Δdbf2Δvma2* double deletion strain could also provide further evidence of the intermediary proteins that take part in the signal transduction pathway of sorbic acid resistance.

In this study, using quinacrine fluorescent staining we have presented evidence that *DBF2* deletion results in a drastic decrease in vacuolar acidification. It would be interesting to measure vacuolar H^+-ATPase activity in *Δdbf2* mutant strain to determine whether the activity of the kinase is required to regulate the activity of the enzyme or the assembly of a functional enzyme. In addition to identifying if Dbf2p regulates the activity of the vacuolar H^+-ATPase, we could also determine the effect of exposure to sorbic acid on the activity of the enzyme. Also studies of changes in the vacuolar pH of *Δdbf2* and *Δvma2* mutant strains in response to sorbic acid stress could provide further evidence that Dbf2p is required to regulate the pH_i of the vauole. Changes in vacuolar pH could be monitored by measuring the change in pH-dependent fluorescence intensity of carboxyl-di-chloro-fluorescein diacetate (CDCFA) using a fluorimeter as described in previous studies (Bracey et al., 1998; Preston et al., 1989; Vindelov and Arneborg, 2002).

Identifying phosphorylation sites on Vma2p using mass spectrometry could provide putative regulatory residues for the mechanism of sorbic acid tolerance via the Dbf2p kinase. Site-directed mutagenesis of these residues could further identify the key regulatory sites that have significant effect on the activity of the enzyme under stress conditions.

Knowledge about the localization of proteins in the cell provides important information of their function. Thus, localization studies of Dbf2p and Vma2p proteins under sorbic acid stress conditions would possibly provide further evidence of their function in regulating the stress response. Proteins of interest could be tagged with green fluorescent protein (GFP) and are commercially available (Invitrogen). High-resolution confocal microscopy can be used to monitor the localisation in living yeast cells.

Structural data on the Dbf2p kinase might prove useful information to identify potential targets. In this study we have tried to express and purify the recombinant protein in an

E. coli expression system but while the expression yield was high the protein was insoluble. Thus it could be challenging to attempt to purify the Dbf2p kinase using a different expression system (i.e. the yeast *pichia pastoris* system, baculovirus or mammalian system) and try to crystalise the protein.

In this work proteomic studies have been performed to identify candidate proteins involved in sorbic acid adaptation. However, 2D-PAGE is insufficiently sensitive to detect low-abundance proteins and shows poor resolution of hydrophobic, membrane-bound proteins. These groups of proteins may represent some of the most important proteins within the cell. For example, regulatory proteins such as transcription factors are often expressed at low levels whereas ion transporters are integral membrane proteins and are highly hydrophobic and insoluble. Thus, identifying ways of studying the expression of these proteins at the level of the proteome is very challenging. We have shown in this study that a functional vacuole is important for the sorbic acid adaptation mechanism. Many gene deletions involved in vacuolar protein sorting and assembly of the vacuolar H^+-ATPase displayed marked sorbate sensitivity. We could use the commercially available yeast TAP-tagged fusion library (Open Biosystems) in combination with protein fractionation via liquid iso-electric focusing (ProTeam, Fast Flow Electrophoresis (FFE)) to perform targeted protein expression analysis of changes in the protein content of the yeast vacuole and vacuolar membrane in response to sorbic acid stress.

Finally, on a more global approach changes in gene expression due to the deletion of *DBF2* in the presence of sorbic acid could be studied. The data generated could complement the phosphoproteome and proteome analysis performed in this thesis and would give more information as to which genes are regulated by the Dbf2p kinase. Gene expression data would also complement the vacuolar protein expression analysis (described above) and allow us to establish if Dbf2p is regulating the expression of any of the other sorbate-sensitive vacuolar genes.

The work presented in this thesis is a significant step forward in gaining a better understanding of the mechanism of sorbic acid stress adaptation in the budding yeast *S. cerevisiae*. This work has highlighted two, novel key proteins involved in sorbic acid tolerance. In this study we propose a novel mechanism of regulation of sorbic acid

adaptation via the B subunit of the vacuolar H^+-ATPase (Vma2p) and the Dbf2p kinase. We have presented strong evidence supporting the hypothesis that a functional vacuolar H^+-ATPase is crucial for sorbic acid adaptation and that its role is linked to the Dbf2p kinase. In light of the results presented here further studies to identify the intermediary proteins that are involved in this signal transduction pathway that mediates sorbic acid resistance are of significant interest in the field.

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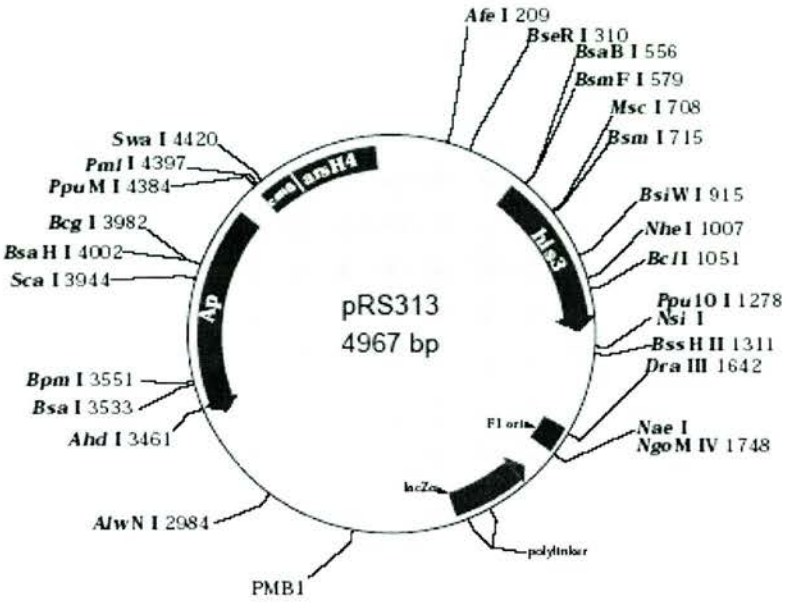
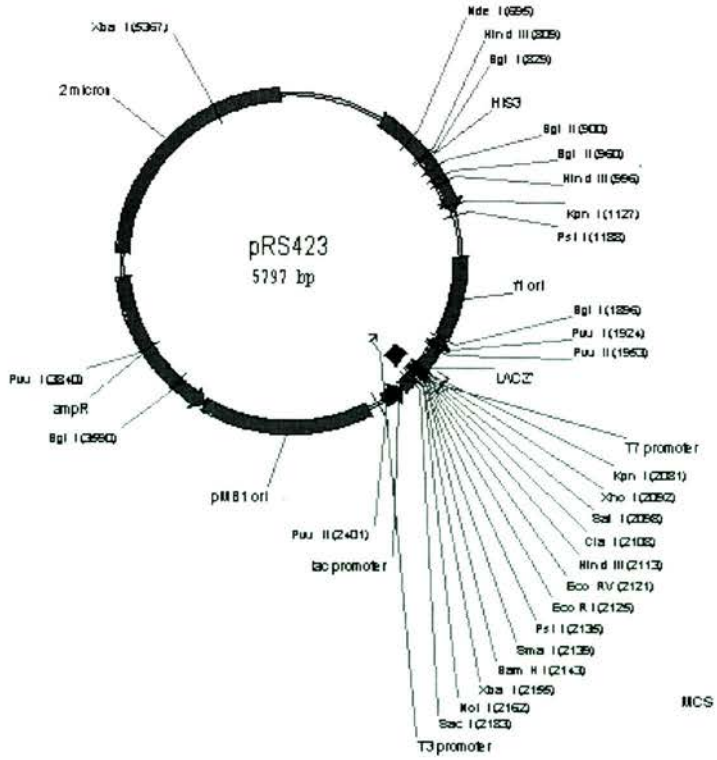
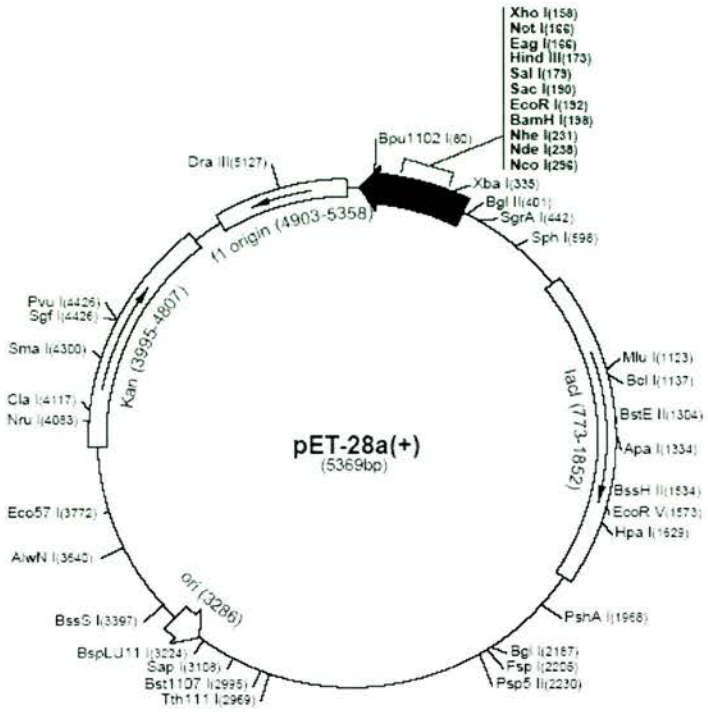
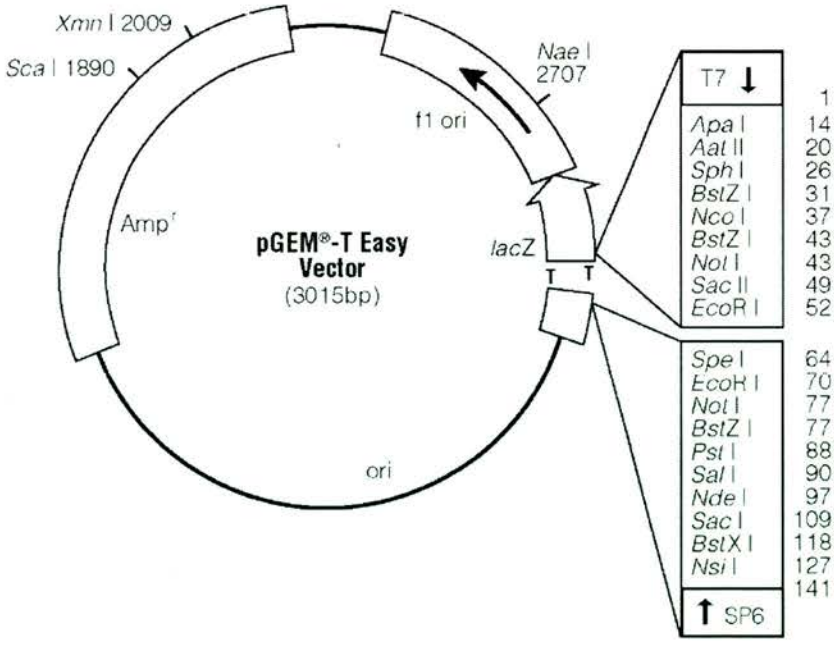
Appendix I

Maps of the pRS313, pRS423 shuttle vectors {Sikorski, 1989 #142} and the commercially available pET28c (Novagen) and pGEM-T Easy (Promega) are listed.






Xho I(158)
Not I(166)
Eag I(166)
Hind III(173)
Sal I(179)
Sac I(190)
EcoR I(192)
BamH I(198)
Nhe I(231)
Nde I(238)
Nco I(296)
Bpu1102 I(60)
Xba I(335)
Bgl II(401)
SgrA I(442)
Sph I(598)
Mlu I(1123)
Bcl I(1137)
BstE II(1304)
Apa I(1334)
BssH II(1534)
EcoR V(1573)
Hpa I(1629)
PshA I(1968)
Bgl I(2167)
Fsp I(2205)
Psp5 II(2230)
Tth111 I(2969)
Bst1107 I(2995)
Sap I(3108)
BspLU11 I(3224)
BssS I(3397)
AlwN I(3640)
Eco57 I(3772)
Cla I(4117)
Nru I(4083)
Sma I(4300)
Pvu I(4426)
Sgf I(4426)
Dra III(5127)
f1 origin (4903-5358)
Kan (3995-4807)
lacI (771-1852)
ori (3286)
pET-28a(+)
(5369bp)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lacZ
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ori
T7
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Aat II 20
Sph I 26
BstZ I 31
Nco I 37
BstZ I 43
Not I 43
Sac II 49
EcoR I 52
Spe I 64
EcoR I 70
Not I 77
BstZ I 77
Pst I 88
Sal I 90
Nde I 97
Sac I 109
BstX I 118
Nsi I 127
141
SP6