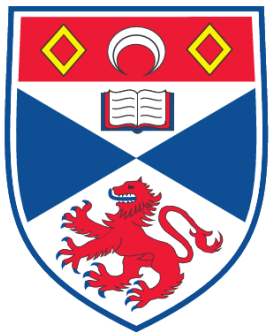
DETERMINATION OF THE MOLECULAR AND PHYSIOLOGICAL BASIS OF CITRIC ACID TOLERANCE IN SPOILAGE YEAST

Lynne McGuire

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



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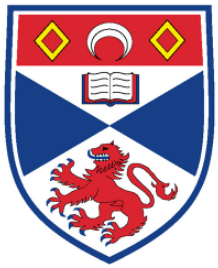

A Thesis submitted for the degree of Doctor of Philosophy at the University of St Andrews
May 2009

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Abstract

The ability of yeasts to grow and adapt under extreme environmental conditions including within the presence of weak organic acid preservatives has led to substantial economic losses through manufactured food and beverage spoilage. The food industry has employed the use of various weak organic acids such as sorbic, benzoic and acetic acid as preservatives to help prevent spoilage by yeasts and moulds. The mechanisms by which *S. cerevisiae* is able to adapt to these weak organic acids have been extensively studied. A lesser studied weak organic acid preservative is citric acid. The aim of this study was to gain further information on the mechanisms of citric acid adaptation and through this identify potential targets for new preservation strategies.

Current knowledge indicates the involvement of the HOG pathway in citric acid adaptation. A citric acid sensitivity screen from a previous study also isolated a SR protein kinase Sky1p, involved in polyamine metabolism, which has been connected with other crucial cellular processes including modulation of ion homeostasis and osmotic shock.

In this study we have undertaken a systematic screen for genes that confer increased sensitivity to citric acid paying particular attention to those involved in polyamine metabolism and those known to encode proteins which have evidence of interactions with Sky1p. Many of the deletion strains tested exhibited hypersensitivity to citric acid including Δ*sky1*. Protein-protein interaction maps for Sky1p highlighted an interesting secondary interacting protein Nmd5p, an importin crucial for the nuclear localization of Hog1p. This information suggested there may be the possibility of linkage between Sky1p and Hog1p and their roles in citric acid tolerance, perhaps through Nmd5p. This provided an incentive to perform a range of experiments to test this theory.

Proteomic and phosphoproteomic analyses were carried out to study protein expression and phosphorylation changes in response to citric acid stress. Comparative proteomic analyses for Δ*sky1*, Δ*hog1* and BY4741a with and without citric acid identified four instances of analogous protein expression responses in both Δ*sky1* and Δ*hog1*, suggesting functional overlap upon exposure to citric acid.

Epistasis studies of Δ*hog*1Δ*sky1* suggested that the two protein kinases do not function on the same pathway. However, overexpression analyses did suggest some functional interaction between Hog1p and Sky1p in mediating citric acid resistance since overexpression of Sky1p in Δ*hog1* resulted in partial rescue of growth. Further supporting evidence for some functional interaction or linkage was provided by Hog1p phosphorylation and localisation studies. Δ*sky1* exhibited dual phosphorylation of Hog1p in the absence of citric acid stress; implying that loss of *SKY1* results in dual phosphorylation of Hog1p by either prompting phosphorylation or perhaps by interfering with dephosphorylation of Hog1p. Localisation studies of Hog1p proved that like osmotic stress, citric acid stress results in nuclear translocation of Hog1p and deletion of *SKY1* seemed to interfere with this localisation to some extent.

In light of the results attained in this study we believe we have evidence to propose a novel role for Sky1p in mediating resistance to citric acid and that there is also substantial evidence to suggest that Sky1p shares some functional redundancy and perhaps functional linkage with Hog1p in citric acid adaptation.

Acknowledgements

I would like to start off by thanking my supervisor Dr Peter Coote for all his help, guidance and encouragement over the years. I must also thank Catherine Botting and Alex Houston for working their magic and identifying the vast number of protein spots I sent their way. Thanks go to Dr Jim Kinghorn and Dr Sheila Unkles for kindly allowing me to use their radioactivity lab and for all their invaluable advice with my uptake experiments. Thank you to all in MFW lab for all the great banter and also for kindly sharing much of their equipment – I did clock up some healthy mileage on those PCR machines! To all the PJC group past and present, I give a huge Thank You. To Clare Lawrence, my mentor, for giving me the best start a Ph.D. student could wish for and to Paul for his proof reading skills and advice. To Shirley who as well as being a great friend, also managed to turn our lab into a well oiled and organised machine. Betty, thank you for your great humour and making the lab a place of fun as well as work and for accessing those papers when I could not. Big thanks to my 'lab buddy', Mark. I never guessed when you first came into the lab for your Honours project that I had met one of my greatest friends. I really do miss you eventhough it did seem that I was always the one who ended up making the coffee (and washing your revolting mug!). There are so many friends outside the lab to thank; Kelly, Lynn, Cheryl, Lizzie and Laura, who gave me some welcome restbite from work when I needed it and also endured my 'yeast chat' and managed to look relatively interested. Thanks go to my big bros Gary & Martin for all their encouragement and support and to Nicky, Shannon & Rylan; hopefully Auntie Lynne will have more time for playing now. The two people I must thank the most are my parents. Thank you for giving me the greatest start in life I could have asked for and for always being there. Whenever things weren't working you were always there telling me 'stick in, you'll get there' and you were right! Thank you both so very very much.

Abbreviations

2-D	2-Dimension
2-DE	2-Dimension electrophoresis
ABC	ATP-binding cassette
Amp	Ampicillin
APS	Ammonium Persulfate
ATP	Adenosine triphosphate
CA	Citric acid
CIP	Calf intestinal phosphatase
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
FFE	Free flow electrophoresis
GFP	Green fluorescent protein
HOG	High osmolarity glycerol
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
LB	Luria Bertani medium
LN	Natural logarithm
MAPK	Mitogen activated protein kinase
MB	Malt extract broth
mRNA	Messenger ribonucleic acid
OD_{600}	Optical density at 600 nm
ORF	Open Reading Frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SC	Synthetic complete medium
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGD	Saccharomyces cerevisiae database
SRPK	Serine and arginine rich protein kinase
STRE	Stress response element
TCA	Tricarboxylic acid
TEMED	Tetramethylethylenediamine
V-ATPase	Vacuolar ATPase
WT	Wild-type
YEPD	Yeast extract broth
Bq	Bequerel

Contents

List of figures

List of tables

Chapter 1

1. Introduction

1.1. Food Spoilage and Preservation

1.1.1. Food Spoilage – impact and causes

Food spoilage results in a considerable annual economic loss to food and beverage manufacturers. It has been estimated that food losses due to microbial spoilage in the UK alone exceed £100m each year (Gould, 1989). Even though this statistic is dated, the current problem is known to be even more acute and highlights the great economic damage that microbial spoilage inflicts on businesses each year. In 2001, microbial contamination was responsible for the highest proportion of the total food and beverage recalls in Australia, totaling 33% (Figure 1). It is for these reasons that investigation into improving preservation methods against these microorganisms is of interest and commercial importance.

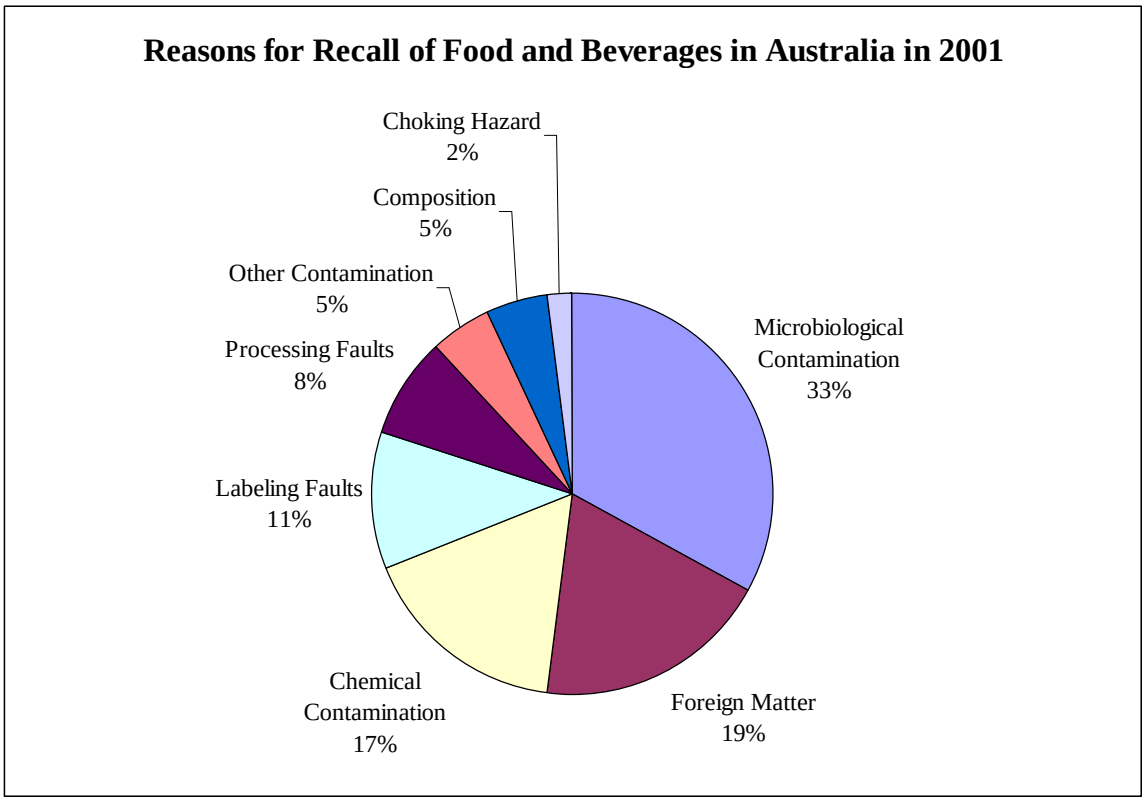


Figure 1.1: Percentages of food and beverage recalls in Australia for 2001. Microbial contamination constituted 33% of the total food and beverage recalls in Australia in 2001. GeneralCologne Re. (January 2003). Loss & litigation report: Recall of food and beverage.

Food spoilage can be considered as any changes, which renders products unacceptable for human consumption (Hayes, 1985). It can be caused by a wide range of reactions including physical, chemical, enzymatic or microbial (Huis in't Veld, 1996). Microbial action is a common cause of food spoilage and also the most common cause of foodborne illness (Steele, 2004). So-called perishable foods such as fresh fruit and vegetables; poultry, meats and fish; dairy products and bakery products, are most susceptible to microbial spoilage. Microorganisms predominantly responsible for food spoilage include bacteria and fungi (moulds & yeasts).

Many bacteria that can cause signs of food spoilage are not harmful upon ingestion but do produce 'off'-flavours and smells, and changes in appearance and texture. For example: *Pseudomonas lundenis*, a gram-negative rod bacterium that causes spoilage of milk, cheese, meat and fish (Gennari and Dragotto, 1992) and *Bronchotrix thermosphacta* causes spoilage but does not produce toxins. In contrast, there are a small percentage of pathogenic bacteria which can cause illness which do not generally affect the taste, smell, or appearance of food. These include *Escherichia. coli* O157:H7 and *Salmonella* and the toxin producing bacteria *Clostridium botulinum* and *Staphylococcus aureus* (Marianski and Marianski, 2008).

Food spoilage caused by yeast is usually recognisable by changes in appearance of the product. Such characteristic features include; production of slime, pigmented growth on surface areas, fermentation of sugars producing gas, recognizable by expansion of flexible packaging (Legan and Voysey, 1991); or production of acid or alcohol giving 'off'-odours and flavours (Huis in't Veld, 1996). Very few yeast species are considered as genuine pathogens to humans. *Candida albicans, Cryptococcus neoformans* and

Malassezia fur fur can cause infection but are not classed as ‘spoilage’ yeasts (Deak, 2007).

The percentage of yeast species associated with food spoilage is less than 10%. Of the known ~120 yeast species, about 10 are responsible for spoilage of foods that have been processed and packaged according to the standards of good manufacturing practice (GMP) (Pitt and Hocking, 1985). Amongst them, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Zygosaccharomyces lentus* and *Zygosaccharomyces rouxii* are reported to be mainly responsible for the significant economic losses caused by food spoilage. This is due to their ability to be physiologically viable in relatively extreme environments. (Neves *et al*, 1994). These extreme environments refer to the presence of weak acid preservatives, low pH, and low water activity, which are sufficient to prevent growth of other spoilage microorganisms such as bacteria.

The yeasts of the *Zygosaccharomyces* genus are notorious as spoilage agents within the food industry with *Z. bailli* considered as the main cause of serious spoilage of bottled wines (Deak, 2007). *Z. bailli* is characterized by its extreme resistance to acetic acid and preservatives, whereas *Z. rouxii* can tolerate high concentrations of sugar and salt (Steele, 2004). It is these characteristics which allow them to grow on low pH and high sugar content food stuffs such as fruit juices, wines and chocolate and in the presence of weak organic acid preservatives (Table 1.1). Another member of the Zygosaccharomyces genus also found to cause food spoilage is *Z. lentus*. It is capable of growth in a wide range of foods, particularly low pH, high sugar foods and drinks. Studies suggest that this species is likely to be more significant than *Z. bailli* in the spoilage of chilled products (Steels *et al*., 1999).

Table 1.1: Food stuffs and beverages affected by spoilage yeast

Examples of food spoilage yeast and typically affected products	
Organism	**Product**
Saccharomyces cerevisiae	Soft drinks, dressings, wine, beer, mayonnaise, bread
Zygosaccharomyces rouxii	Soy sauce, fondants, chocolate
Zygosaccharomyces bailii	Dressings, soft drinks, wine
Zygosaccharomyces lentus	Fruit juice, wine, ketchup

Data were taken from Smits and Brul, 2005; Deak, 2007; Steels *et al*., 1999.

Saccharomyces cerevisiae strains are widely used in the industrial production of beer, wines and bakery food stuffs. However, at the same time it is also one of the most common spoilage yeasts found in foods and beverages affecting those food products with low pH and high sugar content (Steele, 2004). Spoilage of wine, beer, and bread by 'wild' strains of *S. cerevisae* is a great problem, highlighting that differences exist between commercially beneficial strains used in industry and those which can cause detrimental effects in food and beverage production (Deak, 2007; Jespersen and Jakobsen, 1996; Legan and Voysey, 1991).

Most food spoilage yeast including *Z. bailii*, *Z. lentus*, and *Z. rouxii* belong to the *ascomycetes* phylum and are thus closely related to the widely studied unicellular model fungus *S. cerevisiae* (Smits and Brul, 2005). With its genome completely sequenced, this extremely well characterized microorganism has been the organism of choice for many studies looking at how spoilage yeasts are able to grow in such extreme environments particularly in the presence of weak organic acid preservatives.

1.1.2. Preservation techniques

Smoking, freezing, and drying have been used to preserve food since the Neolithic period (Carolina Biological Supply Company, 2003). Also, many natural compounds in plants, herbs and spices, such as garlic and onion were found to influence microbial stability and improve both smell and taste (Lawson, 1996). Such techniques are still used today. However, with the increase of knowledge into the growth and metabolism of these microorganisms, chemical agents which interfere and prevent growth have been employed.

The use of chemical preservatives both directly in food stuffs and within wrapping materials is extensive. Such steps were necessary to satisfy the modern day consumer. Contrary to past practice, where foodstuffs were grown and sold locally, products are produced globally and transported hundreds of thousands of miles to their consumer market. This new system of marketing, together with greater consumer demand for more convenience foods, has led to the development of more effective preservation procedures to protect food from spoiling (Holyoak, 1999).

Progress in the development of new preservation methods has been slow, despite the recognized need for additives, as their safety has been under scrutiny. Since the 19th century many inexpensive chemicals known to exhibit strong antimicrobial properties have been used as effective food preservatives. Some, such as salicylic acid and formaldehyde were subsequently abandoned when undesirable physiological and biochemical properties were discovered. More recently consumers have demanded more ‘natural’ products and subsequent changes in legislation in many countries has resulted in the abandonment of these chemical additives for more natural methods of preservation

including the use of weak organic acids (Brul and Coote, 1999). Sorbic acid, benzoic acid, and p-hydroxy benzoic acid esters have proved to be of use in various food preservation systems and their use has been officially permitted in almost all the countries of the world (Thakur *et al*., 1994).

1.2. Weak Acids as Preservatives

The use of weak acid preservatives in foods and beverages to inhibit the growth of microorganisms extends back many centuries. The employment of sulphur dioxide through burning sulphur in the preservation of cider was described as far back as 1670 (Pilkington and Rose, 1989). Today, this method still prevails in the preservation of wine. Other weak acid preservatives include; propionic acid in bread, acetic acid in pickles and in recent times the acids: sorbic and benzoic have been extensively used in soft drinks (Chichester and Tanner, 1972). The monocarboxylic acids; sorbic acid, benzoic acid, and acetic acid, and the tri-carboxylic acid; citric acid, are some of the most commonly used preservatives in the food and beverage industry today.

1.2.1. Sorbic acid

Sorbic acid is a naturally occurring weak acid. It appears as white crystals and is used either on its own or as the sodium, potassium or calcium salts known as sorbates. It was first isolated from the oil of unripened Rowanberries (Mountain Ash) by a German chemist A.W Hofmann during 1859 in London (Sofos and Busta, 1981). However, it was not until approximately 90 years later, in the 1930's that its antimicrobial properties were recognized. Then it was not until the 1950's that sorbic acid became widely used as a preservative.

Sorbic acid is a straight chain trans-trans α,β-unstaurated monocarboxylic acid with the chemical formulae $CH_3CH=CHOH=CHOOH$, a molecular weight of 112.13 and a pKa of 4.76. The most common sorbate products used in food preservation are sorbic acid itself (E200) and potassium sorbate (E202). Sorbates are used in the preservation of many foodstuffs including dairy products, fruit juices, wine, mayonnaise and salad dressings (Sofos and Busta, 1981).

1.2.2. Benzoic acid

Benzoic acid is a colourless crystalline solid and the simplest aromatic carboxylic acid with the chemical formulae C_6H_5COOH, a molecular weight 121.11 and a pKa of 4.19. Its name was derived from gum benzoin, a balsamic resin obtained from the bark of several species of trees in the genus Styrax, which was for a long time the only source for benzoic acid. It can be used as a food preservative either in the free acid form (E210) or its salts; sodium benzoate (E211), potassium benzoate (E212) and calcium benzoate (E213). Typical levels of use for benzoic acid as a preservative in food are between 0.05-0.1%. Some concern has been expressed that benzoic acid and its salts may react with ascorbic acid (vitamin C) in some soft drinks, forming small quantities of the carcinogen benzene (Van Poucke *et al*., 2008).

1.2.3. Acetic acid

Acetic acid, also known as ethanoic acid and is recognised for giving vinegar its characteristic sour taste and pungent smell. It is one of the simplest carboxylic acids (the second-simplest, next to formic acid). It has the structural formulae CH_3COOH, a molecular weight of 60.05 and its pKa is 4.75. Pure, water-free acetic acid (glacial acetic acid) is a colourless liquid that absorbs water from the environment, and freezes below 16.7°C to a colourless crystalline solid. It is a natural acid, present in most fruits and can be produced by bacterial fermentation and thus present in all fermented products. Commercially acetic acid is produced by bacterial fermentation of sugar, molasses or alcohol or by chemical synthesis from acetealdehyde. In the food industry acetic acid is used under the food additive code E260 as an acidity regulator and also as a preservative. It can be found in many food and drink products including; beer, bread, cheese, chutney, pickles, salad cream, brown sauce, fruit sauce, tinned baby food, sardines and tomatoes.

1.2.4. Citric acid

Citric acid is the most widely used organic acidulant and pH control agent used in the food and beverage industry. It is a tri-protic acid, containing three carboxylic acid groups with pKa values of 3.13, 4.76 and 6.4 respectively. Citric acid has a distinctive sharp taste and is found in the juice of lemons and other sour fruits. In manufactured foods and beverages citric acid is most often used as a flavour adjunct to improve taste. It is also used as a pH control agent and preservative, often in conjunction with other weak-acid preservatives, such as sorbic or benzoic acid, to prevent microbial growth and as a chelating agent to prevent food spoilage. It is commonly used in acid foods and beverages with a pH <4.6, such as mayonnaise, salad dressing and fruit drinks. In addition, it is also incorporated into low acid foods, whose pH >4.6 (Gardner and Howlett, 1972), such as; processed soft cheese and some desserts (Wright and Hughes, 1975).

The addition of acids, such as citric acid, as a preservative, is effective against bacterial growth due to the fact that most bacteria will not grow at low pH levels. Their growth range is between pH 4-9 (Gould G.W., 1989). Yeasts and moulds however, present a different problem. They pose a heightened threat due to their ability to adapt and grow under extreme conditions such as low pH. *Saccharomyces cerevisiae,* also known as ‘baker’s’ or ‘budding yeast’, is a strain of yeast which causes spoilage of foods with high sugar content and low pH such as jams, jellies and soft drinks.

1.2.5. Mode of action of weak organic acid preservatives

Generally, weak acid preservatives do not actively kill-micro-organisms but inhibit growth, inducing significantly extended lag phases (Lambert and Stratford, 1999). The more acidic the pH-value induced by weak acids, the more potent their antimicrobial action. This is due to the proportion of undissociated acid (RCOOH), which increases as pH declines, and it is this undissociated acid, which possesses the majority of the antimicrobial activity (Lambert and Stratford, 1998), although some activity by anions has been suggested (Eklund, 1989). At low pH values commonly found in food and beverages such as fruit juices, jams, mayonnaise, cheeses and soft drinks, acetic (pKa 4.75), sorbic (pKa 4.76) and benzoic (pKa 4.19) exist mainly in their undissociated state (RCOOH). The general perception of how weak acids inhibit microbial growth begins with the diffusion of the protonated, uncharged, undissociated acid molecule across the plasma membrane into the cytoplasm. Here the acid molecule encounters a pH near to neutrality, causing dissociation into charged ions, which cannot readily diffuse back across the plasma membrane. This results in accumulation of anions ($RCOO^-$) within the cell, with the release of a proton upon each dissociation, the cytoplasm becomes increasingly acidic (Lambert and Stratford, 1999). This acidification, if it occurs, may prevent growth via inhibition of glycolysis (Krebs *et al.* 1983), by prevention of active transport (Freese *et al.*, 1973) and/or through interference with signal transduction (Thevelein, 1994). Changes in intracellular pH have been reported to interfere with cell cycle control and the proliferation capacity of cells (Anand and Prasad, 1989).

Inhibition of growth by preservatives has also been proposed to be due to membrane disruption. The more hydrophobic sorbate has been shown to inhibit *S. cerevisiae* through disordering of the membrane structure, studies have showed that this was not the case for

acetate. (Bracey *et al*., 1998; Stratford and Anslow, 1996). In order to completely inhibit the growth of *S. cerevisiae* (pH 4.5), very high concentrations of acetic acid (80-150 mM) are needed. However, only 1-3 mM of the more liposoluble sorbate, which has an identical pKa and therefore degree of dissociation to acetate, can achieve this same level of inhibition (Piper *et al*., 1998; Stratford and Anslow, 1996).

Summarising these previous studies it is possible to define a hierarchy of weak organic acid inhibition levels between these three monocarboxylic acids with concentrations of acetate needed to completely inhibit *S. cerevisiae* being highest > 80 mM, with benzoic acid secondary completely inhibiting *S. cerevisiae* with > 2 mM (Hazan *et al*., 2004) to the low levels of sorbate < 3 mM needed in comparison to induce complete inhibition. It is clear that the two moderately lipophilic weak acids benzoate and sorbate are far more effective than the less lipophilic acetate at inhibition of *S. cerevisiae*, substantiated further by their YPC_{50} values; the concentration required to reduce the biomass yield to 50% of the reference condition (Table 1.2) (Abbott *et al*., 2007).

Table 1.2: Comparison of weak organic acid properties.

Acid	**Formula**	**Pk_a**	**YPC_{50}**
Acetic	CH_3COOH	4.75	105.0
Benzoic	C_6H_5COOH	4.19	1.3
Sorbic	$CH_3CH=CHCH=CHCOOH$	4.76	2.0

Data taken from Abbott *et al*., 2007.

1.3. Weak organic acid adaptation and resistance mechanisms

Immediately following weak acid addition the cells usually exit the cell cycle and enter a long period of stasis (lag phase). Eventually, after several hours, they resume growth (Piper *et al*., 1997). They are now weak acid adapted, in that they will not display transient growth arrest into fresh medium containing the same levels of weak acid (Piper *et al*., 2001). Upon exposure to weak acids the plasma membrane H^+-ATPase that regulates pHi homeostasis in yeast cells is activated and plays an essential role in optimal adaptation to preservatives (Holyoak *et al*., 1996). Weak acid exposure also results in the strong induction of two plasma membrane proteins Pdr12p and Hsp30p (Piper *et al*., 2001). A model illustrating the mechanisms by which *S. cerevisiae* cells may adapt to weak acid stress, involving the plasma membrane H^+-ATPase, Pdr12p and Hsp30p has been proposed (Figure 2). Uncharged weak acids (RCOOH) first enter the cell freely by diffusion. There in the higher pH environment of the cytoplasm, they dissociate giving anions (RCOO-) and protons (H+), which being charged accumulate inside the cell, resulting in cytoplasmic acidification. The resulting protons are extruded from the cell by Pma1p (plasma membrane H^+-ATPase), reducing organic acid levels and maintaining the electrochemical potential difference across the membrane. The anions are effluxed by induced Pdr12p in adapting cells (Piper *et al*., 2001). This mechanism is extremely energy expensive since two molecules of ATP are consumed for every weak acid molecule that enters the cell. Hsp30p has been shown to down regulate the activity of the plasma membrane H^+-ATPase and is suggested to play a role in conservation of ATP under conditions of prolonged stress (Piper *et al*., 1997). It is the combined effort of the H^+-ATPase pump and Pdr12p transporter which is vital for the restoration of homeostasis within the cell to the point where metabolic activity and cell growth can resume.

However, H^+-ATPase activity and the induction of the Pdr12p transporter facilitating anion extrusion alone would be pointless without simultaneous limitation to the diffusional uptake of the dissociated acid (Piper *et al*., 1998). Without any limitation on acid uptake, acid could potentially diffuse into the cytosol as quickly as the Pma1p and Pdr12p pumps it out creating a futile cycle which is both high energy demanding but would also result in a considerable intracellular concentration of protons. It is presently unknown how adapted cells restrict this weak acid diffusion but it there are suggestions that it may result from changes in the cell wall or membrane (de Nobel and Barnett, 1991).

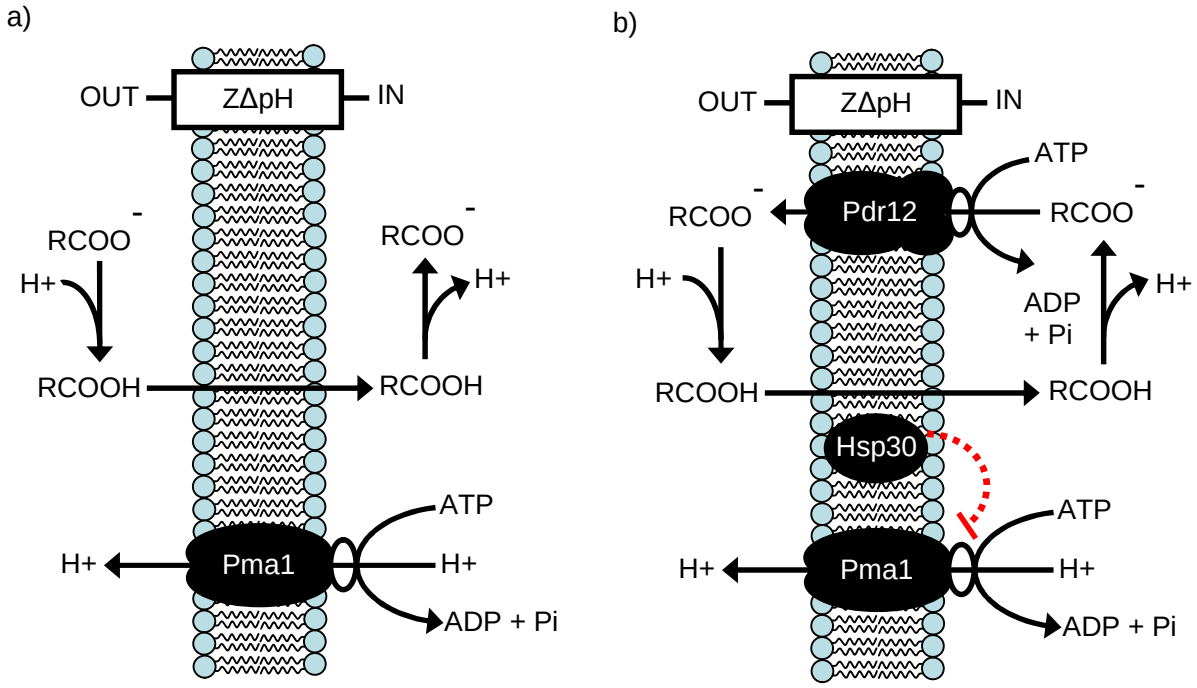


Figure 1.2: Schematic model of the effects of weak acids on unadapted and adapted yeast cells.
In both unadapted (a) and adapted (b) cells, the uncharged form of the weak acid (RCOOH) enters the cell by diffusion. The electrochemical potential difference across the plasma membrane (ZΔpH) is controlled by the proton (H+) extrusion by Pma1p. Pdr12p is induced in adapting cells and effluxes anions (RCOO-). Hsp30p down regulates the activity of Pma1p suggesting an energy conservation role, limiting excessive ATP consumption by plasma membrane H^+-ATPase during prolonged weak acid stress exposure (Model adapted from Piper *et al*., 1998).

1.3.1. The plasma membrane H+-ATPase

The plasma membrane H^+-ATPase of *S. cerevisiae*, encoded by *PMA1* is an ATP-driven proton efflux pump. It serves to generate an electrochemical proton gradient required for cell growth and development. This proton gradient drives nutrient uptake and regulates ion and pH balance (Serrano, 1991). The H+-ATPase is one of the most abundant proteins in the yeast plasma membrane and is composed of a single 100 kDa polypeptide, firmly embedded in the lipid bilayer by 10 hydrophobic α helices (Serrano *et al*., 1986). Estimations suggest that the ATPase consumes 10-15% of the ATP produced during cell growth and has a reaction stoichiometry of 1 H^+ proton translocated per ATP hydrolysed (Lutensko and Kaplan, 1995; Lutensko and Kaplan, 1996).

The plasma membrane H^+-ATPase has been implicated in response to several stresses. Enzyme activity is increased under heat shock (Piper *et al*., 1997), in the presence of ethanol (Monteiro *et al*., 1994; Rosa and Sa Corriea, 1996), suboptimal temperatures (Coote *et al*., 1994), while removal of glucose and low temperature reduced its activity (Mason *et al*., 1998). Upon exposure to weak acids the plasma membrane H^+-ATPase is strongly activated as revealed from measurements of H^+-ATPase activity in purified plasma membranes (Piper *et al*., 1997; Viegas and Sa Correia, 1991). In addition, reduced expression of the gene encoding H^+-ATPase (*PMA1*) results in an increased sensitivity of cells to weak acids (Holyoak *et al*., 1996). The increased activity of the H^+-ATPase under weak acid stress is extremely energetically expensive due to the high consumption of cellular ATP by the plasma membrane H^+-ATPase. For each weak acid molecule that enters the cell at least 1 ATP is being consumed. This is reflected in the dramatic reductions in biomass yield for cultures grown in the presence of weak acid stress (Warth, 1989; Viegas and Sa Correia, 1991).

1.3.2. The heat shock protein Hsp30

S. cerevisiae has a single highly hydrophobic integral plasma membrane heat shock protein (HSP), Hsp30p. Hsp30p is induced by weak organic acid stress and also several other stresses including heat shock, ethanol exposure, glucose limitation and severe osmotic stress (Piper *et al*., 1997). Plasma membrane H^+-ATPase activities of heat shocked and weak acid-adapted *HSP30* mutant and WT cells, revealed that Hsp30p induction leads to a down-regulation of the stress-stimulation of this H^+-ATPase. As aforementioned, plasma membrane H^+-ATPase activity is known to consume a substantial fraction of ATP generated by the cell. Hsp30-inducing stresses such as weak organic acid stress would stimulate the H^+-ATPase further, increasing this ATP usage. Studies have suggested that Hsp30p might therefore provide an energy conservation function, limiting excessive ATP consumption by the plasma membrane H^+-ATPase during prolonged stress exposure (Piper *et al*., 1997).

1.3.3. The Pdr12 transporter

Pdr12, a 171 kDa membrane ABC transporter, is induced very strongly by weak organic acid stress (maximally by 1 mM sorbic acid in pH 4.5 cultures and by 8 mM sorbic acid in pH 6.8 cultures). Pdr12p induction in sorbate-stressed cells is so great, that levels of this transporter come close to those of the most abundant plasma membrane protein, the plasma membrane H^+-ATPase, whereas in unstressed cells it is present at very low levels (Piper *et al*., 2001).

Pdr12p appears to function as an efflux pump for weak organic carboxylate anions and is essential for the development of weak organic acid resistance by mediating the energy-dependent extrusion of water-soluble carboxylate anions from the cell, thereby lowering intracellular levels of the weak organic acid. In vivo studies have shown Pdr12p lowers the intracellular levels of benzoate and fluorescein diacetate through energy-dependent extrusion from the cell (Piper *et al*., 1998). In Δ*pdr12* cells weak acid resistance is severely compromised as they are unable to catalyse this efflux (Holyoak *et al*., 1999). Strains lacking the Pdr12p transporter are hypersensitive to water-soluble monocarboxylic acids of relatively short aliphatic carbon chain length such as sorbate and benzoate. They are also sensitive to short-chain alkanols (Piper *et al*., 2001). However, Δ*pdr12* cells are neither sensitive to dicarboxylic acids, nor to the highly lipophilic, long-chain fatty acids and alcohols whose toxic effects are thought are primarily due to a detergent distruption effect on membranes (Piper *et al*., 2001). It seems, therefore that Pdr12p imparts resistance to those organic acids or alcohols that are reasonably soluble in both lipid and aqueous phases (Holyoak *et al*., 2000).

1.3.4. Different weak organic acids induce unique stress responses

The inhibition of growth does not simply constitute the toxic effect of high hydrogen ion concentration but is also dependent on the chemical nature of the acid to which the organism is exposed (Lawrence *et al.* 2004). Even when applied at identical pH values, different weak organic acids operate differently to inhibit growth (Salmond *et al.*, 1984).

The way in which *S. cerevisiae* adapts to the presence of weak acids has shown that although the component of 'acid stress' involved in adaptation to high levels of hydrogen ions and low pH may be conserved. Other inhibitory effects of weak acids must be considered to be entirely dependant on the unique chemistry of each individual acid. From this we would expect to find that different weak acids induce different stress responses specific to that compound. This hypothesis can be supported by data published on the effects of sorbic acid, acetic acid and to some extent citric acid on *S. cerevisiae*. Previous research into the effects of sorbic acid on *S. cerevisiae* has identified a role for the Pdr12 ABC transporter in the development of weak organic resistance (Piper *et al*., 1998). The recently identified transcription factor, War1p, has been implicated in activating *PDR12* through an uncharacterised signal transduction event that elicits weak organic acid stress (Kren *et al*., 2003). This work also identified that *PDR12* induction requires a unique cis-acting weak acid response element (WARE) in the *PDR12* promoter. The transcriptional response to acetic acid is not well characterized, but it is known that resistance is mediated by *AZR1*, a different plasma membrane transporter (Tenreiro *et al.*, 2000). More recently research has shown that unlike the classical weak organic acids (benzoic, sorbic and acetic acid), citric acid induced growth inhibition of *S. cerevisiae* and *Zygosaccharomyces bailii* increased with increasing pH values (Nielsen and Arneborg., 2007).

1.3.4.1. Sorbic acid response

The weak acid stress responses in *S. cerevisiae* elicited by sorbic acid have been more extensively studied than any other of the weak organic acids employed in preservation methods. Research has shown that optimal adaptation to sorbic acid stress requires the activation of the plasma membrane H+-ATPase, necessary for proton extrusion and induction of the ATP-binding cassette (ABC) efflux pump Pdr12p, essential for counteracting intracellular anion accumulation. Also identified as having a vital role in the sorbic acid stress response is War1p, a novel transcription factor of the Zn_2Cys_6 family and the stress activated regulator of *PDR12* (Kren *et al*., 2003). Sorbic acid stress is believed to activate War1p via phosphorylation, the signaling pathway mediating this phosphorylation has yet to be identified (Kren *et al*., 2003; Schuller *et al*., 2004)

A serine-threonine protein kinase Dbf2p was also found to be hypersensitive to sorbic acid. However, the presence or absence of Dbf2p did not affect expression of Pdr12p, suggesting that the kinase mediates sorbic acid tolerance via a novel mechanism (Makrantoni *et al*., 2007). In two previous studies by de Nobel *et al.* (2001) and Schuller *et al*. (2004), more than 250 genes were shown to be affected by sorbic acid stress, a sign of the complexity of the cellular response. Side-by-side comparisons of the effects of sorbic acid and other weak organic acids such as acetic acid and benzoic acid have shown that many of the same mechanisms allowing adaptation are employed. However, research has also shown that different weak acids induce different stress responses specific to that compound (Lawrence *et al*., 2004).

1.3.4.2. Benzoic acid response

The general concept of how sorbic acid acts upon *S. cerevisiae* cells has also been proven to apply to benzoic acid; the undissociated acid (RCOOH) diffuses across the membrane where it dissociates and the anion accumulation results in the cytoplasm becoming increasingly acidic (Piper *et al*., 2001). *S. cerevisiae* counteracts these inhibitory effects by inducing the plasma membrane H^+-ATPase, necessary for proton extrusion and by induction of the ATP-binding cassette (ABC) efflux pump Pdr12p, essential for counteracting intracellular anion accumulation. Additionally, there has been some suggestion that benzoic acid, like other lipohilic acids, influences the membrane either directly via membrane proteins (Sheu *et al*., 1972) or by changing membrane fluidity. The *SPI1* gene, encoding a glycosylphospatidylinositol-anchored cell wall protein, has been shown to play a prominent role in benzoic acid resistance in yeast. Increased expression of a cell *SPI1* gene under benzoic acid stress decreases cell wall porosity. Decreased cell wall porosity, in turn, reduces access to the plasma membrane, reducing membrane damage, intracellular acidification, and viability loss. The decrease in cell wall porosity was more evident for the equivalent inhibitory concentrations of benzoic acid than of acetic acid, attributable to the more lipophilic disposition of benzoic acid (Simoes *et al*., 2006).

As with sorbic acid, it is unlikely that one single mechanism is responsible for growth inhibition seen with benzoic acid.

1.3.4.3. Acetic acid response

Similar resistance mechanisms employed by *S. cerevisiae* under sorbic acid and benzoic acid stress are also induced in acetic acid stress conditions. The plasma membrane H^+-ATPase and the ATP-binding cassette (ABC) efflux pump Pdr12p are also induced by acetic acid stress. There are however, stress responses resulting in adaptation which seem to be specific to acetic acid. The *AZR1* gene which encodes Azr1p, a plasma membrane putative transporter, facilitates yeast adaptation to acetic acid. It however has no detectable effect on the resistance of yeast cells to either benzoic or sorbic acid. The mode by which Azr1p confers resistance to acetic acid is yet unknown but research suggest that is not due to efflux of acetate anions via the transporter (Tenreiro *et al*., 2000). It has also been suggested that acetic acid stress effects metal metabolism in yeast. An expression analysis on the response to acetic acid stress revealed *AFT1* was induced under acetic acid stress. Also many genes involved in metal metabolism, regulated by Aft1p, were induced under acetic acid adaptation (Kawahata *et al*., 2006). From the same study, functional screening indicated that loss of the V-ATPase and HOG MAPK proteins caused acetic acid sensitivity. A recent study has since implicated Hog1p in the phosphorylation of the aquaglyceroporin Fps1p, triggering the endocytosis and degradation of the Fps1p channel. This loss of Fps1p is important for the acquisition of resistance to acetic acid, as it eliminates the channel for the passive diffusional entry of this acid into the cells (Mollapour and Piper, 2007). It seems that only acetic acid is small enough to diffuse through the Fps1p channel. Other carboxylate preservatives such as sorbic acid and benzoic acid are too large to traverse the Fps1p pore (Mollapour *et al*., 2008).

It is apparent that even though benzoic, sorbic and acetic acid are all lipophillic monocarboxylic acids, they do not act identically on *S. cerevisiae* and the resistance mechanisms activated in response to them differ from one acid to the other.

1.3.4.4. Citric acid response

Citric acid is an intermediary metabolite of the tri-carboxylic (TCA) cycle and is a key component of normal respiratory metabolism in yeast. There are four genes listed in the *Saccharomyces* Genome database (http://www.yeastgenome.org/) under the gene ontology heading of Citrate Metabolism; *ACO1*, *CIT1*, *CIT2* and *CIT3*. Deletion of either *ACO1* (encoding aconitate hydratase) or *CIT1* (encoding citrate synthase) results in a growth defect in the presence of a non-fermentable carbon source indicating the importance of citric acid metabolism to the generation of energy via oxidative phosphorylation (Lawrence *et al.*, 2004). Research has suggested that the up-regulation of TCA cycle enzymes, such as Mdh1p and Idh1p, in the presence of citric acid, may be attempting to remove excess citric acid by increasing the rate of metabolism through the TCA cycle (Lawrence *et al.*, 2004). Apart from the research by Lawrence *et al*, which implicated the involvement of the high osmolarity glycerol (HOG) pathway in regulating the cellular response to citric acid stress, very little has been published on the effect of citric acid on *S. cerevisiae* or the way in which adaptation occurs (Lawrence *et al.*, 2004). Research into the effect of citric acid on growth of *Clostridium botulinum* has revealed that growth inhibition by citric acid was attributable to chelation of divalent metal ions by citrate. The addition of Ca^{2+}, Mg^{2+}, Mn^{2+} or Fe^{2+} was found to prevent or reduce inhibition by citrate (Graham and Lund, 1986). The regulation of cation homeostasis appears to play a crucial role in adaptation to citric acid stress which suggests there could be a possibility that components of, or linked to, polyamine metabolism could be involved. This is supported by data attained by Lawrence *et al*, where they found citric acid sensitive phenotypes for Δ*sky1* and Δ*ptk2* while screening the entire 'distruptome' of *S. cerevisiae* (Lawrence *et al.*, 2004). Sky1p and Ptk2p are protein kinases involved in polyamine metabolism.

1.3.4.5. Citric acid chelation

Chelation is the ability of compounds such as citric acid to form ring structures with metal ions preventing them from reacting with other materials or acting as a catalyst for reactions. Citric acid is the strongest chelating agent of all the common food acids and is used in the binding of metal ions in food stuffs to prevent spoilage. Copper and iron ions in food products catalyse oxidative reactions such as fat rancidity and oxidative browning and the formation of off-flavours and fishy odours (Hui and Khachatourians, 1995).

In organic acids, the greater the number of carboxylic acid groups present on the molecule, the greater the susceptibility for metal ion chelation. EDTA has four, citric acid with three, succinic acid with two and lactic acid with one. Stability constant values for citric acid's affinity for various metal ions, compared to other chelating acids has been provided in table 1.3. It is clear that chelation of Fe^{3+} and the divalent metal ions is more highly favoured by each acid than the monovalent ions.

Table 1.3: Chelation properties of acidulants used in food. Stability constant values for acid/metal complexes quoted are the log of equilibrium constant at 20-25°C.

Metal ion	**Acid**			
	EDTA	Citric	Succinic	Lactic
K^{+}	0.96	0.59	-	-
Na^{+}	1.79-2.61	0.70	0.30	-
Mg^{2+}	8.69	3.16-3.96	-	0.73
Ca^{2+}	10.45-10.59	3.40-3.55	1.20	0.90
Mn^{2+}	12.88-13.64	2.84-3.72	-	0.92
Cu^{2+}	18.80-19.13	5.90	2.93	2.49-2.55
Zn^{2+}	15.94-17.50	4.98	1.76-3.22	1.61
Fe^{3+}	23.75-25.15	11.40	6.88	6.40

Data from Russell, N. J., and Gould, G. W. (2003).

This ability of citric acid to bind and sequester metal ions has led to studies researching the effect that this chelation may have on microorganisms such as *Schizosaccharomyces pombe* (Walker and Duffus, 1979) and *Clostridium botulinum* (Graham and Lund, 1986). At 50 mM levels of citric acid, growth of the fission yeast *S. pombe* is rapidly and completely inhibited. Since citric acid is known to be a powerful chelating agent for Mg^{2+} (O'Sullivan, 1969), Walker and Duffus concluded that the inhibition of *S. pombe* cell growth was a consequence of citric acid sequestering free Mg^{2+}. The methods used in this study cannot confirm that it was Mg^{2+} being chelated. Perhaps growth inhibition was a result of the removal of other metal ions? A latter study carried out by Graham and Lund studying the effect of citric acid on the growth of the bacteria *C. botulinum* also concluded that chelation of metal ions by citric acid inhibited organism growth. The addition of 60 mM citric acid resulted in a decrease in free Ca^{2+} concentration in the medium and consequently resulted in the inhibition of cell growth. However upon addition of 12 mM CaCl the concentration of free Ca^{2+} was restored and the inhibition decreased. At pH 5.2 citric acid chelates Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+}. The resultant inhibitory effect on *C. botulinum* growth was shown be prevented or reduced by the addition of any one of these ions (Graham and Lund, 1986), highlighting the importance of these metal ions for the cell growth.

Yeast cells require a wide range of metals for their successful growth and for metabolic regulation. Yeasts need metals for maintaining structural integrity of cells and organelles, for cell division and growth, for energy maintenance and as protectants under environmental stresses (Walker, 2004). The cationic nutrients which play essential structural and functional roles in *S. cerevisiae* cells are potassium, magnesium, calcium and zinc.

Potassium cations are intracellularly the most abundant in yeast and are essential for osmoregulation, and divalent cation and phosphate uptake regulation (Jones and Greenfield, 1994). Enzymes involved in carbohydrate metabolism, protein biosynthesis and oxidative phosphorylation also rely on the availability of potassium as their cofactor.

The most abundant intracellular divalent cation is magnesium and is absolutely essential for yeast growth. Cells deficient in magnesium will not complete mitosis and there is no other metal which can take its place in this role (Walker, 1994). Being an essential cofactor for over 300 enzymes, many of which are involved in metabolic and bioenergetic pathways, magnesium plays a multifaceted role crucial in ensuring yeast cell growth.

Calcium can be considered as a trace metal with intracellular levels of free calcium being low (sub-micromolar). Its role however, is still important with small changes in intracellular calcium having the ability to trigger cascades of protein kinase activity, leading ultimately to initiation of vital processes such as the onset of mitosis (Walker, 2004).

Other trace cations such as iron, zinc, nickel, manganese and copper are essential cofactors required by metalloenzymes and haem-proteins. Media deficient in zinc has been shown to result in slow or incomplete fermentations of yeast in the brewing industry (Walker, 2004).

In order to take up these metals from their growth environment, yeast cells must transport them in their free ionized forms. Thus, physico-chemical constraints such as addition of

chelators such as citric acid impede metal ion uptake by yeast with a knock-on effect on the vital processes which require these metal cations (discussed above).

The chelating effects of citric acid on *S. cerevisiae* are not well characterised. However, the previous studies in *S. pombe* and *C. botulinum* suggest that chelation of important metal cations such as Mg^{2+}, K^{+} and/or Ca^{2+} is probable and may cause growth inhibition as a result. Lawrence's study of citric acid adaptation also suggested the possibility that citric acid chelation of Ca^{2+} from the medium may cause growth inhibition in *S. cerevisiae* and that the addition of $CaCl_2$ rescued cell growth (Lawrence *et al*., 2004). Unpublished data from Lawrence suggested that addition of Fe^{2+} and Fe^{3+} to the media decreased growth inhibition caused by citric acid. This is also plausible since citric acid is known to be a powerful iron chelator (White *et al*., 1997). The chelating activity of citric acid for iron ions in media is further supported by the sensitivity of Δ*rcs1* in response to citric acid stress (Lawrence *et al*., 2004). *RCS1* encodes a transcription factor known to activate transcription in response to iron deprivation. Thus, if iron levels in media are hugely reduced by citric acid chelation, expression of Rcs1p will be essential.

Citric acid's chelating properties should be considered during the study of citric acid adaptation in *S. cerevisiae*. The sequestering of these vital metal ions by citric acid may be the cause of growth inhibition in *S. cerevisiae* rather than the conventional weak organic acid model; where cytoplasmic acidification by diffusion of undissociated acid across the plasma membrane results in growth inhibition.

1.4. The high-osmolarity glycerol mitogen-activated protein kinase pathway

Cells respond to diverse stresses by adapting their physiology to environmental conditions. Most cells adapt to increased osmolarity by accumulating compatible solutes to balance the cellular osmotic pressure with the external environment. *S. cerevisiae* uses glycerol as a compatible solute (O'Rourke *et al.*, 2002). The high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway in yeast was named due to the discovery of its role in osmotic stress, whereby this cascade pathway up-regulated glycerol biosynthesis. Since then the HOG pathway has also been implicated in response to heat stress (Winkler *et al.*, 2002), weak acid stress induced by citric acid (Lawrence *et al.*, 2004), and also acetic acid stress (Mollapour and Piper, 2007).

MAP Kinase pathways are highly conserved signalling modules found in both higher and lower eukaryotic cells. There they play essential roles in the response to environmental signals, hormones, growth factors and cytokines. They control cell growth, morphogenesis, proliferation, and stress responses, and they are involved in many disease processes (Hohmann, 2002). MAP kinase cascades are composed of three consecutively activated tiers of kinases: MAPKKK, MAPKK and MAPK (de Nadal *et al.*, 2002) (Figure 1.3).

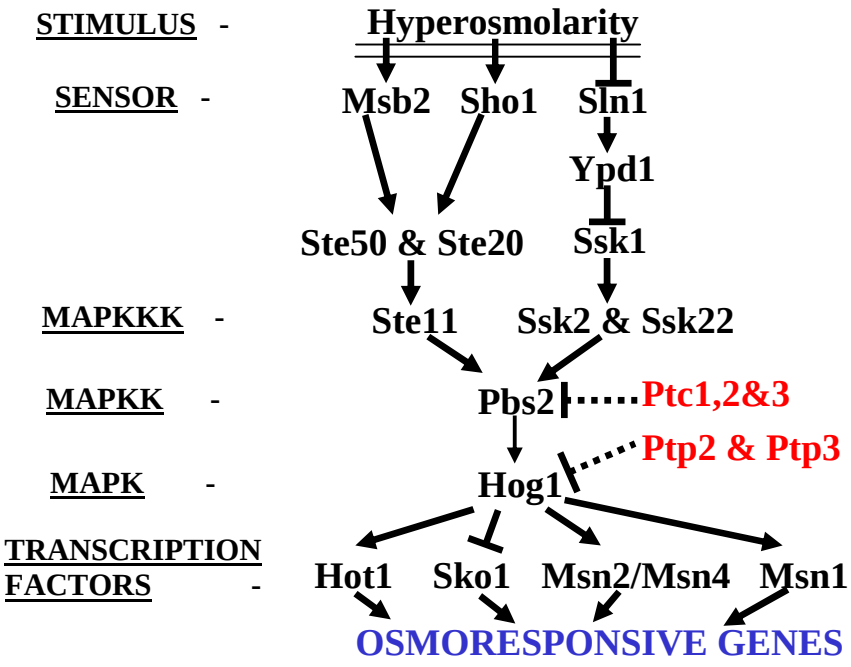


Figure 1.3: The HOG MAPK pathway.
Summary of the HOG MAPK pathway highlighting the three sensory branches regulating a down stream cascade, resulting in the phosphorylation of Hog1p, which in turn regulates the expression of numerous genes by controlling several transcriptional activators and repressors. Ptc1,2&3 and Ptp2 & Ptp3 act as negative regulators of Pbs2p and Hog1p respectively.

The HOG pathway senses osmotic stress via two membrane-bound regulators, each of which regulates a down stream cascade. One branch is the two-component system containing Sln1p, a plasma membrane localised histidine kinase response regulator protein; Ypd1p, a histidine kinase; and Ssk1p, a second response regulator protein. Sln1p-Ypd1p-Ssk1p negatively regulates the downstream MAPK cascade comprising the MAPKKKs Ssk2p and Ssk22p, the MAPKK Pbs2p, and the MAPK Hog1p (Winkler *et al.*, 2002). The second branch contains membrane bound protein Sho1p. Sho1p transduces signals via the small G protein Cdc42p, the p21-activated kinase Ste20p, Ste50p, and the MAPKKK Ste11p, resulting in the activation of Pbs2p and subsequent phosphorylation of Hog1p on Thr174 and Tyr176 residues (Winkler *et al.*, 2002). Recently evidence has been

presented for a third osmosensing branch of the HOG pathway that requires the protein Msb2p. This branch regulates Hog1p via the same constituent proteins as the Sho1p osmosensing branch (O'Rourke and Herskowitz., 2002). Once phosphorylated Hog1p is imported into the nucleus, regulating the expression of numerous genes by controlling several transcriptional activators and repressors (O'Rourke *et al*., 2002).

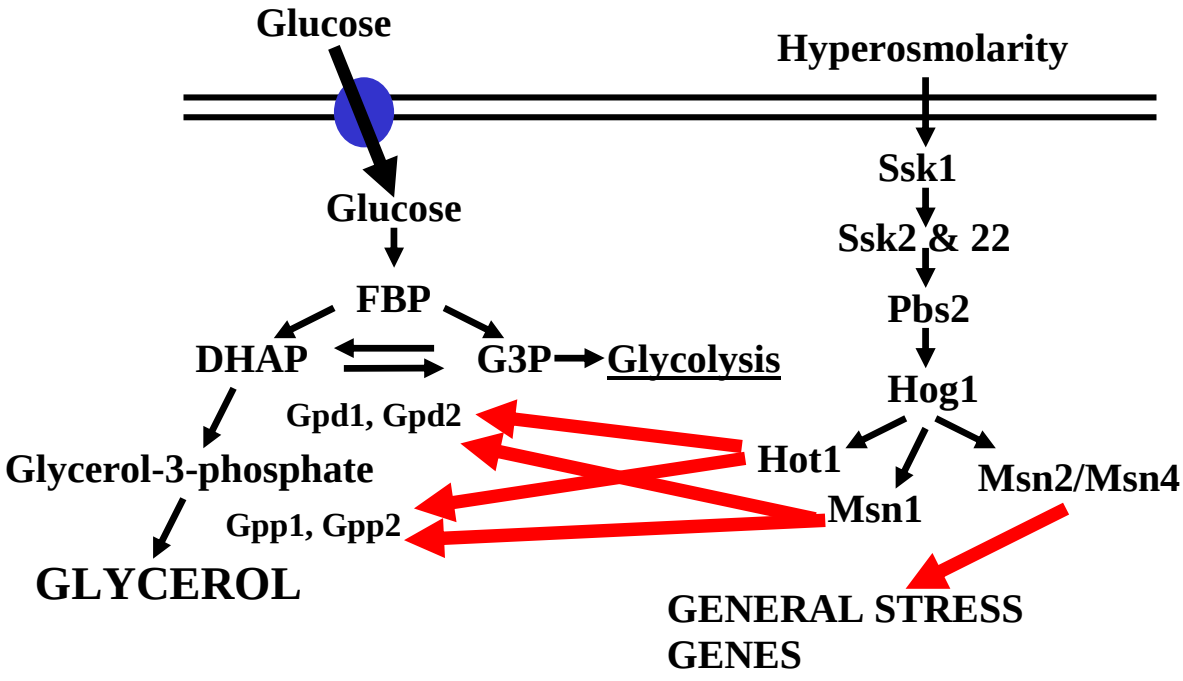


Figure 1.4: Trancription factors activated by the Hog pathway up-regulate glycerol biosynthesis upon cell hyperosmolarity. Phosphorylation of Hog1p activates the transcription factors Hot1p and Msn1p whose activation affects the expression of key enzymes in glycerol biosynthesis Gdp1&2p and Gpp1&2p. Hog1p activation of Msn2p and Msn4p results in expression changes of general stress response genes resulting in cell osmoadaptation.

In yeast, five transcription factors have been proposed to be controlled by the Hog1p kinase activity. Hot1p, Smp1p, Msn2p and Msn4p activate, whereas Sko1p represses or activates, different subsets of osmotic inducible and Hog1p-regulated genes (Nadal *et al.*, 2002). Hot1p affects the expression of a small subset of Hog1p target genes. These include the genes encoding the two key enzymes in glycerol biosynthesis, *GPD1* and *GPP2* (Figure 1.4). To some extent Msn1p also affects the expression of these enzymes but unlike Hot1p it is not crucial for glycerol biosynthesis. Msn2p and Msn4p regulate transcription of general stress response genes via stress response elements (STREs) (Hohmann, 2002). It is the action of these transcription factors, although not yet fully understood, which results in the osmoadaptation of *S. cerevisiae* (Figure 1.4).

Following the publication of research by Winkler *et al* (2002), the HOG pathway was also shown to be activated by heat stress. Sho1, previously identified as a membrane bound osmosensor, was shown to be required for heat stress activation of Hog1p. Sln1p, the second osmosensor was not involved in heat stress activation of Hog1p. This implied that Sho1p and Sln1p could discriminate between different stresses. Hog1p is not essential during heat stress as shown via deletion of *HOG1*, which only slowed recovery compared to the wild type (Winkler *et al.*, 2002). These findings emphasise the importance of the HOG pathway in roles other than osmoadaptation as previously shown.

Most recently the HOG pathway has been implicated in the adaptation of *S. cerevisiae* to citric acid. Lawrence *et al* have screened the yeast genome deletion set to identify genes mediating resistance to citric acid stress. They identified key components of the molecular response of *S. cerevisiae* to citric acid via analysis of differential protein expression and changes in transcriptome induced by citric acid exposure (Lawrence *et al.*, 2004). Their

results concluded that there is a new role for the HOG MAPK pathway in yeast in regulating adaptation to citric acid stress. Protein expression analysis revealed that cells grown in the presence of citric acid up-regulated both isoforms of glycerol-3-phosphatase, Gpp1p and Gpp2p. Analysis of changes in gene expression induced by citric acid revealed the up-regulation of *GPP2*, and *GPD1* which encodes glycerol-3-phosphate dehyrogenase (Lawrence *et al*,. 2004). The expression of these glycerol biosynthesis genes are known to be regulated by the HOG pathway (Figure 1.4) (Hohmann, 2002). These findings clearly implicated the involvement of the HOG pathway in the response to citric acid stress. Using an antibody that specifically detected the phosphorylation of both Thr174 and Tyr176 on Hog1p, Lawrence proved that the presence of citric acid results in dual phosphorylation, thus activation of Hog1p. This result reiterated the involvement of the HOG pathway in citric acid stress. The possibility that the inhibitory effect of citric acid at high concentrations may be due to osmotic shock was discounted as equivalent osmolarities of sorbitol, KCl, and NaCl to that of citric acid were tested against Δ*hog1* and wild type strains. No significant inhibition of growth was detected in either strain proving that the inhibitory effect of citric acid is not due to osmotic stress (Lawrence *et al*, 2004). Thus, the HOG pathway must be activated by some, as yet unknown, inhibitory effect of citric acid.

Only gene deletions on the Sln1p branch of the HOG pathway displayed sensitivity to citric acid suggesting that adaptation to citric acid only occurs via the Sln1p branch of the HOG pathway. This exemplifies the complexity of the role of HOG pathway in stress response as it known that both Sln1p and Sho1p branches are utilized in osmotic stress (Hohmann, 2002). However, only the Sho1p branch is involved in heat stress (Winkler *et al*, 2002) and the Sln1p branch is solely responsible for citric acid stress adaptation

(Lawrence *et al,* 2004). Of all the known HOG-dependent transcription factors, only deletion of *MSN4* resulted in sensitivity to citric acid. The importance of this transcription factor with respect to citric acid adaptation was reiterated when a number of genes known to be controlled by Msn2p and Msn4p were shown to have been up-regulated in the presence of citric acid, such as *CTT1*, *ALD3*, *PNC1*, *DDR48* and *YDL204w* (Lawrence *et al,* 2004). The transcription factor Hot1p known to be important for mediating enhanced glycerol biosynthesis in osmotic stress situations (O'Rourke *et al,* 2002), does not exhibit sensitivity when deleted under exposure to citric acid. Neither does Δ*msn1* which also mediates expression of *GPD1* and *GPP2*. This suggests that these transcription factors are not as important for glycerol biosynthesis enhancement under citric acid stress (Lawrence *et al,* 2004). It is apparent that the high-osmolarity glycerol (HOG) MAPK pathway plays a larger role in yeast homeostasis than merely adaptation to osmotic stress.

1.5. Polyamine Transport

The polyamine transport system involves the three known polyamines: spermine, spermidine and their precursor putriscine. These ubiquitous polycations have been demonstrated to be essential for various cellular functions such as growth, proliferation, differentiation, transformation and apoptosis (Kahana, 2007). The significant importance of these polycations in these crucial cellular processes has led to the theory that polyamine transport may in fact be involved in the adaptation of *S. cerevisiae* to low pH. Although most cells are capable of transporting polyamines, the mechanism that regulates polyamine transport in eukaryotes is still largely unknown. There appears to be at least two polyamine transport systems that can recognise all three polyamines. A vacuolar membrane transporter (*TPO1*), which excretes spermidine has also been identified. A distinctive feature of the polyamine transport system in *S. cerevisiae* is that it is strongly inhibited by Mg^{2+}. Thus in a Mg^{2+} - limited medium, polyamines, especially spermine, overaccumulate in cells and are toxic for growth. Igarashi and Kashiwagi (1999) isolated a mutant (*YTM22-8*) whose growth was tolerant to spermine in Mg^{2+} limited medium. The mutant was defective in polyamine uptake and did not accumulate spermine. The genes *PTK1* and *PTK2* were found to restore spermine sensitivity when cloned into the mutant. These genes encode putative serine/threonine protein kinase. This was suggestive that spermine uptake in yeast was regulated by phosphorylation and dephosphorylation by protein kinases (Igarashi, and Kashiwagi, 1999). It was the discovery of a unique protein kinase, Sky1p, which would give further evidence that this was so.

1.6. The SR Protein Kinase Sky1p

A surprising finding was published in 1999, when an SR protein kinase (SRPK), now referred to as Sky1p, was discovered in *S. cerevisiae*. SR proteins are a family of mRNA splicing factors characterised by one or two N-terminal RNA recognition motifs and a C-terminal tail enriched in alternating serine(S) and arginine (R) dipeptides. SRPKs regulate the interaction and nuclear distribution of SR proteins. Sky1p showed a similar structure to other protein kinases (Figure 1.5). The discovery of this SRPK was surprising due to the fact that it had been generally assumed that budding yeasts did not have an SR system (Yun and Fu, 2000).

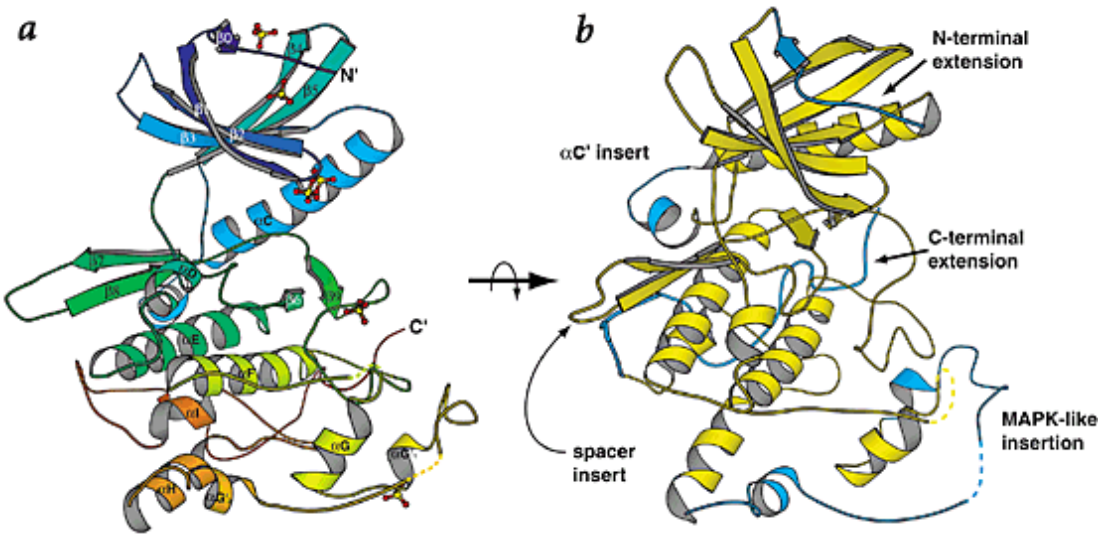


Figure 1.5: Overall structure of Sky1p.
a. The overall structure of Sky1p shows similarity to other protein kinase structures. Sky1p has a small lobe consisting of mainly β-strands and a large lobe that is mainly α-helical. The large `spacer' insertion common to the SRPK family members lies between β 7 and β8 on the back side of the catalytic cleft near the hinge between the two lobes. Breaks in the model are represented by dotted lines. Sulfates from crystallization are shown in ball-and-stick representation. b, Nonkinase core segments of Sky1p. The orientation in a. was rotated along the x-axis by −40° to highlight nonkinase core segments of the protein, which are light blue (Nolen *et al*, 2001).

The recognition of this SRPK led to the discovery of its involvement in the modulation of many cellular processes including polyamine transport, ion homeostasis and osmotic shock. Erez and Kahana (2001) established the involvement of *SKY1* in regulation of polyamine transport. They found that like the *PTK2* deletion, deletion of *SKY1* confers tolerance to spermine by dramatically reducing its uptake into the cell. Spermidine and putrescine uptake were also severely inhibited by the *SKY1* deletion. It was also evident that *SKY1* and *PTK2* were not only linked by their involvement in polyamine transport but also in regulation of salt homeostasis. Deletion of either *SKY1* or *PTK2* results in an increase in tolerance to LiCl and NaCl. Although both deletions increased salt tolerance, it emerged that the *SKY1* disruption, but not *PTK2* disruption, increased sensitivity to osmotic shock caused by 1.5M KCl or 1.5M sorbitol. A Δ*ptk2*Δ*sky1* mutant displayed spermine tolerance which proved greater than the additive tolerance of the single mutants, therefore concluding that the two kinases act in two parallel signalling pathways (Erez and Kahana, 2001). This involvement of *SKY1* with osmotic shock and its sensitivity to citric acid (Lawrence *et al*, 2004), provided further motive for the study of its possible involvement in citric acid adaptation.

The possibility of *SKY1* involvement with adaptation to low pH is reiterated in a study where deletions of *SKY1* or *PTK2* in Δ*trk1*Δ*trk2* mutant cells exerted a dual effect on ion homeostasis. Trk1p and Trk2p are transporters, which utilize the H^+ generated membrane potential to transport K^+ into the cell, creating an opposite gradient and relieving membrane polarisation. Deletion of *SKY1* in Δ*trk1*Δ*trk2* mutant cells were shown to have improved growth on low pH media in a Trk1,2p dependent manner (Erez and Kahana, 2002). The involvement of *SKY1* in the modulation of salt tolerance, membrane potential and the Trk1,2p potassium transporter generated a hypothesis for the mechanism of *SKY1*

action: the processing and/or nuclear export of the mRNA for some cation transporters including Trk1,2p could be modulated by Sky1p through its known action on SR proteins. Interestingly, nuclear mRNA export via the Sky1p/Npl3p pathway is inhibited during heat shock and high salt stress, whereas mRNAs for heat shock proteins are exported through distinct pathways after stress. Therefore the balance between both pathways, perhaps modulated by Sky1p, may be important for expression of stress tolerance genes (Forment *et al.* 2002).

1.7. *Saccharomyces cerevisiae* as a model organism for analysis

Apart from the fact *S. cerevisiae* is one of the major causative strains of food spoilage, it was also the first unicellular eukaryotic organism to be completely sequenced thus a more attractive model to use for food preservation research. The entire genome sequencing of *S. cerevisiae* (strain S288C) was carried out by an International Consortium involving more than 100 laboratories from Europe, USA, Canada and Japan and released by GenBank in 1996 (EMBL-EBI, 2003). This simple, unicellular eukaryote has developed into a unique powerful model system for biological research. Its advantageous features are include cheap and easy cultivation, short generation times, and a detailed genetic and biochemical knowledge accumulated through many years of research and the ease of the application of molecular techniques for its genetic manipulation. Based on these features, this fungus provides a highly suitable system to study biological processes such as the adaptation of yeast to low pH.

The laboratory strain used in this and many other studies BY4741a is a derivative of the strain S288C. BY4741a is used widely since it is the parent strain used in the knockout library (Winzeler *et al.,* 1999), the TAP library (Ho *et al.,* 2002) and the GFP fusion library (Huh *et al.,* 2003). Strains such as BY4741a are based on *S. cerevisiae* S288C, where by commonly used selectable marker genes are deleted. These strains allow the use of vectors by minimizing or eliminating the homology with corresponding marker genes without significantly affecting adjacent gene expression (Brachmann *et al.,* 1998).

1.8. Aims of this study

The purpose of this research was to gain further fundamental understanding of how spoilage yeasts adapt to preservative stress with specific reference to citric acid stress. This study concentrated on the molecular level responses and aimed to identify other novel components that contribute to weak organic acid adaptation in *S. cerevisiae*. This knowledge may offer potential new methods to combat spoilage or provide a better understanding of how it can be prevented.

Chapter 2

2. Materials and methods

2.1. Chemicals and Suppliers

General laboratory chemicals were of analytical grade and purchased from Sigma-Aldrich Company Ltd., Dorset, UK, Fisher Scientific Ltd., Leicestershire, UK, and Melford Laboratories Ltd., Suffolk, UK. Polymerase Chain Reaction (PCR) DNA oligo nucleotide primers were synthesised by MWG Biotech (Eurofins MWG Operon, London, UK). Restriction enzymes, T4 DNA ligase, Calf intestinal phosphatase (CIP), Taq polymerase and 1kb DNA ladder were from Promega Ltd., Hampshire, UK.

Plasmid DNA extraction kits (QIAprep Spin Miniprep), PCR purification and gel extraction kit (QIAquick) were from QIAGEN Ltd., West Sussex, UK. Complete protease inhibitor cocktail, Ribonuclease A (RNAse A), lyticase and salmon sperm carrier DNA were from Sigma-Aldrich Company Ltd., Dorset, UK. [1,5-^{14}C] Citric acid was purchased from GE Healthcare Ltd., Bucks, UK.

Precast 12.5% Tris-HCl polyacrylamide gels, prestained SDS-PAGE standards broad range, 10X TGS (Tris/Glycine/SDS) electrophoresis buffer were from Bio-Rad Laboratories Ltd., Hertfordshire, UK. MagicMark™ western protein standards were from Invitrogen Ltd., Paisley, UK. Immobiline™ DryStrips, IPG buffer, DeStreak™ rehydration solution, Hybond™ ECL™ Nitrocellulose membrane and ECL™ Advance Western Blotting Detection Kit (GE Healthcare, Bucks, UK).

All disposable plasticware was obtained from Greiner Bio-One Ltd., Gloucestershire, UK. PCR tubes from Abgene Ltd., Epsom, UK and VivaSpin concentrators from Vivascience Ltd., Stonehouse, UK. Malt broth components (Difco™ malt extract broth) and Bacto™

branded media components were purchased from Becton, Dickinson and Company (BD Biosciences, Oxford, UK). Agar was purchased from Duchefa Biochemie B.V., Harlem, Netherlands. Silver stain kits were supplied by GE Healthcare Ltd., Bucks, UK and SYPRO® Ruby stain were obtained from Genomic Solutions, Michigan, USA. Coomassie gel stain was from Bio-rad Laboratories Ltd. Hertfordshire, UK and Bradford reagent (Coomassie Plus-The Better Bradford Assay) was purchased from Pierce (Perbio Science, Northumberland, UK).

2.2. Strains, growth media and growth conditions

All growth media were sterilised by autoclaving at 121°C for 20min at 15 PSI. Media, buffers and solutions were prepared using ultra pure deionised water (Elga system, Millipore, Herts, UK).

2.2.1. Yeast strains and growth media

The strain of *Saccharomyces cerevisiae* used in this study was BY4741a, (Table 1; Research Genetics, Huntsville, USA). The gene deletions used were from Research Genetics BY4741 *MAT*a haploid genome deletion set, which contains 4,847 nonessential open reading frames (ORFs) deletions disrupted by the KanMX cassette (http://www-sequence.stanford.edu/). All other yeast strains used in this study are listed in Table 2.1.

Yeast strains were grown in liquid malt extract (MB) media, pH 3.5 (0.6% malt extract (BD Biosciences, Oxford, UK), 1% glucose, 0.12% yeast extract) and solid MB media, pH 3.5 containing 2% (w/v) agar. The pH was adjusted using 10 M NaOH (Fisher Scientific Ltd., Leicestershire, UK).

Gene deletions (incorporating the KanMX disruption cassette) were selected on YEPD agar (2% (w/v) glucose, 1% (w/v) bactopeptone, 1% (w/v) yeast extract, 2% agar) + 150 μg/ml geneticin (Sigma-Aldrich, Dorset, UK). Geneticin resistance indicated the presence of the KanMX as a marker. MB agar plates were used when growing strains in the presence of citric acid. When citric acid was added to media, the pH was adjusted to pH 3.5. Growth was measured visually from agar plates incubated for 2 days at 30°C or in liquid culture by change in optical density (OD_{600}).

Malt broth media was favoured over YEPD media for growth assays since addition of citric acid seemed to result in some sort of precipitation.

Table 2.1: Strains used in this study.

Strains	Genotype	Source
BY4741a	MATa, his 3Δ1, leu 2Δ0, met 15 Δ0, ura 3Δ0	Research Genetics
BY-Deletions	BY4741a::KanMX4 deletions of 4,847 genes	Research Genetics
Δ*nmd5*	BY4741a Δnmd5::KanMX4	This study
Δ*sky1*Δ*hog1*	BY4741a Δhog1::KanMX4Δsky1::loxP	This study (P.Dennison)
Hog1-GFP	BY4741a HOG1::GFP1	Invitrogen
Δ*sky1*-Hog1-GFP	BY4741a Δsky1::KanMX4 HOG1::GFP1	This study (P.Dennison)
Sky1 comp	BY4741a Δsky1::KanMX4 [pRS313::SKY1]	This study
Δ*hog1* ovexp SKY1	BY4741a Δhog1::KanMX4 [pRS423::SKY1]	This study
Δ*sky1* ovexp HOG1	BY4741a Δsky1::KanMX4 [pRS423::HOG1]	This study
Wt + 313	BY4741a [pRS313]	This study
Wt + 423	BY4741a [pRS423]	This study

2.2.2. Yeast growth conditions

Single yeast strain colonies were picked from plates with sterile yellow tips and inoculated into 5 ml of YEPD, MB or SC containing the appropriate supplements. Overnight incubation at 30°C in an orbital incubator at 200 rpm provided starter cultures. These were used for inoculation and cells were then incubated according to individual protocols. For short term storage, cells were streaked to single colonies on to YEPD, MB or SC agar, containing the appropriate supplements, and stored at 4°C for up to two months. For long term storage, glycerol stocks were prepared. Sterile glycerol to a concentration of 15% (v/v) was added to liquid culture, vortexed and stored at -80°C.

2.2.3. Escherichia coli strain, Growth media and growth conditions

The *Escherichia coli* strain used for all DNA cloning procedures and transformations was DH5α (Invitrogen Ltd., Paisley, UK).

DH5-α Genotype: F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-

Strains were grown in Luria-Bertani (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. Single *E. coli* colonies were picked from a fresh LB-ampicillin (LB-Amp) (100μg/ml ampicillin) (Sigma-Aldrich Company Ltd., Dorset, UK) plate, inoculated into 10 ml of LB-Amp, and grown to stationary phase overnight at 37°C in an orbital shaking incubator at 200 rpm.

For short-term storage, strains were streaked onto LB plates, supplemented with ampicillin if appropriate, to obtain single colonies. For long term storage, sterile glycerol was added to a final concentration of 30% (v/v) to an overnight culture, vortexed and stored at -80°C.

2.3. Yeast Growth assays

2.3.1. Screening the 'distruptome'

Strains were inoculated in 3 ml MB media and incubated overnight at 30°C with shaking at 200 rpm. Sensitivity assays were performed by spotting 10-fold serial dilutions of an exponentially growing starter culture. The OD at 600 nm was determined for each culture and subsequently dilutions were calculated to give an OD_{600nm} of 0.1. Serial dilutions were prepared giving a concentration range of 10^0 - 10^{-5}. The varying concentrations for each deletion strain were spotted (10 μl) onto a set of MB agar plates with differing citric acid concentrations, 0 mM, 100 mM, 200 mM, 300 mM, and 400 mM [pH 3.5]. The plates were then incubated at 30°C for 3 days before analysis. Growth comparisons were made between the WT and deletion strains in response to increasing levels of citric acid concentration. Spotting assays were carried out in duplicate for each strain.

2.3.2. Growth assays of *S. cerevisiae* strains

Duplicate starter cultures were inoculated in 5 ml MB media and grown at 30°C with shaking at 200 rpm overnight. For each strain, the duplicate starter cultures were used to inoculate 25 ml MB [pH 3.5] or 25 ml MB + 300 mM citric acid [pH 3.5] to an optical density at 600nm, (OD_{600nm}) of approximately 0.05. The flasks were then incubated at 30°C with shaking at 200 rpm. Culture growth was monitored at hourly intervals via optical density (OD_{600nm}) readings over a period of 8 hours using a spectrophotometer, (NovaspecII, Amersham Pharmacia Biotech, Buckinghamshire, UK). The ODs were presented graphically with OD_{600nm} against time and LN OD_{600nm} against time showing the equation of the line. This enabled the percentage (%) sensitivity or resistance of the strain to citric acid to be calculated.

2.4. Cloning

2.4.1. *S. cerevisiae* gene cloning for complementation studies

For complementation studies the *S. cerevisiae* gene: *SKY1* was cloned under the control of its own promoter first into pGEM-T® easy vector (Promega Ltd., Hampshire, UK) and afterwards into the single-copy pRS313 vector (Sikorski and Hieter, 1989) according to standard methods (Sambrook, 1989).

2.4.1.1. PCR amplification of *SKY1*

SKY1 was amplified from *S. cerevisiae* BY4741a strain genomic DNA with Taq polymerase (Promega Ltd., Hampshire, UK). Reaction mix; 1 µl genomic DNA template, 5 µl 10 x polymerase buffer, 2 µl 10 mM dNTP mix, 4 µl $MgCl_2$, 2 µl of 10 µM forward primer stock, 2 µl of 10 µM reverse primer stock, 0.5 µl Taq polymerase and ultra-pure water to a final volume of 50 µl. The oligonucleotide primers used in this study are listed in Table 2.2. The samples were placed in a Thermo Hybaid PCR Express cycler (Franklin, Massachusetts, USA). The program was designed specifically with annealing temperatures and extension times determined by the predicted Tm of the primers and the length of the expected product, resectively. The conditions used were; 94°C for 300 s, 94°C for 30 s, 48°C for 30 s and 72°C for 120 s the preceeding three stages were repeated for 30 cycles, finishing with 72°C for 420 s. Controls were included with no template and/or missing each primer. The PCR products were run on a 1% agarose gel at 90 V. The resulting amplified *SKY1* DNA (3 kb band) was excised from the gel and extracted using a PCR gel extraction kit (QIAGEN Ltd., West Sussex, UK), according to manufacturer's instructions.

2.4.1.2. Cloning *SKY1* into pGEM-T® Easy vector

The amplified *SKY1* gene was ligated into the linearised pGEM-T® Easy Vector (Promega Ltd., Hampshire, UK) (Appendix II) via the A overhangs added onto the *SKY1* PCR product by Taq polymerase. For the ligation reaction a control and three varying concentrations of *SKY1* were prepared. Control: [1 x ligation buffer, 100 ng pGem-T® Easy Vector, 1U T4 ligase (All Promega Ltd., Hampshire, UK)]. Samples: [1 x ligation buffer, 100 ng p-Gem-T® Easy Vector, 1U T4 ligase, and 50 ng, 100 ng or 200 ng *SKY1* insert DNA]. The ligation reaction was incubated overnight at 4°C.

10 µl ligation mix was added to 100 µl competent *E. coli* cells in a 1.5 ml microfuge tube. The cells were then left of ice for 30 min, before heat shocking the cells at 42°C for exactly 90s. The cells were then placed on ice for a further 2 min. 500 µl LB was added to cells, which were subsequently incubated at 37°C for 1 hour. The cells were harvested by centrifuging at 13,000 rpm for 30 s at room temperature. The supernatant was removed and the cells were resuspended in 100 µl of dH_2O. The cell suspension was then plated onto LB + 100 µg/ml ampicillin agar plates and incubated overnight at 37°C.

PCR was carried out in order to check the *SKY1* gene insertion using the method described previously (Section 2.4.1.1). 5 µl of *E.coli* culture prepared from colonies from the *SKY1* transformations (grown in LB), was added instead of Template DNA. The PCR products were run on a 1% agarose gel. The PCR products which exhibited *SKY1* amplification, confirmed insertion of *SKY1* gene into the pGem-T® Easy Vector.

The plasmid DNA was isolated using a Miniprep kit, (QIAprep Spin, QIAGEN Ltd., West Sussex, UK) following the manufacturer's instructions.

2.4.1.3. Cloning of *SKY1* into pRS313

To clone *SKY1* into pRS313 (Appendix II), the pGEM-T plasmid was cut at two sites, either end of *SKY1* using the restriction digest enzyme Not*I* (Promega Ltd., Hampshire, UK).

Digestion of *SKY1*-pGEM-T plasmid and pRS313 was carried out using 1 x reaction buffer D, 0.5U NotI, 1μg *SKY1* pGEM-T plasmid or pRS313 DNA. The digest was incubated at 37°C overnight. To dephosphorylate the 5' overhangs and prevent re-ligation of pRS313, alkaline phosphatase (CIAP) (Promega Ltd., Hampshire, UK) was used. 1 x reaction buffer, 0.5U CIAP and 5 μg pRS313 DNA and was incubated at 37°C for 1 hour.

The digested pRS313 vector and *SKY1* p-Gem-T plasmid were run on a 1% agarose gel and the *SKY1* gene (3.0 Kb fragment) and digested pRS313 DNA bands were excised from the gel. The DNA was isolated using QIAquick Gel extraction kit (QIAGEN Ltd., West Sussex, UK) following the manufacturer's instructions.

2.4.1.4. Ligation of the *SKY1* Gene and pRS313 Vector

The *SKY1* gene and pRS313 vector ligation was prepared with a control and two differing concentrations of vector and *SKY1* DNA.

Control: [1 x reaction buffer, 100ng pRS313 vector, and 1U ligase (Promega Ltd., UK)]

Sample 1: [1 x reaction buffer, 100ng pRS313 vector, 50ng *SKY1* DNA, and 1U ligase]

Sample 2: [1 x reaction buffer, 50ng pRS313 vector, 100ng *SKY1* DNA, and 1U ligase]

The samples were left for two hours at room temperature to ensure maximal ligation, before transformation into competent yeast cells.

2.4.1.5. Yeast Transformation

WT and Δ*sky1* cultures (50 ml MB) were incubated at 30°C overnight with shaking at 200 rpm to an OD_{600} ~ 0.5. The cultures were transferred into separate 50 ml falcon tube, and the cells harvested by centrifuging at 3,000 rpm for 5 mins at room temperature ('Biofuge Primo', Heraeus Instruments, Hanau, Germany). Supernatants were discarded and the pellets washed in 10 ml dH_2O. The cells were harvested again, at 3000 rpm for 5 mins, at room temperature, and the supernatant discarded. Cells were resuspended in 1 ml 100 mM Lithium Acetate (Sigma-Aldrich Company Ltd., Dorset, UK) and transferred into 1.5 ml microfuge tube. The cells were pelleted at 13,000 rpm for 30 s and the LiAc removed via pipette. The cells were resuspended with 100 mM LiAc to a final volume of 500 ml. Samples were vortexed and 50 µl samples pipetted into new microfuge tubes. The resultant WT and Δ*sky1* competent cells were harvested by centrifuging at 13,000 rpm for 30 s and the LiAc removed via pipette.

The WT competent cells were transformed with 5, 10 and 15 µl empty pRS313 vector made up to 75 µl with dH_2O. Δ*sky1* competent cells were transformed with 5, 10, and 15 µl pRS313-SKY1 plasmid DNA and empty pRS313 vector (made up to 75 µl with dH_2O).

The transformation mix was added to the cells in order:

240 µl 50% PEG (w/v)

36 µl 1 M LiAc

5 µl Carrier DNA (Sigma)

75 µl DNA + dH_2O

The tubes were vortexed vigorously until cell pellet completely mixed, then incubated at 30°C for 30 mins. They were then heat shocked in a water bath at 42°C for 15 mins. The cells were centrifuged at 13,000 rpm for 1 min and the transformation mix removed. The pellet was resuspended in 200 µl dH_2O and plated onto YEPD + geneticin. The plates were incubated at 30°C for several days until colonies appeared.

Cloning was confirmed using PCR and spotting analyses comparing growth of WT + pRS313, Δ*sky1* + pRS313, and Δ*sky1* + pRS313::*SKY1* were carried out as per 'spotting methods' with the exception of being tested on selective synthetic complete medium (SC-His, pH 3.5) with 0-300 mM citric acid.

2.4.2. Cloning of *S. cerevisiae SKY1* and *HOG1* for overexpression studies

SKY1 and *HOG1* were cloned under the control of their own promoters into the 2µ-based multicopy vector pRS423 (Sikorski and Heiter, 1989) according to standard methods (Sambrook, 1989). The methodology used for the complementation of *SKY1* is almost exactly the same for the overexpression of *HOG1* and *SKY1*, only differences being that the multicopy vector pRS423 is used and that pRS423::*HOG1* and pRS423::*SKY1* were transformed into their opposing deletion strains.

2.4.3. Deletion of NMD5 by homologous recombination using the KanMX cassette

One step PCR cloning by homologous recombination was used for deletion of *NMD5*. PCR using 59-mer and 58-mer oligonucleotides NMD5for and NMD5rev (Table 2.2) was carried out to construct a KanMX casette flanked by 40bp up/down stream of *NMD5*, nmd5::KanMX. Homologous recombination was utilised to insert the KanMX cassette in place of *NMD5*. This method is similar to that used in creating the Yeast Knock-out (YKO) deletion collection (http://www-sequence.stanford.edu/).

The nmd5::KanMX disruption cassette sample was amplified using PCR, cleaned using a PCR purification kit (QIAGEN Ltd., West Sussex, UK) to remove residual primers and nucleotides, then transformed into yeast and selected on YEPD agar + 150 µg/ml geneticin. PCR was carried out to confirm the *NMD5* deletion by the presence of a 1.4 kb band representing the amplification of the KanMX cassette.

Table 2.2: Primers used in this study

Target DNA	*Bp*	*Direction*	*Length (nt)*	*Tm (°C)*	*Sites*	*Nucleotide sequence*
pRS313 & pRS423						
SKY1	**3079**	**Fwd**	**17**	**55.2**	**NotI**	TCT CGA TGC TCA TCG CC
		Rev	**18**	**53.7**	**NotI**	ATC ATC TTC CAC CAG GAC
HOG1	**1870**	**Fwd**	**21**	**54.3**	**NotI**	ATT TTG ACA TAC AGG AGT GCC
		Rev	**18**	**54.5**	**NotI**	CAA GAA AAT CCA ATG CGG
Homologous recombination						
NMD5		**Fwd**	**58**	**83.2**		CGA AGA CAT TTT ATT CTC GAT GGA TAT TAC AGA ATT GTT ACG TAC GCT GCA GGT CGA C
		Rev	**59**	**85.2**		AAT CGC CAT TTA ATT CAA TTA GGC ATT CAT AAT TCC CAT GAT CGA TGA ATT CGA GCT CG

2.5. Protein analysis

2.5.1. Protein extraction

Duplicate 150 ml BY4741a, Δ*sky1* and Δ*hog1* cultures were grown to OD_{600} ~ 0.5 in MB +/- 300 mM citric acid [pH 3.5]. Cells were harvested by centrifugation ('Biofuge Primo', Heraeus Instruments, Hanau, Germany), for 5 min at 3,500 rpm at room temperature. The resulting pellet was resuspended in 50 ml dH_2O and centrifuged for a further 5 min at 3,500 rpm then resuspended in 1 ml sample buffer [8 M Urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 40 mM Tris base], plus 2 µl fungal protease inhibitor (Sigma-Aldrich Company Ltd., Dorset, UK) per 1 ml sample buffer. The cell suspension was transferred to a 3 ml screw top tube containing 2 ml 425-600 µM diameter glass beads (Sigma-Aldrich Company Ltd., Dorset, UK), which was then placed in the mini-bead beater (Biospec Products, Inc., Oklahoma, USA) for 1 min at 3,000 rpm then placed on ice for 1 min to disrupt the cells. This was repeated four times. The samples were left on ice for 2 hours and then bead beating was repeated a further two times before the suspension was transferred to a 1.5 ml eppendorf. In order to remove cell debris, the suspension was centrifuged (Mini Spin®, Eppendorf Ltd., Cambridge, UK), at 13,000 rpm at room temperature for 1 min. The protein samples (supernatant) were then divided into 100 µl aliquots and stored at -80°C.

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

2.5.2.1. Protein estimation using SDS-PAGE

To determine the approximate amount of protein required for 2-DE gel analysis, protein samples were run on SDS PAGE, compared to a reference gel and calculated empirically. One dimensional SDS PAGE was carried out using 12% acrylamide precast gels (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Neat*, 1 in 2, 1 in 5 and 1 in 10 protein extract samples were prepared, diluted using SDS sample buffer [glycerol, 20% SDS, β-mercaptoethanol, 0.5 M Tris (pH 6.8), bromophenol blue]. The gel was loaded with 3 µl of pre-stained SDS standard broad range protein marker (Bio-Rad Laboratories Ltd., Hertfordshire, UK) and 10 µl of each sample. * 2 µl sample buffer added to 10 µl protein extract for neat sample.

Electrophoresis was performed in 20% (w/v) SDS running buffer [25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS, pH 8.3] (Bio-Rad Laboratories Ltd., Hertfordshire, UK), at a constant current of 50 mA, until the dye front was at the bottom of the gel. The gel was rinsed in dH_2O three times for 5 min each. It was stained with Colloidal Coomassie Blue (Bio-Rad Laboratories Ltd., Hertfordshire, UK) for 1 hour, and then destained for 30 min in dH_2O.

The gels were dried using a gel dryer and aquired using an Image Scanner (Amersham Pharmacia Biotech, Freiburg, Germany) and using ImageMaster LabScanV3.00.

2.5.2.2. Protein loading the IPG strip

The approximate amount of protein for each sample required for 2-DE (calculated empirically by comparison of the SDS PAGE) was made up to 400 µl with sample buffer [8 M Urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 40 mM Tris base, trace bromophenol blue]. The 400 µl protein sample was then pipetted into the channel of the Dry Strip reswelling tray, 18 cm IPG strips (pH range 4-7 and 6-11 used) (Amersham Pharmacia Biotech, Freiburg, Germany) lowered gel side down into the channel. The IPG strip was moved back and forth to ensure even distribution of the protein sample. The strip was subsequently covered with 2 ml Dry Strip Covering Fluid (Amersham Pharmacia Biotech, Freiburg, Germany), and left to rehydrate overnight.

2.5.2.3. Isoelectric Focusing

A layer of oil was poured onto the isoelectric focusing unit and the IPG platform placed on top, avoiding bubbles. The temperature of the equipment was set to 20°C prior to plating of IPG strips. Another layer of oil was poured onto the platform and the isoelectric focusing tray placed on top.

The rehydrated IPG strips were washed in dH_2O to remove the oil. They were then placed into the grooves on the isoelectric focusing tray with the acid end (point) towards the anode (+ve end). Two 3 mm wide electrode pads were cut to 11 cm in length and each strip was moistened with 500 µl dH_2O. Excess water was removed by blotting with paper towel and the electrode pads were placed on either end of the IPG strips, ensuring contact with the gel. The electrodes were then placed on top of the pads. The strips were then covered with dry strip covering fluid.

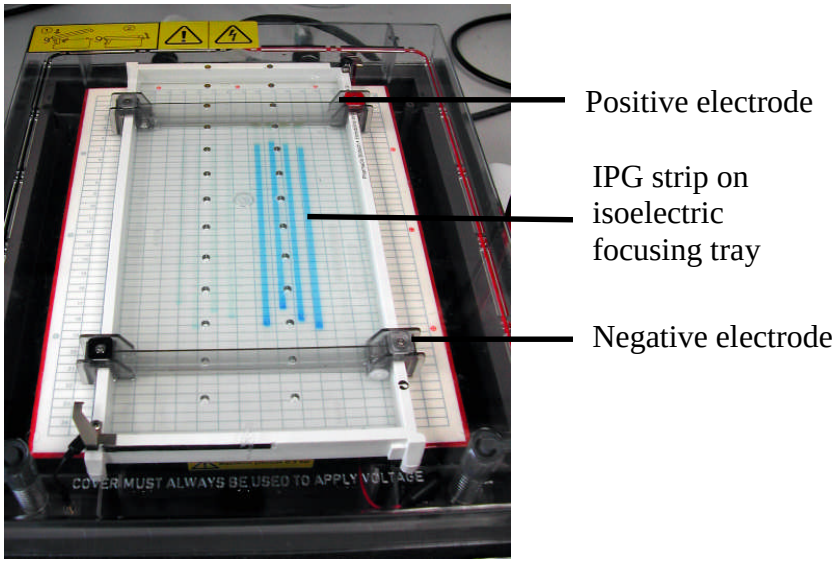


Figure 2.1: MultiphorII isoelectric focusing unit.

The IEF programme used was: 1 h at 150 V, 2 mA, 5 W; 1 h at 300 V, 2 mA, 5 W; 90 min at 3500 V, 2 mA, 5 W; and 20.5 h at 3500 V, 2 mA, 5 W.

The isoelectric focusing unit used was the MultiphorII manufactured by Amersham Pharmacia Biotech, Freiburg, Germany (Figure 2.1).

Following isoelectric focusing, the IPG strips were removed and were stored at -80°C.

2.5.2.4. 2nd Dimension – SDS PAGE

10% acrylamide gels used for the second dimension were cast using a multiple gel caster (Amersham Pharmacia Biotech, Freiburg, Germany). Care was taken to ensure the caster was packed tightly before addition of the gel solution [10% Duracryl (Genomic Solutions Inc., Huntingdon, UK), 0.4 M Tris pH 8.8 (Sigma-Aldrich Company Ltd., Dorset, UK), 0.1% SDS (Sigma-Aldrich Company Ltd., Dorset, UK), 0.1% APS (Fisher Scientific Ltd., Leicestershire, UK), 0.01% TEMED (Sigma-Aldrich Company Ltd., Dorset, UK)]. Before addition of the APS and TEMED, the gel solution was de-gassed using a vacuum for 30 mins. The APS and TEMED were added and slowly stirred for 1 min. The gel solution was then poured slowly into the gel caster, to avoid bubbles, using a funnel.

Displacing solution [0.375 M Tris pH 8.8, 50% glycerol and trace bromophenol blue] was added to the gel castor to move the gel solution into the gel cassettes, to approximately 3-10 mm from top of cassette. 2 ml dH_2O was pipetted across the top of the gel to ensure a level top. The gels were left to set for 1-2 hrs. Gels were removed from the castor and stored in dH_2O, until ready to use. The tank (Hoefer DALT 2-D electrophoresis system, Amersham Pharmacia Biotech, Freiburg, Germany) was filled with 10% 10 x SDS running (Bio-Rad Laboratories Ltd., Hertfordshire, UK) and set to 10°C.

IPG strips were placed in equilibration buffer [0.05 M Tris-Cl pH 8.8, 6 M urea, 30% glycerol, 2% SDS] with 1% DTT and incubated at room temperature, with shaking at 200 rpm for 15 mins. This would preserve the reduced state of unalkylated proteins. The solution was poured off and equilibration buffer with 0.25% iodoacetamide was added, with shaking at 200 rpm for 10 mins. This results in alkylation of thiol groups to prevent their re-oxidation. The solution was discarded and the IPG strips were placed on top of the gels. 5 µl pre-stained SDS broad range marker proteins (Bio-Rad Laboratories Ltd., Hertfordshire, UK) was applied to IEF sample application paper, this was placed in contact with the gel, to the left of the IPG strip. The IPG strip and marker were sealed onto the gel using Agarose sealing solution [SDS running buffer, 2.5% low melting point agarose (Sigma-Aldrich Company Ltd., Dorset, UK), trace bromophenol blue]. Once set the gel cassettes were placed into the 2D Electrophoresis tank and the gels were run overnight at 100 Volts at 20°C, until the dye front was near the end of the gel. The gel was removed from the cassette and stained using Silver stain or Sypro stain (Both from Genomic Solutions Inc., Huntingdon, UK), using the manufacturers instruction, with the exception that glutaraldehyde was not added to fixative 2 when silver staining. Washes with dH_2O were also extended to 5 x 12 min apposed to 4 x 15 mins.

Silver staining was used for analysis of gels and Sypro stain was used for spot identification by mass spectrometry. 10 times more protein was loaded for IPG strip rehydration when staining with Sypro to ensure a high protein concentration for spot identification.

The silver stain gels were scanned using an Image Scanner from Amersham Pharmacia Biotech, Freiburg, Germany and the computer package; ImageMaster LabScanV3.00. The Sypro gels were scanned using FLA-5000 scanner (Fujifilm Co. Ltd., Japan) at an excitation wavelength of 473 nm.

The identification of up- and down-regulated proteins was performed by mass spectrometry (Section 2.5.4).

2.5.3. Phosphoproteome analysis by 2-D electrophoresis

Affinity capture of phosphoproteins was performed using the PhosphoProtein Purification kit from QIAGEN Ltd., West Sussex, UK, according to the manufacturer's instructions with some alterations (Makrantoni *et al.*, 2005). Protein was extracted from duplicate (6 L) BY4741a cultures grown in MB, [pH 3.5] either in presence or absence of 150 mM citric acid to an OD_{600} of 0.5. Cells were subsequently harvested and washed in ice cold ddH_2O. Cell pellets were resuspended as per manufacturer's instructions. The resuspended cells were then added to equal volume of 425-600 μm diameter glass beads (Sigma-Aldrich Company Ltd., Dorset, UK) in 30 ml size bead beater vessel (Biospec Products Inc., Oklahoma, USA). Cells were lysed as previously described (Section 2.5.1). The resulting protein preparations were 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 Ltd., West Sussex, UK) prior to loading onto the column. After the entire sample had passed through, the column was washed twice and the bound proteins were eluted. Comparative 2-D electrophoresis was performed using a MultiphorII apparatus for isoelectric focussing (IEF) and a Hoefer-DALT apparatus (Amersham Pharmacia Biotech, Freiburg, Germany) as previously described (Section 2.5.2.3). Immobilized pH gradient 18 cm pH range 4-7 (Amersham Pharmacia Biotech, Freiburg, Germany) were used for IEF. The second dimension was performed as previously described (Section 2.5.2.4). The 2-D gels were stained using Silver Stain (GE Healthcare Ltd, Bucks, UK). Protein spots showing reproducible changes in protein abundance (present on both gel sets from duplicate experiments) were excised and sent for identification by mass spectrometry.

2.5.4. Mass spectrometry

Protein spots of interest were excised from the gels and identified using mass spectrometry, courtesy of Dr. C. Botting & Mr. A. Houston. Proteins were digested in the gel with trypsin (Promega Ltd., Hampshire, UK) using an Investigator Progest digestion robot (Genomic Solutions Inc., Huntingdon, UK) as previously described (Lawrence *et al*., 2004). Half of the sample was desalted and concentrated using a micro C18 column (0.2 µl of ZipTip) (Millipore, Gloucestershire, UK). The peptides were eluted directly from the tip onto the target in 1.5 µl α-cyano-4-hydroxycinnamic acid (saturated stock in acetonitrile-0.2 % trifluoroacetic acid [60:40]). Spectra were obtained on a Micromass TofSpec 2E instrument (Micromass, Manchester, UK), equipped with a 337 nm wavelength laser and operated in reflection mode. The data were calibrated using the tryptic peptides of β-galactosidase (Sigma-Aldrich Company Ltd., Dorset, UK) and lock mass corrected using a Glu-Fibrinopeptide B spike. Monoisotopic peptide masses were selected using BioLynx ProteinProbe (Micromass, Manchester, UK) 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. Western blotting analysis of Hog1p phosphorylation in Δ*sky1*

Duplicate 300 ml cultures of BY4741/a, Δ*sky1* and Δ*hog1* were grown in MB, pH 3.5 to mid-exponential phase of OD_{600} of 0.4. Three 100 ml cultures were harvested and resuspended in 50 ml; MB, [pH 3.5] (control); MB plus 300 mM citric acid [pH 3.5]; and MB plus 0.4 M NaCl [pH 4.5]. Cultures were incubated for at 30°C, 200 rpm, for 20 min before being harvested and washed in cold ddH_2O. The cell pellet was resuspended in lysis buffer [8 M Urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 40 mM Tris base], plus 2 µl fungal protease inhibitor (Sigma-Aldrich Company Ltd., Dorset, UK) per 1 ml sample buffer and lysed as previously described (Section 2.5.1). The concentrations of proteins were determined empirically and adjusted accordingly.

Following sample analysis by SDS-PAGE and incubation in transfer buffer for 15 min, proteins were transferred to nitrocellulose membranes pre-soaked in transfer buffer, and run at 400 mA for 30 min. Ponceau S solution stain (Sigma-Aldrich Company Ltd., Dorset, UK) was used to check the protein transfer then washed in ddH_2O before blocking. The membrane was blocked overnight at 4°C (10 % (w/v) skimmed milk powder, 0.1 % (v/v) Tween 20 in PBS) to block non-specific protein binding sites. The protein of interest, Hog1p, was subsequently detected with primary antibody Phospho-p38 MAP Kinase (Thr180/Tyr182) antibody (#9211, Cell Signalling Technology Inc., New England Biolabs Ltd., Hertfordshire, UK) for detection of phosphorylated Hog1p and the primary antibody Hog1 (yC-20) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) for the Hog1p level detection by incubating 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 1-2 h (Sigma-Aldrich Company Ltd., Dorset, UK). The membrane was then washed as above before bands were visualized by enhanced chemiluminescence (ECL) according to manufacturer's instructions (Amersham ECL advance kit, Amersham Biosciences, Buckinghamshire, UK). Images were taken with a Luminescent Image Analyser LAS-1000plus (Fujifilm Co. Ltd., Japan). Actin was used as a control blot; following Hog1p phosphorylation detection, the membrane was stripped using stripping buffer [2 % SDS, 100 mM β-mercaptoethanol, 50 mM Tris, pH 6.8] at 50°C for 30 min with gentle shaking, washed in PBS x 4, blocked as before then incubated in Actin Antibody (Mouse monoclonal to beta Actin, mAbcam 8224, Abcam Plc., Cambridge, UK) overnight at 4°C. Subsequently the blot was probed with the appropriate secondary antibody (Sigma-Aldrich Company Ltd., Dorset, UK) for 1 hour, washed as before and visualized by ECL.

Table 2.3 Antibodies used in this study.

Antibody	Target Protein	Source
Phospho-p38 MAP Kinase (Thr180/Tyr182) antibody	Hog1p (Thr180/Tyr182)	Cell Signalling Tech., New England Biolabs Ltd.
Hog1 (yC-20)	Hog1p	Santa Cruz Biotechnology Inc.
beta Actin antibody (mAbcam 8224)	Act1p	Abcam Plc.
Anti-mouse IgG, HRP-linked antibody	2°Ab for Act1p	Sigma Aldrich
Anti-goat IgG, HRP-linked antibody	2°Ab for Hog1p	Sigma Aldrich
Anti-rabbit IgG, HRP-linked antibody	2°Ab for p38/Hog1p	Sigma Aldrich

2.7. Detection of Hog1p translocation to the nucleus using HOG1-GFP constructs

Duplicate 50 ml cultures of Hog1-GFP and Δ*sky1*-Hog1-GFP (Table 2.1) were grown to mid-exponential phase in MB [std pH]. Cultures were split into 10 ml aliquots and cells were harvested by centrifugation at 3,000 rpm. Cells were resuspended in duplicate with four different medium (10 ml); MB [pH 5.5], MB [pH 3.5], MB + 0.4M NaCl [pH 5.5] and MB + 300 mM citric acid [pH 3.5]. Cells grown in MB + NaCl were used as a positive control for Hog1p nuclear translocation with MB [pH 5.5] as the corresponding negative control (Ferrigno *et al.*, 1998). The cultures were incubated at 30°C, 200 rpm for 2 min. Samples (1 ml) from each culture were taken and harvested at 13,000 rpm and washed once with PBS. The cells were resuspended in 1 ml paraformaldehyde (Sigma-Aldrich Company Ltd., Dorset, UK) and set aside for 10 min. The cells were harvested and the paraformaldehyde removed. The cells were washed twice with PBS and resuspended in 50 µl PBS. Varying concentrations of cells were added with 10 µl moviol + 1 µg/ml DAPI (Sigma-Aldrich Company Ltd., Dorset, UK) to microscope slides with cover slips 1/10, 2/5 and 1/5. The slides were stored overnight at 4°C to set before viewing using fluorescent microscopy. Images were captured on an Olympus IX70 DeltaVision microscope (Applied Precision Inc., Washington, USA). SoftWoRx Explorer 1.3 (Applied Precision Inc., Washington USA) was used for image processing and analysis.

2.8. Citric acid uptake assay

2.8.1. Enzymatic approach

Yeast cells were grown in 500 ml MB to OD_{600} 0.4. Cells were pelleted, washed in ddH_2O, and resuspended in 1 ml 80% (v/v) ethanol in 15 ml centrifuge tubes. To lyse the cells the suspension was boiled at 80°C for 20 min with frequent vigorous vortexing. The cell debris with denatured proteins was pelleted, the supernatant was passed through a 0.45µm filter and filtrate used in the estimation of citric acid content via an enzymatic reaction (Citric acid assay kit, R-Biopharm, GmBh, Darmstadt, Germany). 300µl filtrate was made up to 1 ml with reaction mix (2 mM Tris-Cl (pH 8.2), 4 nM $ZnCl_2$, 0.06 units ml^{-1} malate dehydrogenase (MDH), 0.2 mM NADH). A stable initial OD_{340} was noted, the reaction was started by addition of 1 unit citrate lyase and a stable final OD_{340} was recorded. Citric acid standards of known concentration were tested and a standard curve plotted using Excel. Extrapolation of the curve allows estimation of citrate content of cells.

2.8.2. Radioactivity approach - Citric acid uptake assay using [1,5-^{14}C] Citric acid

Triplicate cultures of yeast strains BY4741/a, *Δsky1*, *Δhog1* and *ΔhogΔsky1* were grown to mid-exponential phase in MB, pH 3.5 to an OD600 0.5 (50 ml). Cells were harvested, washed twice with cold sterile dH2O and resuspended in either 50 ml fresh medium MB for one experiment and MB (minus glucose) for the other. 20 ml cultures were incubated, with 100 rpm shaking at 30°C.

Prior to addition of [1,5-^{14}C] Citric acid, a 1 ml background sample was filtered through wet glass microfibre filters (Whatman® GF/C, GE Healthcare Ltd., Bucks, UK) at reduced pressure and washed twice with 10 ml cold sterile dH2O. The filter was then

placed in a scintillation vial with 2 ml scintillant (Ecoscint, National Diagnostics, Georgia, USA). The cell suspension was then quickly mixed with 20 µl [1,5-^{14}C] Citric acid, which represents 74 MBq/mmol (2 mCi/mmol). At time point 0, a 1 ml sample was taken and filtered using a 0.22 µ filter attached to a 2 ml syringe. The supernatant was collected to provide an overall count. Over 60 min, samples (1 ml) were taken at 1 min intervals, filtered and washed as before. Radioactivity was measured in a liquid scintillation counter (Beckman Coulter Ltd., High Wycombe, UK).

2.9. Tecan FFE

Microsomal preparations were obtained from duplicate 500 ml cultures of WT, with and without 300 mM citric acid, harvested in mid exponential growth (OD_{600} 0.4). Cell pellets were resuspended in 10 ml lysis buffer [50 mM Tris-HCL (pH 8.5), 1 mM EDTA, 0.4 M sorbitol, fungal protease inhibitor cocktail (Sigma-Aldrich Company Ltd., Dorset, UK)]. The cell suspension was transferred to a 20 ml bead beater vessel containing 10 ml 425-600 μ diameter glass beads (Sigma-Aldrich Company Ltd., Dorset, UK). Cells were disrupted using a bead beater (Biospec) for 5 x 1 min, with 1 min between bead beating on ice. Lysate was removed and transferred to a 15 ml centrifuge tube. Cellular debris was collected by centrifugation at 4°C twice for 10 min at 3,000 rpm ('Biofuge Primo', Heraeus Instruments, Hanau, Germany). Microsomes were pelleted by centrifugation at 4°C for 45 min at 32,000 rpm (Beckman Coulter L-90K Optima ultracentrifuge, Beckman type 70 Ti rotor, Beckman Coulter Ltd., High Wycombe, UK) and were resuspended in sample buffer [8 M urea, 2 M thio-urea, 4% CHAPS, 50 mM DTT, 40 mM Tris base, 1% Pharmalyte 3-10 (Amersham Pharmacia Biotech, Freiburg, Germany), fungal protease inhibitor cocktail (Sigma-Aldrich Company Ltd., Dorset, UK)]. The microsomal preparations were homogenised by shearing microsomes through a 27-guage needle ten times and stored at -80°C.

Figure 2.2: The Tecan ProTeam™ FFE Unit.

A 1D SDS PAGE gel was run to compare the protein concentration of the duplicate WT and WT + 300 mM citric acid samples to ensure equal loading onto the Tecan Proteam™ FFE (Free Flow Electrophoresis) unit (Tecan Ltd., Reading, UK). The Tecan FFE unit was run as per manufacturer's instructions, whereby the microsomal protein sample loaded was fractionated by pI and the resultant fractions collected in deep well 96-well plates. The pH of each fraction was recorded using a micro-pH electrode (Biotrode, Hamilton Co., Nevada, USA) and the OD_{280} for each fraction was recorded using 96-well UV plate and plate reader (KC4 Powerwave XS, Bio-Tek Instruments Ltd., Bedfordshire, UK). Sets of 15 fractions were pooled and protein concentrated using a protein concentration kit (Calbiochem, Merck Chemicals Ltd., Nottingham, UK). This step also removed much of the urea from the samples. Further concentration was undertaken via acetone precipitation, which also facilitated the removal of high salt concentration created by the protein concentration kit. This was carried out twice and the resultant concentrated protein was run on a 1D SDS PAGE gel and stained with Sypro Ruby (Genomic Solutions Inc., Huntingdon, UK). Ten bands of protein were cut out and sent off for identification by mass spectrometry.

A second run of the Tecan FFE was performed using >10 fold more microsomal protein from a 10 L culture. All steps to extract microsomal protein were carried out as previously described.

2.10. Computer analysis

Commonly used programs and databases are listed below:

Saccharomyces Genome Database (SGD): http://www.yeastgenome.org/
SGD is a scientific database providing molecular biology and genetic information for the yeast, *S. cerevisiae*.

BioGRID Interactions Dataset: http://www.thebiogrid.org
BioPIXIE Interactions Dataset: http://pixie.princeton.edu
BioGRID and BioPIXIE are databases providing information on physical and genetic interactions for various different organisms including *S. cerevisiae*.

EMBL-EBI WU-BLAST2 Home Page: http://www.ebi.ac.uk/Tools/blast2/index.html
WU-BLAST2 stands for Washington University Basic Local Alignment Search Tool Version 2.0. This tool can be used to find regions of sequence similarity between organisms providing fuctional and evolutionary clues about the structure and function of your chosen sequence.

Swiss-Prot Protein Knowledgebase: http://www.expasy.ch/sprot/
Swiss-Prot is a protein sequence database which provides high level of annotation icluding information on protein function, its domains structure, post-translational modifications and information of relevant publications.

pDraw32 v1.1: http://acaclone.com.
pDraw32 is a free DNA cloning, sequence analysis and plasmid/DNA plotting software. This software was invaluable for the construction of plasmid maps.

Netprimer: http://www.premierbiosoft.com/netprimer/index.html
Netprimer allows analysis of primers; analyzing primer melting temperature and accurately predicting Tm; also analyzing for all primer secondary structures, including hairpins, self-dimers, and cross-dimers in primer pairs thus enabling successful primer design and optimized PCR conditions.

Oligo Calculator: http://www.pitt.edu/~rsup/OligoCalc.html
Oligo Calculator is a quick and easy tool which determines; the length (bp), melting temperature (Tm), % GC content, and molecular weight of proposed oligo sequence.

NetPhos v2.0 Database: http://www.cbs.dtu.dk/services/NetPhos/
The NetPhos 2.0 server produces neural network predictions for serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins.

All URL's referenced in this and other sections were confirmed as current and functional on 4th December 2008.

Chapter 3

3. Characterisation of gene deletions conferring sensitivity to citric acid

3.1. Introduction

The cellular mechanisms of *S. cerevisiae* involved in adaptation to citric acid stress had not previously been studied until the work of Lawrence *et al*. (2004). They conducted a phenotypic screen of all 4,847 non-essential gene deletions in *S. cerevisiae* BY4741 MATa deletion set (Research Genetics, Huntsville, USA). In *S. cerevisiae*, more than 80% of the approximately 6,200 predicted genes are non-essential (Tong *et al.*, 2001). Non-essential, in this context, means that you can grow the yeast in the lab, when that particular gene is deleted; the yeast may not be happy, and may require special care, but it will survive.

Yeast deletion strains were spotted onto plates in the presence and absence of 400 mM citric acid. Of the 4,847 non essential gene deletions tested, 69 (1.4%) showed sensitivity to citric acid compared to the parent strain. No citric acid-resistant phenotypes were detected. Of the 69 citric acid-sensitive gene deletions, 10 were of particular interest because they encoded known regulatory proteins that could be involved in signaling an adaptive response to citric acid stress. In particular deletion of *MSN4* and *HOG1* (a stress related transcriptional activator, and a component of the HOG MAPK pathway respectively), were both found to be citric acid sensitive. These findings presented evidence of a new role for the HOG MAPK pathway in regulating adaptation to citric acid stress. The screen also identified that loss of *PTK2*, which encodes a protein kinase involved in polyamine uptake, and *SKY1* which encodes a SR protein kinase, shown to be involved in polyamine metabolism, also conferred sensitivity.

In this study we present the results of a more detailed citric acid sensitivity screen, focusing on genes involved in polyamine metabolism and those known to encode proteins

which interact with Sky1p. This study also looks at citric acid uptake in both the parent strain (BY4741a) and specific mutant strains using ^{14}C radio-labeled citric acid and looks at possible citric acid transporters.

3.2. Mutants indicating the importance of polyamine metabolism to citric acid resistance

A subset of genes involved in the regulation of polyamine metabolism have also been shown to be involved in the modulation of osmotic shock (Erez and Kahana, 2000). *SKY1*, which encodes a protein kinase involved in polyamine metabolism, has previously been found to be involved in the regulation of osmotic shock (Erez and Kahana, 2001) and deletion of this gene shows enhanced sensitivity to citric acid (Lawrence *et al*., 2004). Research to determine whether deletion of other genes involved in polyamine metabolism would exhibit sensitivity to citric acid, was undertaken.

Genes which were involved in polyamine transport or encoding proteins know to interact with Sky1p were selected. Additionally, genes with known connections to polyamine transport were also selected. Thus, a total of 53 viable gene deletion strains were tested.

Preliminary investigations into the effect of citric acid on the growth of these 53 deletion strains of were carried out by spotting MB agar plates [pH 3.5], with varying concentrations of citric acid (Section 2.4.1).

3.2.1. ‘Spotting’ Analysis

Sensitivity assays were performed by spotting 10 µl of 10-fold serial dilutions of an exponentially growing culture (OD_{600} of 0.1) on MB agar plates with increasing concentrations of citric acid (0 - 400 mM, pH 3.5) which were incubated for 72 hrs at 30°C. Each strain was spotted in duplicate and analysis revealed 15 gene deletion strains which exhibited a citric acid sensitive phenotype (Figures 3.1 to 3.4). The genes found to exhibit sensitivity to citric acid were: Δ*bim1*, Δ*ctk1*, Δ*dtr1*, Δ*hal3*, Δ*mag2*, Δ*met2*, Δ*mpc54*, Δ*mud1*, Δ*nam8*, Δ*npl3*, Δ*ptk2*, Δ*pub1*, Δ*sky1*, Δ*tpo1*, and Δ*yhr087w*. In particular Δ*sky1* and Δ*yhr087w* displayed the greatest sensitivity to citric acid (Figure 3.2, E).

A number of the 15 deletion strains deemed to exhibit sensitive phenotypes were borderline sensitive. However, further analysis to determine more accurately whether the deletion strain were citric acid sensitive was carried out. The 15 deletion strains isolated from the ‘spotted’ plates, were tested further using liquid culture to obtain growth curve data (Figure 3.5).

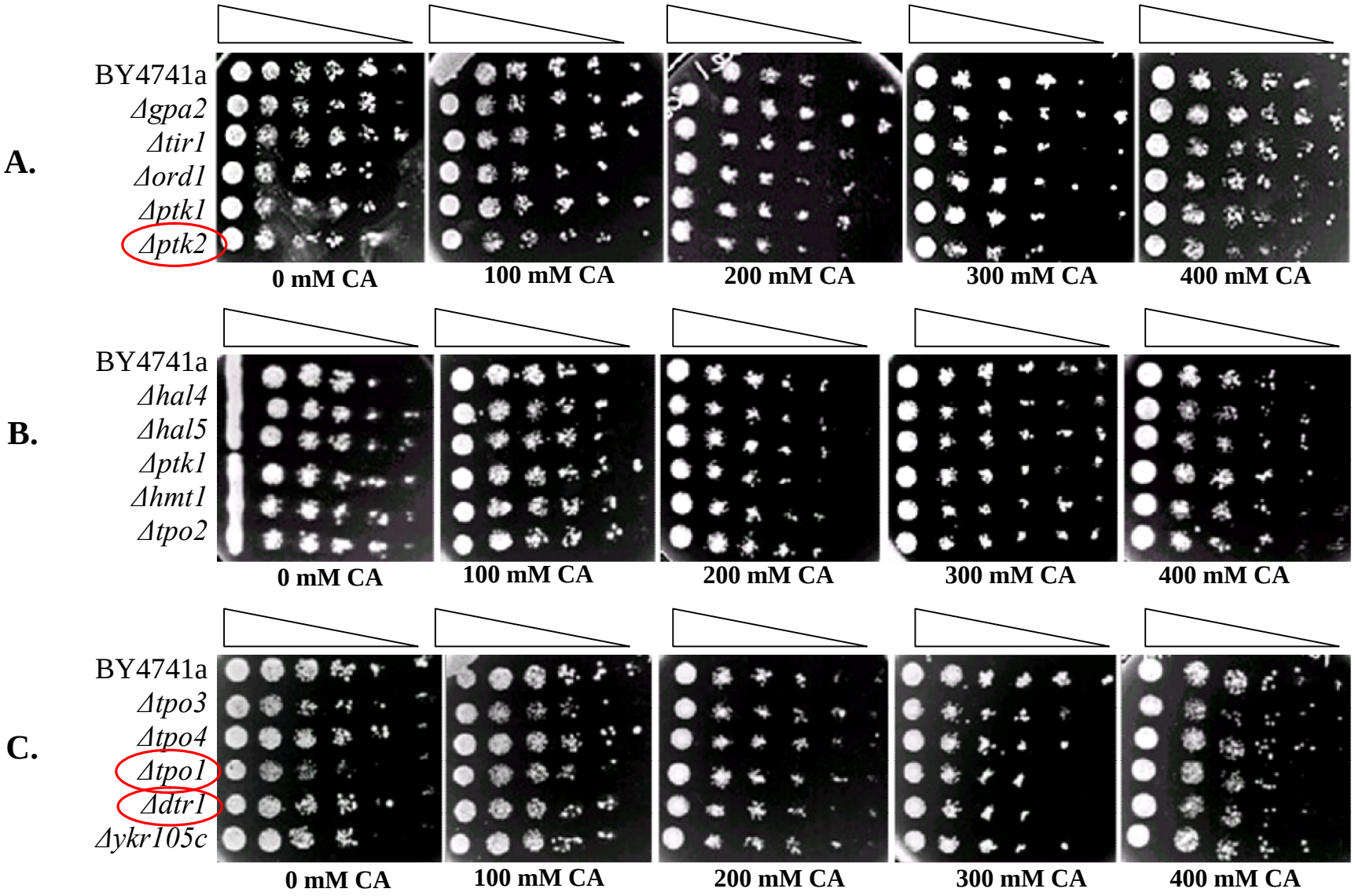


Figure 3.1: Growth of deletion strains under citric acid stress. Growth of wild type (BY4741a) and deletion strains spotted onto MB agar, [pH 3.5] at 10-fold serial dilutions, with various concentrations of citric acid (0 - 400 mM). Deletion strains exhibiting a citric acid sensitive phenotype are highlighted (circled in red). Plates A-C: Sensitive deletions - Δ*ptk2*, Δ*tpo1* and Δ*dtr1*.

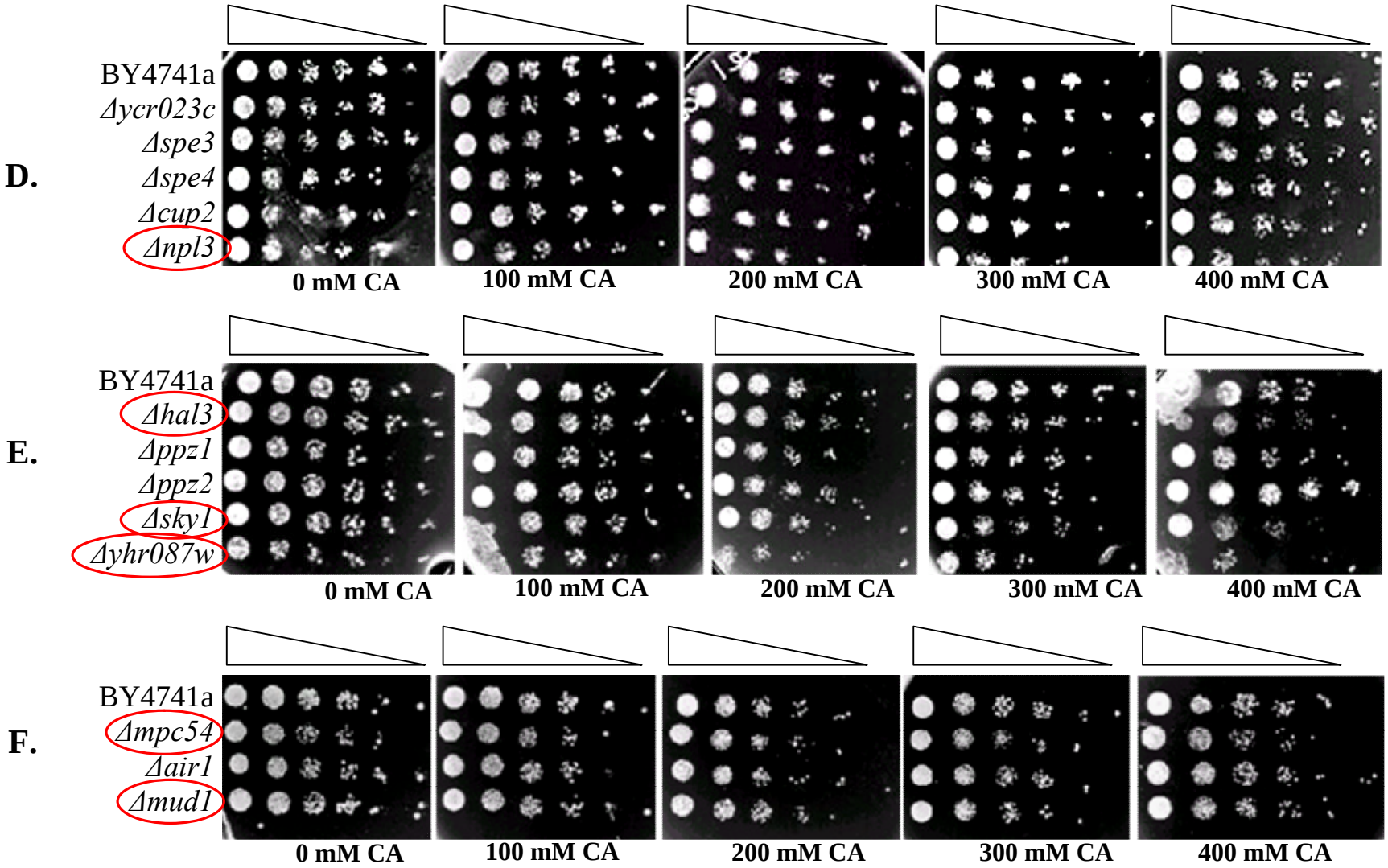


Figure 3.2: Growth of deletion strains under citric acid stress. Growth of wild type (BY4741a) and deletion strains spotted onto MB agar, [pH 3.5] at 10-fold serial dilutions, with various concentrations of citric acid (0 - 400 mM). Deletion strains exhibiting a citric acid sensitive phenotype are highlighted (circled in red). Plates D-F: Sensitive deletions – Δ*npl3*, Δ*hal3*, Δ*sky1*, Δ*yhr087w*, Δ*mpc54* and Δ*mud1*.

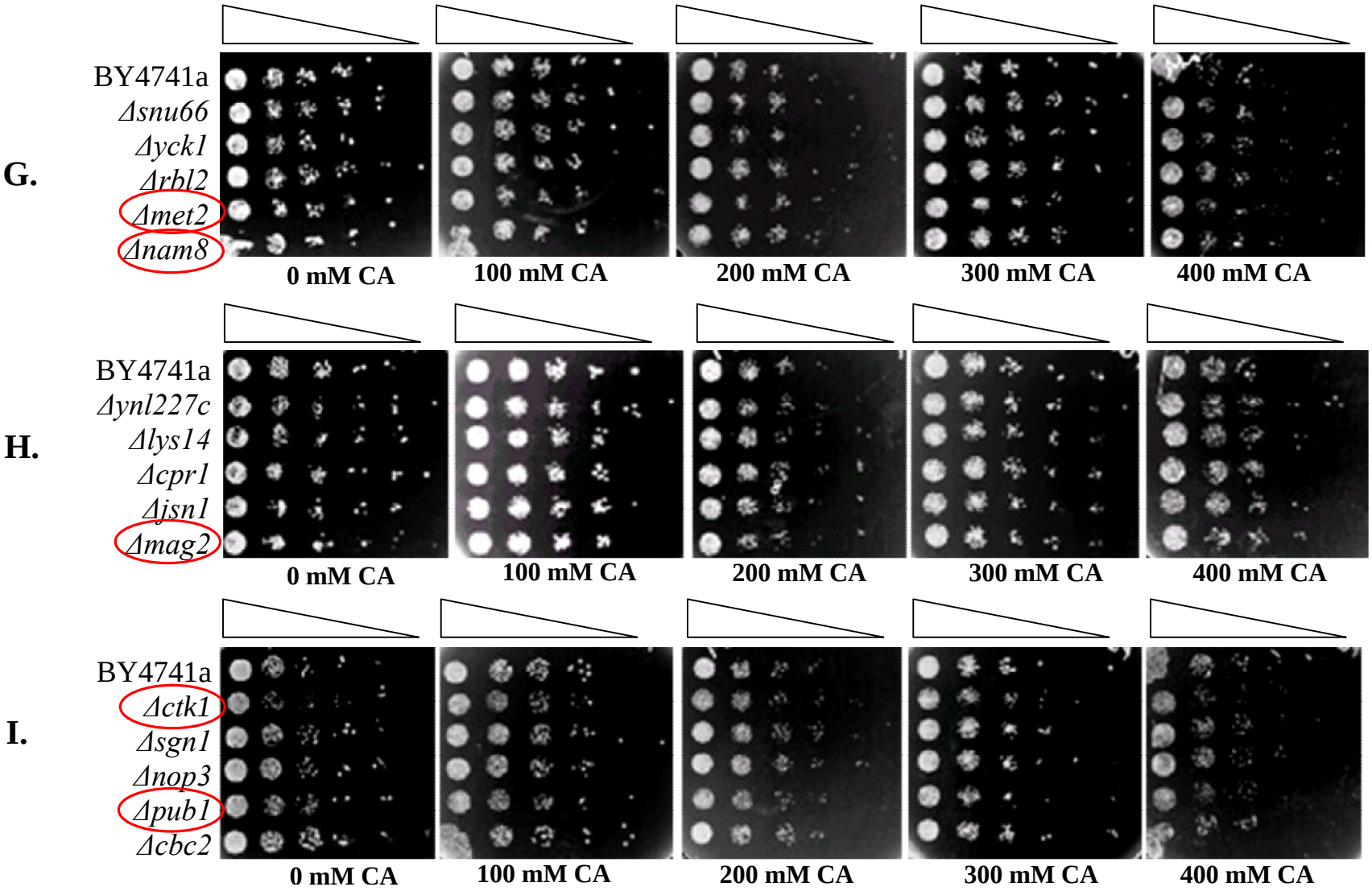


Figure 3.3: Growth of deletion strains under citric acid stress. Growth of wild type (BY4741a) and deletion strains spotted onto MB agar, [pH 3.5] at 10-fold serial dilutions, with various concentrations of citric acid (0 - 400 mM). Deletion strains exhibiting a citric acid sensitive phenotype are highlighted (circled in red). Plates G-I: Sensitive deletions – Δ*met2*, Δ*nam8*, Δ*mag2*, Δ*ctk1*, and Δ*pub1*.

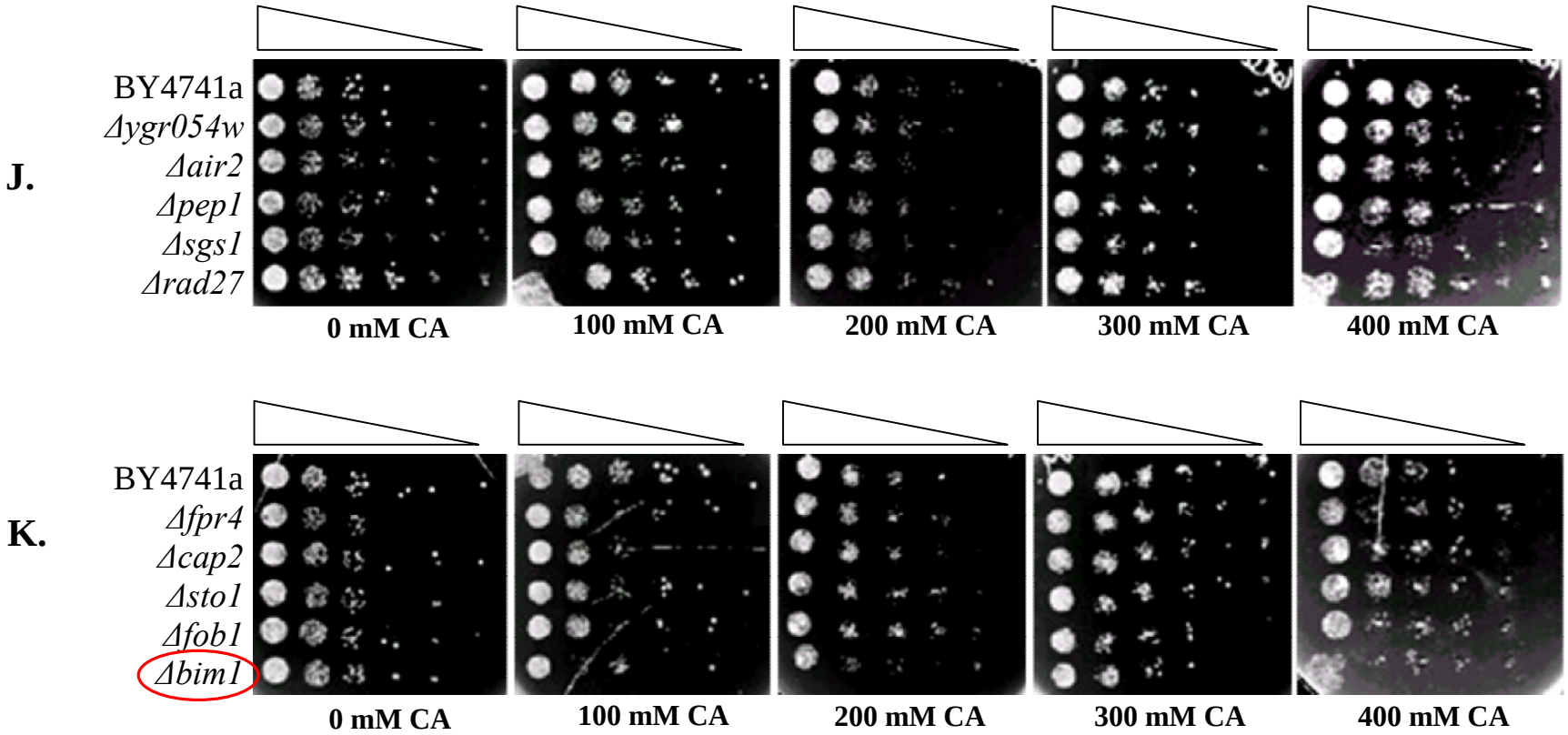


Figure 3.4: Growth of deletion strains under citric acid stress. Growth of wild type (BY4741a) and deletion strains spotted onto MB agar, [pH 3.5] at 10-fold serial dilutions, with various concentrations of citric acid (0 - 400 mM). Deletion strains exhibiting a citric acid sensitive phenotype are highlighted (circled in red). Plates J & K: Sensitive deletions – Δ*bim1*.

3.2.2. Growth Curve Analysis

In order to confirm and quantify the deletion strains' sensitivity to citric acid, duplicate 25 ml cultures of wild type and each deletion strain, with and without 300 mM citric acid, were incubated and optical density readings (OD 600nm) taken every hour. The OD_{600} values against time for each culture were plotted graphically.

A total of 29 deletion strains were investigated, 15 isolated from the 'spotting' screen (Section 3.2.1), and 14 that were selected from relevant literature. Only growth curve data for WT and Δ*sky1* (Figure 3.5 & 3.6) and the calculations used to quantify citric acid sensitivity (Formula 3.1, Table 3.1) have been included in this section. Growth curve data and calculations for all other deletion strains can be found in Appendix I. However, a graphical comparison of % sensitivity for all 29 deletion strains has been plotted (Figure 3.7).

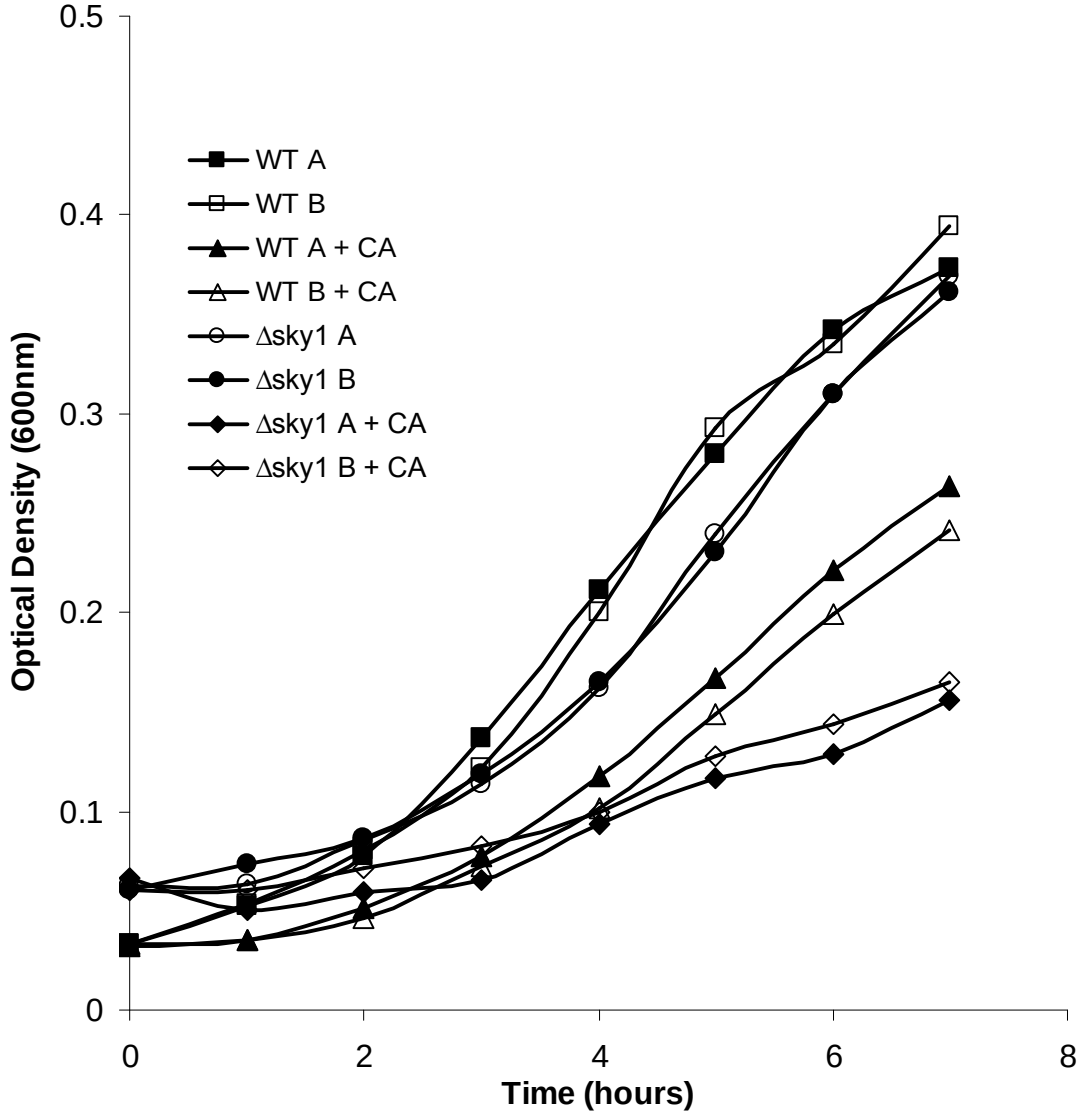


Figure 3.5: Comparison of growth of WT and Δ*sky1*, with and without 300 mM Citric acid. Duplicate liquid cultures of *S. cerevisiae* BY4741a (WT) (■,□) and deletion strain Δ*sky1* (●,○) were grown over a 7 hour period in MB, [pH 3.5] at 30°C. Duplicate cultures of WT (▲,Δ) and Δ*sky1* (♦,◊) were grown along side for 7 hours at 30°C in MB, [pH 3.5] in the presence of 300 mM citric acid. OD_{600} readings were recorded every hour for each culture.

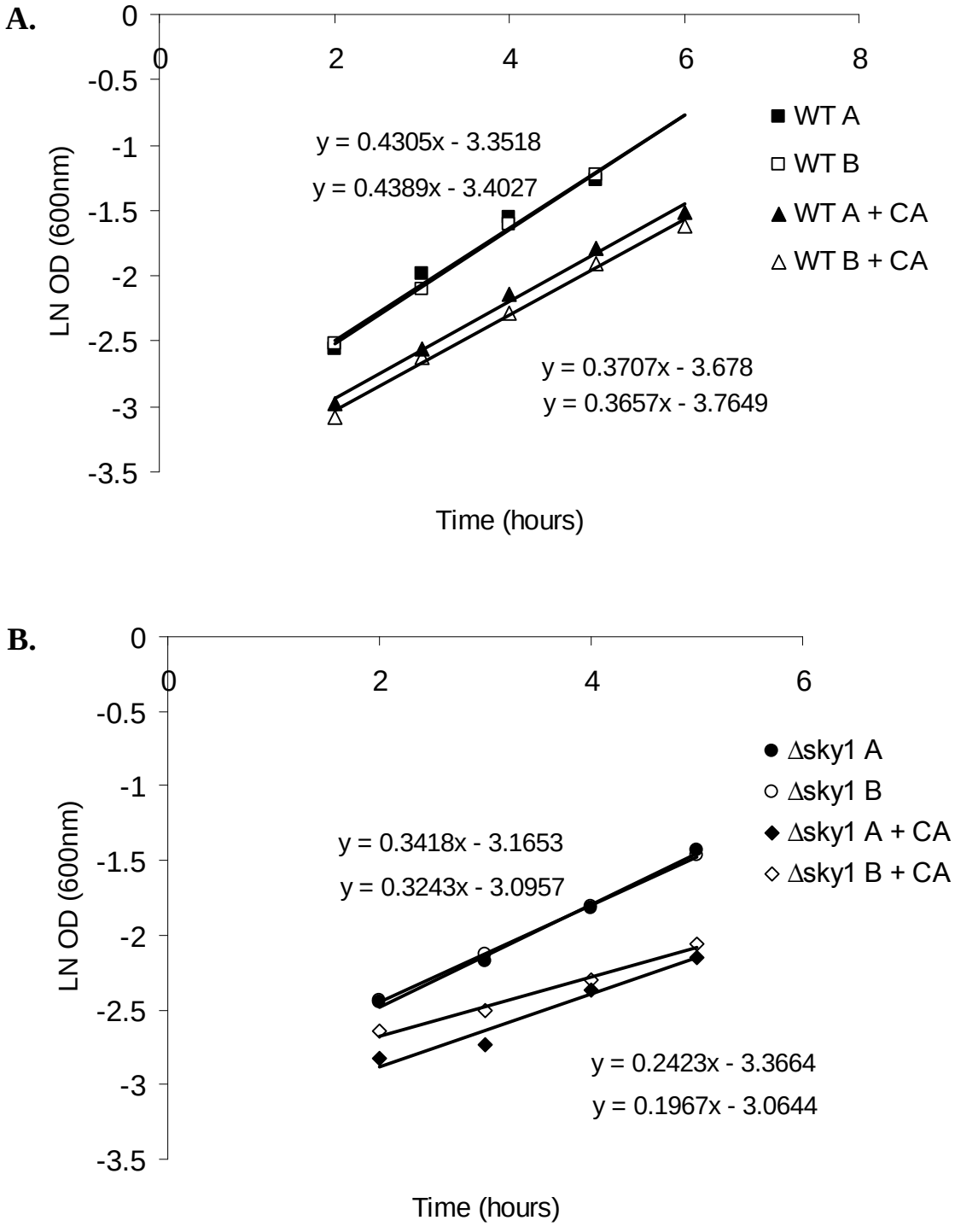


Figure 3.6: Comparison of the growth rate of WT and Δ*sky1*, with and without 300 mM citric acid. The natural log of the OD_{600} was taken for WT (A.) and Δ*sky1* (B.) and plotted against time in hours. Lines of best fit were added and the equation of the line shown. The growth rate for each culture was calculated from their gradient.

Comparison between WT and Δ*sky1* in the presence and absence of 300 mM citric acid, [pH 3.5] showed all eight cultures exhibited a lag phase between 0 – 2 hours, followed by exponential growth (Figure 3.5). The growth rates (ln OD_{600}/hr), for both WT and Δ*sky1* were significantly reduced in the presence of 300 mM citric acid. Duplicates of each culture illustrated reproducibility (Figure 3.5). To calculate the level of sensitivity quantitatively, the natural log of the OD_{600} was plotted against time and lines of best fit added and the equation of the line shown (Figure 3.6).The gradient of each line was used to calculate percentage sensitivity for both WT and deletion strain using the average rate for each duplicate (Formulae 3.1). The difference in sensitivity between the WT and deletion strain was calculated and deletion strains exhibiting greater than 10% difference (approximately 1/3 greater sensitivity than WT) were classed as being 'sensitive' to 300 mM citric acid as in accordance with previous publications (Lawrence *et al*., 2004). Δ*sky1* was calculated to have a sensitivity of 18.8% in 300 mM citric acid compared to WT (Table 3.1).

$$\textbf{\% Sensitivity} = \frac{\textbf{(Av. growth rate w/o CA – Av. growth rate with CA)}}{\textbf{Av. growth rate w/o CA}} \times 100$$

Formulae 3.1: Formula for calculating % sensitivity to citric acid. Growth inhibition induced by 300 mM citric acid was calculated as % sensitivity for WT and deletion strains using the growth rates (ln OD_{600}/hr), calculated from the natural log OD_{600} plots (Figure 3.6).

Table 3.1: Growth rates of WT and Δ*sky1* and % sensitivity to 300 mM citric acid. *Growth rate is the average growth rate for the duplicate cultures. % sensitivity highlighted is the difference between the deletion strain (Δ*sky1*) and WT.

Strain	***Growth Rate - No Citric acid (ln OD_{600}/hr)**	***Growth Rate - 300 mM Citric Acid (ln OD_{600}/hr)**	**% Sensitivity to Citric acid**
WT	0.435	0.368	15.3
Δ*sky1*	0.333	0.220	34.1
		% Sensitivity Difference (Δ – WT)	**18.8**

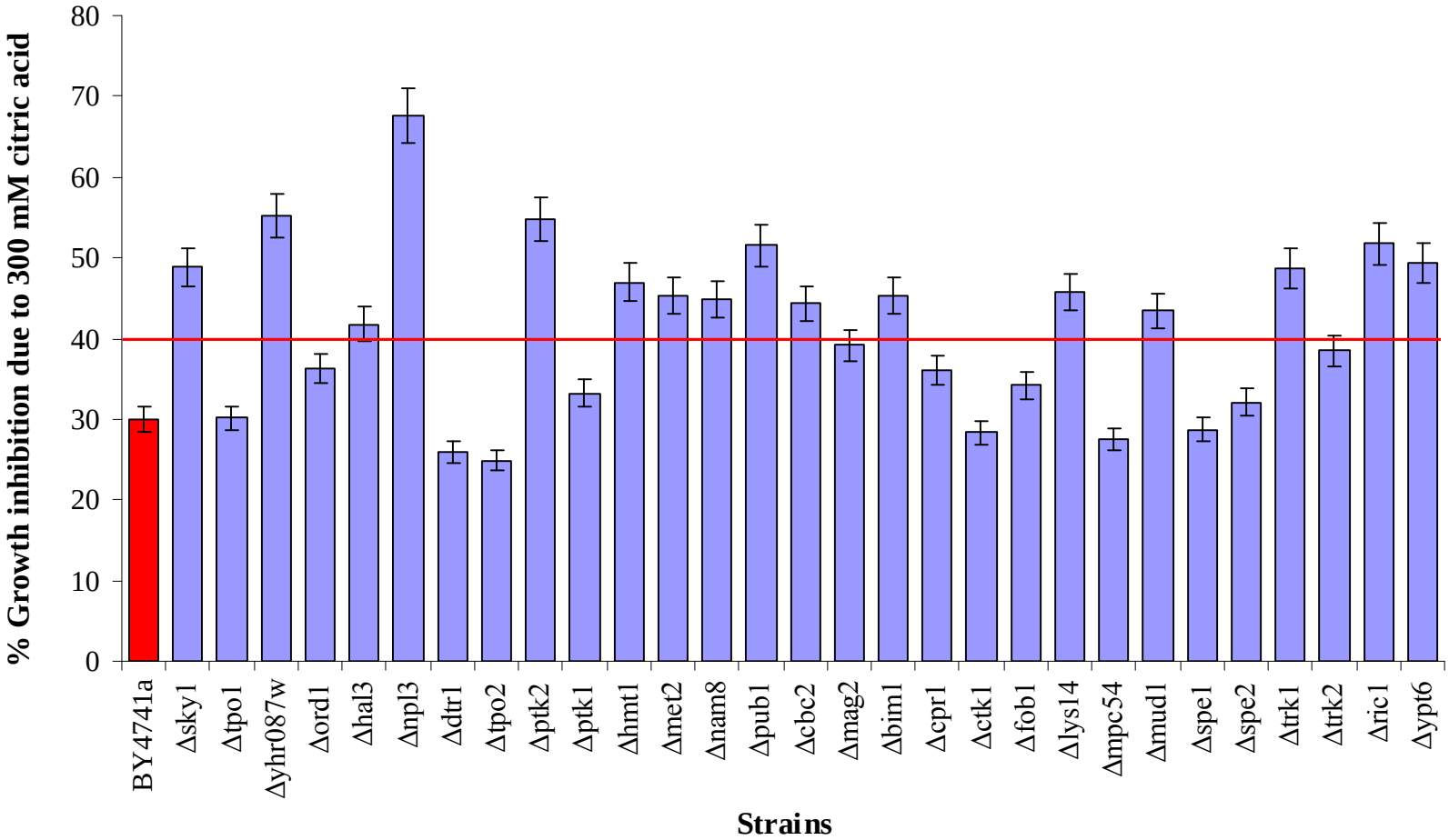


Figure 3.7: Comparison of % sensitivity to 300 mM citric acid for selected gene deletion strains. Deletion strains were grown in liquid media MB, [pH 3.5], with and without 300 mM citric acid. The percentage inhibition (% sensitivity) due to presence of citric acid was calculated. Those exhibiting 10% greater sensitivity than WT were classed as sensitive.

A comparison of WT with all polyamine related gene deletions tested and quantified using growth curves, has been summarized (Figure 3.7). Sixteen of the deletion strains tested exhibited hypersensitivity to 300 mM citric acid with sensitivity greater than 10% of that of WT: Δ*bim1*, Δ*cbc2*, Δ*hal3*, Δ*hmt1*, Δ*lys14*, Δ*met2*, Δ*mud1*, Δ*nam8*, Δ*npl3,* Δ*ptk2*, Δ*pub1*, Δ*ric1*, Δ*sky1*, Δ*trk1*, Δ*YHR087w*, and Δ*ypt6*. A further 7 displayed sensitivity 0.1-10%. The remaining 5 ranged between ⁻1.3-⁻5.1%, growing slightly better than WT under 300 mM citric acid stress but not significantly enough to be classed as resistant (Figure 3.7).

The genes, whose deletion resulted in increased sensitivity to citric acid, and their functions according to the SGD are listed (Table 3.2). Each are either involved in polyamine transport or metabolism, or encode proteins known to interact with Sky1p.

These deletions' sensitivity to citric acid suggested these genes are required for optimal growth in the presence of 300 mM citric acid. They also suggested a possible involvement of polyamine metabolism related genes in the adaptation of *S. cerevisiae* to citric acid.

Table 3.2: Spotting assays and growth curves identified genes required for optimal growth of S. *cerevisiae* in the presence of 300 mM citric acid, [pH 3.5]. Gene deletions sensitive to 300 mM citric acid are listed below with their functions according to SGD.

ORF	GENE	% sensitivity	Function
Cell Homeostasis			
YMR216c	*SKY1*	18.8	SR protein kinase (SRPK) involved in regulating proteins involved in mRNA metabolism and cation homeostasis; similar to human SRPK1
YLR039c	*RIC1*	21.8	Ribosome control
YHR087w	-	25.2	'Unknown'
RNA Metabolic Process			
YKR072c	*HAL3*	11.8	Involved in ion homeostasis and cell cycle progression
YBR034c	*HMT1*	16.9	Nuclear SAM-dependent mono- and asymmetric arginine dimethylating methyltransferase that modifies hnRNPs, including Npl3p
YLR262c	*YPT6*	19.3	GTPase, Ras-like GTP binding protein involved in the secretory pathway
YJR059w	*PTK2*	24.8	Putative serine/threonine protein kinase involved in cell ion homeostasis and polyamine metabolism
YDR432w	*NPL3*	37.6	Nuclear shuttling protein; phosphorylated by Sky1p in the cytoplasm
Transport			
YPL178w	*CBC2*	14.3	RNA cap binding
YHR086w	*NAM8*	14.8	RNA binding
YER016w	*BIM1*	15.2	Involved in microtubule binding
YNL227w	*MET2*	15.3	Homoserine O-acetyltransferase activity
YNL016w	*PUB1*	21.5	Poly(A)$^+$ RNA-binding protein
Amino Acid and Derivative Metabolic Process			
YBR119w	*MUD1*	13.4	U1 snRNP A protein, homolog of human U1-A; involved in nuclear mRNA splicing
YDR034c	*LYS14*	15.8	Transcriptional activator involved in regulation of genes of the lysine biosynthesis pathway
Response to Stress			
YJL129c	*TRK1*	18.7	Involved in potassium ion transport activity, potassium ion homeostasis

3.2.3. *SKY1* mutant and mutants of interacting proteins indicating their importance to citric acid resistance

To confirm that the citric acid sensitive phenotype observed in Δ*sky1* was specifically caused by the distruption of the *SKY1* gene, complementation studies were performed. Initially the *SKY1* gene was PCR amplified using primers SKY1fwd and SKY1rev (table 2.2) and cloned into the pGEM-Teasy vector (Promega Ltd., Hampshire, UK) (Section 2.5.1). This created the plasmid pGEM-TE-SKY1 (Appendix II) which was transformed into *E. coli* DH5α competent cells and selected on LB + ampicillin solid media. *SKY1* was then cleaved from the plasmid via Not*I* digestion and cloned into the Not*I* site of shuttle vector pRS313 (Sikorski and Hieter, 1989). The pRS313-SKY1 plasmid was transformed back into the deletion strain and tested on selective synthetic complete medium (SC-His, pH 3.5) with 0-300 mM citric acid. Δ*sky1* was successfully complemented and incorporation of *SKY1* rescued citric acid sensitivity (Figure 3.8).

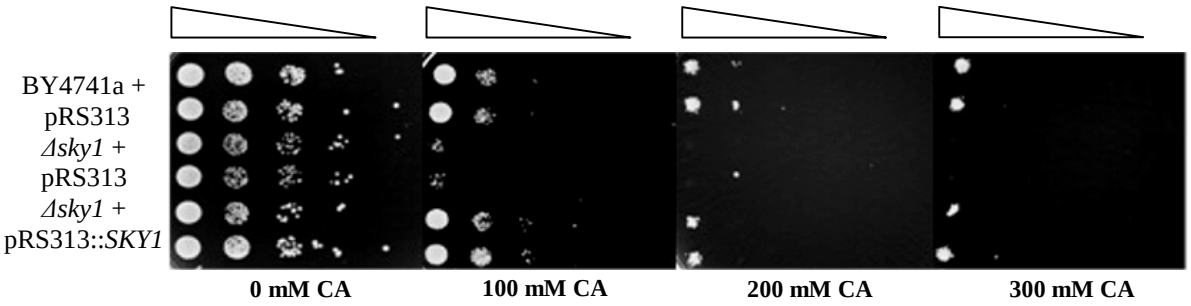


Figure 3.8: *SKY1* complementation study. Growth comparison on selective media (MB-His, pH 3.5) in 0, 100, 200 and 300 mM citric acid. BY4741a transformed with an empty single copy plasmid (pRS313), Δ*sky1* transformed with empty vector (pRS313) and the Δ*sky1* strain was verified using pRS313 incorporating a copy of *SKY1*. Incorporation of *SKY1* rescued citric acid sensitivity, verifying the deletion strain.

Many of the gene deletions regulating the proteins which are known to interact with Sky1p were found to be sensitive to citric acid. The Sky1p interacting protein map (Figure 3.9), illustrates which primary, secondary, tertiary and even quaternary known interacting proteins of Sky1p exhibit sensitivity to citric acid when their encoding gene is deleted. All deletions, if viable, were present in the genome deletion set with the exception of the Δ*nmd5*. Each was tested by spotting and growth curve (Section 3.2.1 & 3.2.2). All of the primary protein interactions of Sky1p exhibited a sensitive phenotype to citric acid with the exceptions of Δ*air1* and Δ*mpc54*. The *MCM6* deletion was inviable. All other interactions proved to be sensitive upon deletion. Δ*nmd5* was not available from the gene deletion library but the deletion was viable (Figure 3.9).

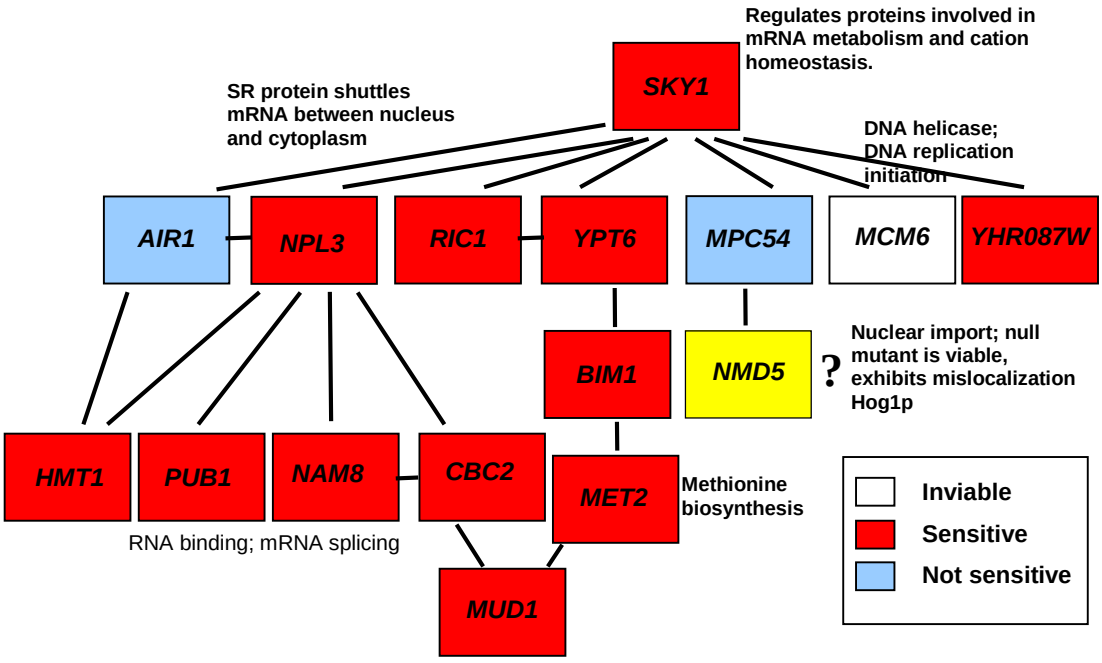


Figure 3.9: Sky1p interacting protein map. The BioGRID protein-protein interactions database (URL: http://www.thebiogrid.org) and BioPIXIE database (URL: http://pixie.princeton.edu/pixie) were used to identify proteins known to interact with Sky1p, and were subsequently tested for sensitivity to citric acid using 'spotting' and growth curve assays (section 3.2.1 & 3.2.2).

3.3. *NMD5* deletion

NMD5 was of interest due to its interaction with *SKY1* and the fact that its deletion results in the mislocalisation of Hog1p (Ferrigno *et al*., 1998). Being linked to both *SKY1* and *HOG1*, both of which have been shown both to be citric acid sensitive upon deletion, suggested that Δ*nmd5* may also show a sensitive phenotype to citric acid. The deletion strain for *NMD5* was not included in our deletion library collection. However, according to the SGD *NMD5* is a non-essential gene, therefore deletion would be viable. Using the KanMX disruption cassette and PCR techniques the deletion was attempted. Primers to amplify the KanMX disruption cassette, as shown below, were designed with 40 bp of sequence either side to target the cassette to integrate and disrupt *NMD5*.

Forward primer:
5' – CGA AGA CAT TTT ATT CTC GAT GGA TAT TAC AGA ATT GTT ACG TAC GCT GCA GGT CGA C – 3'

Reverse primer:
5' – AAT CGC CAT TTA ATT CAA TTA GGC ATT CAT AAT TCC CAT GAT CGA TGA ATT CGA GCT CG – 3'

*The highlighted section indicates the KanMX disruption cassette.

PCR was used to construct a KanMX casette flanked by 40 bp up/down stream of *NMD5*, nmd5::KanMX. Homologous recombination was utilised to insert the KanMX cassette in place of *NMD5* (Figure 3.10). This method is similar to that used in creating the Yeast Knock-out (YKO) deletion collection (http://www-sequence.stanford.edu/).

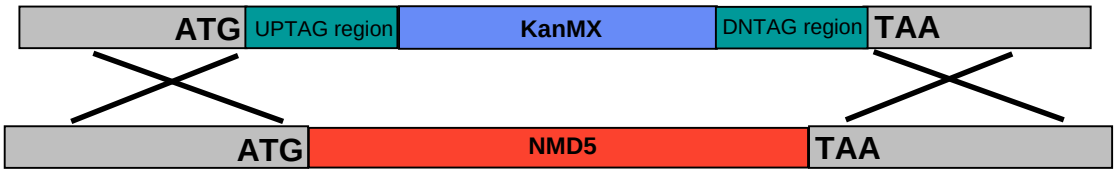


Figure 3.10: PCR-based gene deletion method. PCR techniques amplify KanMX cassette flanked by 40 bp up and down stream of gene for deletion. Homologous recombination is used to insert cassette in place of gene.
(http://www-sequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html)

The nmd5::KanMX disruption cassette sample was cleaned using a PCR purification kit (QIAquick PCR purification kit, QIAGEN Ltd., West Sussex, UK) to remove residual primers and nucleotides, then transformed into yeast and selected on YEPD agar + 150 µg/ml geneticin. Colonies were visible following incubation at 30°C for 5 days. PCR was carried out to confirm the *NMD5* deletion by the presence of a 1.4 kb band representing the amplification of the KanMX cassette (Data not shown).

To investigate whether the *Δnmd5* was sensitive to citric acid, growth curve data was obtained (Figure 3.11). The growth rate of Δ*nmd5* without citric acid is similar to that of the WT, thus the deletion does not seem to have affected growth. However in the presence of 300 mM citric acid Δ*nmd5* exhibits 23.3% additional sensitivity compared to the WT (Table 3.3).

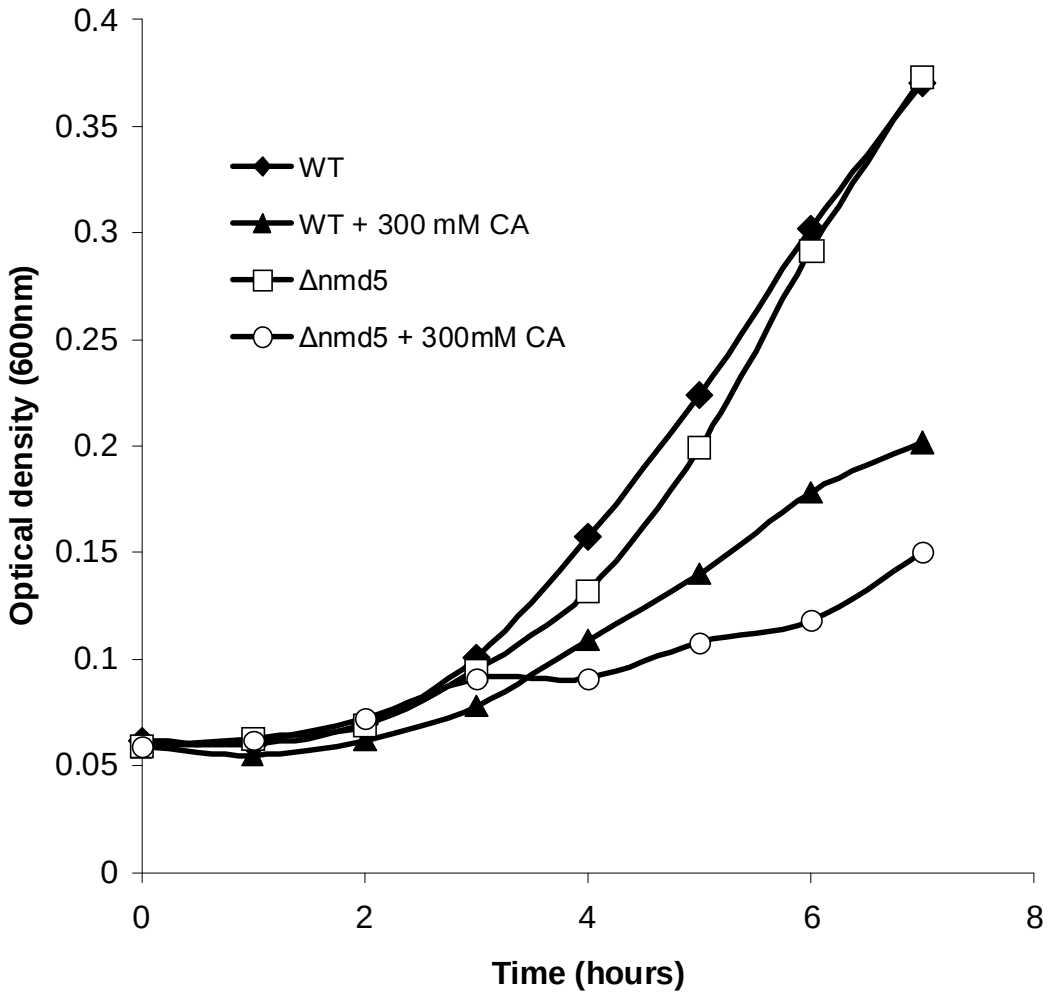


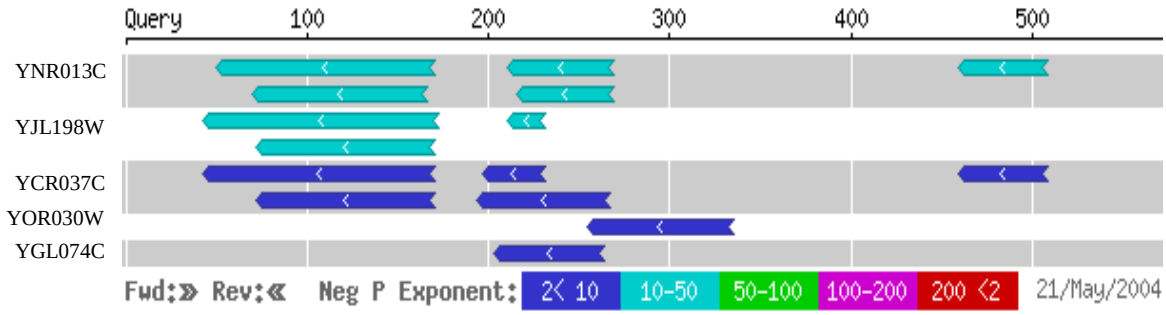
Figure 3.11: Comparison of the growth rate of WT and Δ*nmd5*. MB with and without 300 mM Citric acid. Liquid cultures of *S. cerevisiae* BY4741a (WT) (♦) and deletion strain Δ*nmd5* (□) were grown over a 7 hour period in MB, [pH 3.5] at 30°C. Liquid cultures of WT (▲) and Δ*nmd5* (○) were grown along side for 7 hours at 30°C in MB, [pH 3.5] in the presence of 300 mM citric acid. OD_{600} readings were recorded every hour for each culture.

Table 3.3: Growth rates of WT and Δ*nmd5* and % sensitivity to citric acid
*Growth rate is the average growth rate for the duplicate cultures. % sensitivity highlighted is the difference between Δ*nmd5* and WT.

Strain	Growth Rate - No Citric acid ($\ln OD_{600}$/hr)	Growth Rate - 300 mM Citric Acid ($\ln OD_{600}$/hr)	% Sensitivity to Citric acid
WT	0.370	0.228	38.4
Δ*nmd5*	0.356	0.137	61.6
		% Sensitivity Difference (Δ – WT)	**23.2**

3.4. *PHO87* – possible citric acid transporter

There is no evidence, at present, of a transport system specifically for citric acid in *S. cerevisiae*. Evidence for low and high affinity citric acid transport systems has been found in the closely related yeast *Candida utilis* (Cassio *et al*., 1991). Additionally in higher eukaryotes such as *Drosophila melanogaster* a protein involved in citrate transport has been identified, the 'I'm not dead yet protein' (INDY transporter protein) (Knauf *et al.,* 2002). Following a BLAST comparison between the INDY transporter protein sequence and *S. cerevisiae* proteins, the citrate transporter protein was found to have high sequence similarity to three phosphate transporters: *PHO87*, *PHO90* and *PHO91*. Also a hypothetical transmembrane protein Dfg16p, involved in invasive growth upon nitrogen starvation, and an ORF of unknown function *YGL074C* were identified as having some similarity to INDY but to a lesser extent (Figure 3.12).



Sequences producing High-scoring Segment Pairs:	High score	Smallest sum probability P (N)
YNR013C PHO91 SGDID:S0005296, Chr XIV from 651711-649027	167	9.2e-15
YJL198W PHO90 SGDID:S0003734, Chr X from 60843-63488	181	8.5e-12
YCR037C PHO87 SGDID:S0000633, Chr III from 197180-194409	159	1.3e-0.8
YOR030W DFG16 SGDID:S0005556, Chr XV from 386824-388683	79	0.29
YGL074C YGL074C SGDID:S0003042, Chr VII fro368923-36859	57	0.991

Figure 3.12: WU-Blast2 results for sequence similarity between the INDY protein (*Drosophila melanogaster)* and *Saccharomyces cerevisiae* proteins. Three genes *PHO87*, *PHO90*, and *PHO91*, encoding phosphate transporters, displayed high sequence similarity.

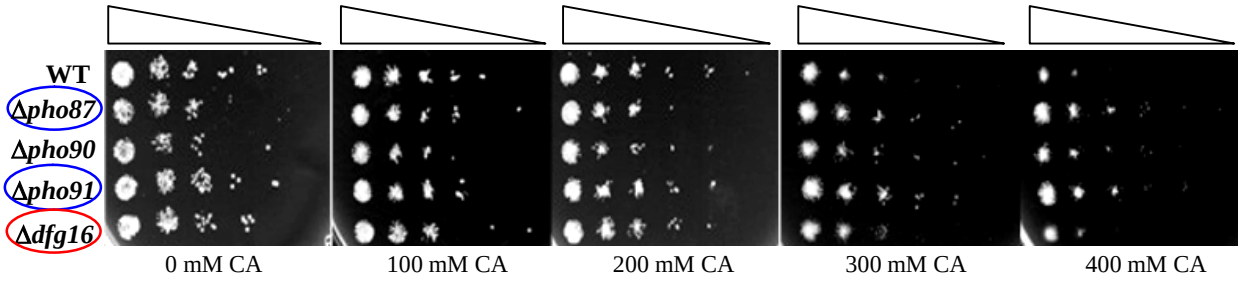


Figure 3.13: Comparison of growth in the presence of citric acid: WT (BY4741a), Δ*pho87*, Δ*pho90*, Δ*pho91* and Δ*dfg16* were subjected to 10-fold serial dilutions and spotted onto MB, [pH 3.5] agar with varying concentrations of citric acid (0 mM – 400 mM). WT strain was used as the control. Resistant phenotypes are circled in blue and sensitive phenotypes in red.

Deletion of *YGL074C* was inviable, however, Δ*pho87*, Δ*pho90*, Δ*pho91* and Δ*dfg16* were viable and were tested on varying concentrations of citric acid (Figure 3.13). Δ*pho87* and Δ*pho91* were shown to be resistant to increasing concentrations of citric acid. Δ*dfg16* proved to be sensitive to citric acid. Δ*pho90* exhibited little change in growth in the presence of citric acid with comparison to WT strain as the control (Figure 3.13).

The resistant phenotypes on agar exhibited by the two phosphate transporter deletion strains, Δ*pho87* and Δ*pho91*, were tested via growth curves (Figure 3.14 – 3.15). Δ*pho91* exhibited a slightly sensitive phenotype to citric acid with 5.6% sensitivity calculated (Table 3.4). Δ*pho87* displayed 4.8% resistance to 300 mM citric acid (Table 3.5). The resistant phenotype for Δ*pho87* suggests it may have a possible role in citric acid transport. It seemed plausible that deletion of Δ*pho87* could be preventing uptake of citric acid into the cell.

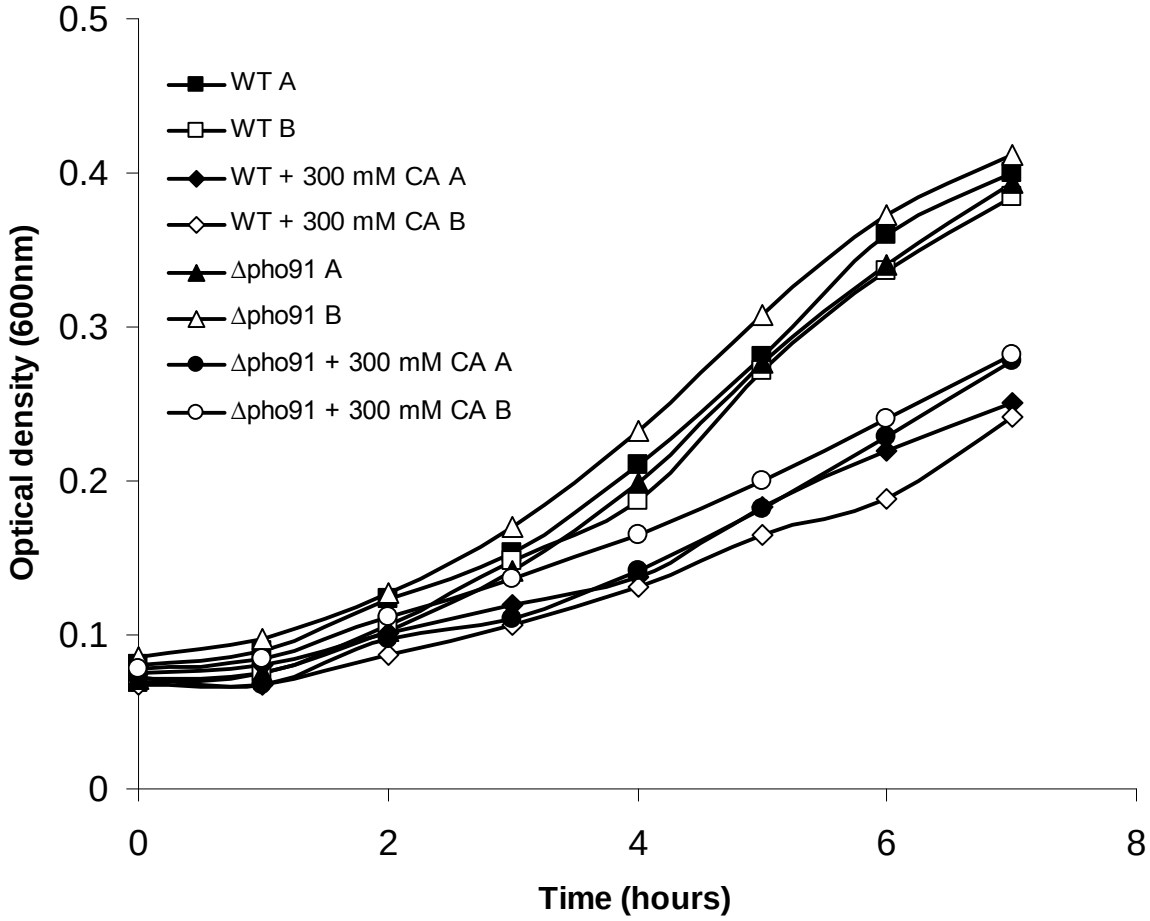


Figure 3.14: Comparison of the growth rate of WT and Δ*pho91*, with and without 300 mM citric acid. Duplicate liquid cultures of *S. cerevisiae* BY4741a (WT) (■,□) and deletion strain Δ*pho91* (▲,Δ) were grown over a 7 hour period in MB, [pH 3.5] at 30°C. Duplicate cultures of WT (♦,◊) and Δ*pho91* (●,○) were grown along side for 7 hours at 30°C in MB, [pH 3.5] in the presence of 300 mM citric acid. OD_{600} readings were recorded every hour for each culture.

Table 3.4: Growth rates of WT and Δ*pho91* and % resistance to citric acid.
*Growth rate is the average growth rate for the duplicate cultures. % sensitivity highlighted is the difference between Δ*pho91* and WT.

Strain	*Growth Rate - No Citric acid ($\ln OD_{600}$/hr)	*Growth Rate - 300 mM Citric Acid ($\ln OD_{600}$/hr)	% Sensitivity to Citric acid
WT	0.290	0.203	30.1
Δ*pho91*	0.314	0.202	35.7
		% Sensitivity Difference (Δ – WT)	**5.6**

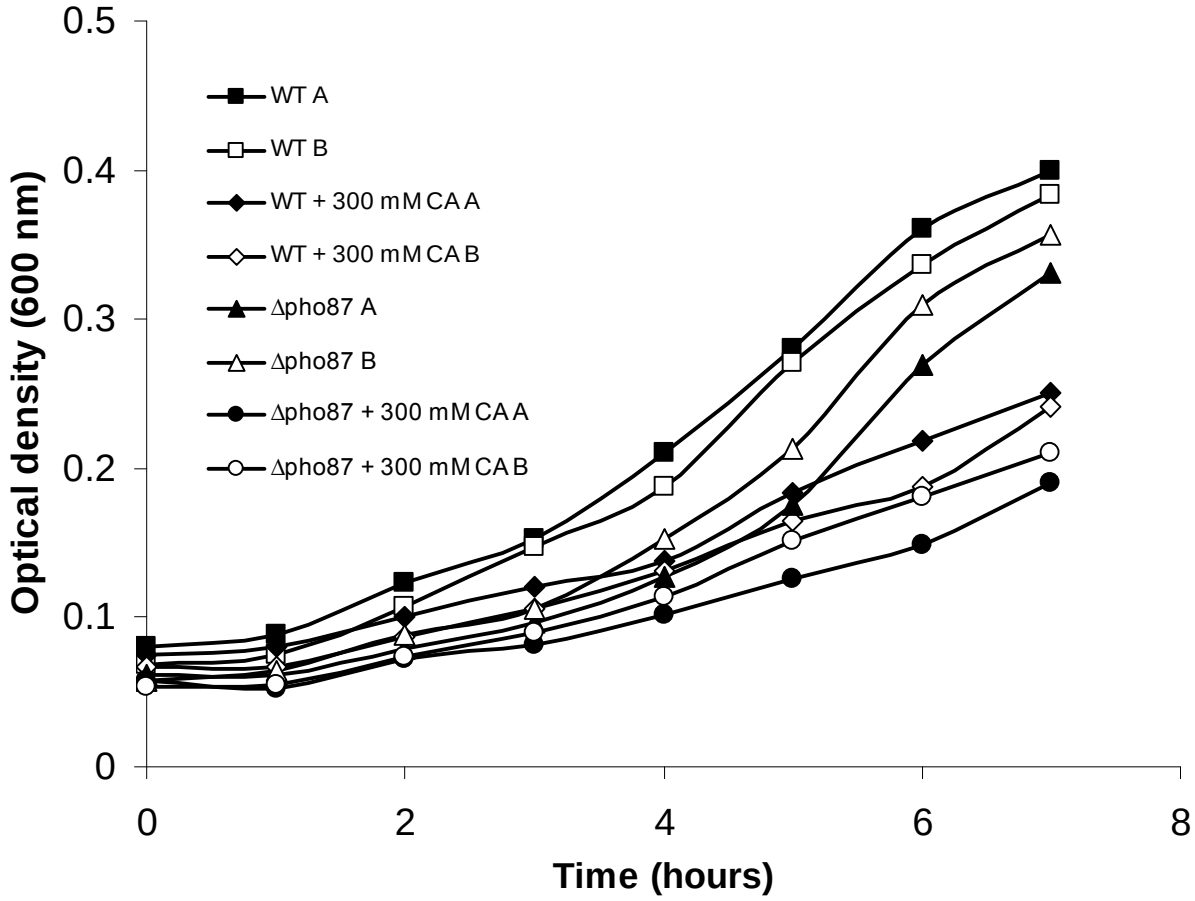


Figure 3.15: Comparison of the growth rate of WT and Δ*pho87*, with and without 300 mM citric acid. Duplicate liquid cultures of *S. cerevisiae* BY4741a (WT) (■,□) and deletion strain Δ*pho87* (▲,Δ) were grown over a 7 hour period in MB, [pH 3.5] at 30°C. Duplicate cultures of WT (♦,◊) and Δ*pho87* (●,○) were grown along side for 7 hours at 30°C in MB, [pH 3.5] in the presence of 300 mM citric acid. OD_{600} readings were recorded every hour for each culture.

Table 3.5: Growth rates of WT and Δ*pho87* and % resistance to citric acid.
*Growth rate is the average growth rate for the duplicate cultures. % sensitivity highlighted is the difference between Δ*pho87* and WT.

Strain	*Growth Rate - No Citric acid ($ln\ OD_{600}$/hr)	*Growth Rate - 300 mM Citric Acid ($ln\ OD_{600}$/hr)	% Sensitivity to Citric acid
WT	0.290	0.203	30.1
Δ*pho87*	0.285	0.213	25.3
		% Resistance Difference (Δ – WT)	**4.8**

3.5. Citric acid uptake assay

3.5.1. Enzymatic assay

There is very little known about exogenous transport of citric acid in *S. cerevisiae*. Thus we attempted to determine if citric acid enters yeast cells in a similar fashion to other weak acids. The use of a time course could determine how quickly citric acid enters the cell. Also, the effect of specific deletion strains on citric acid uptake could also be studied.

An enzymatic assay (Citric acid assay kit, R-Biopharm, GmBh, Darmstadt, Germany) measuring the change in NADH oxidation upon the addition of citrate lyase, via OD_{340}, was used to measure the citrate content of yeast cells. Initial data using the assay was obtained using known concentrations of citric acid and a standard curve plotted. Results suggested that the enzymatic assay was an effective and accurate method (Figure 3.16).

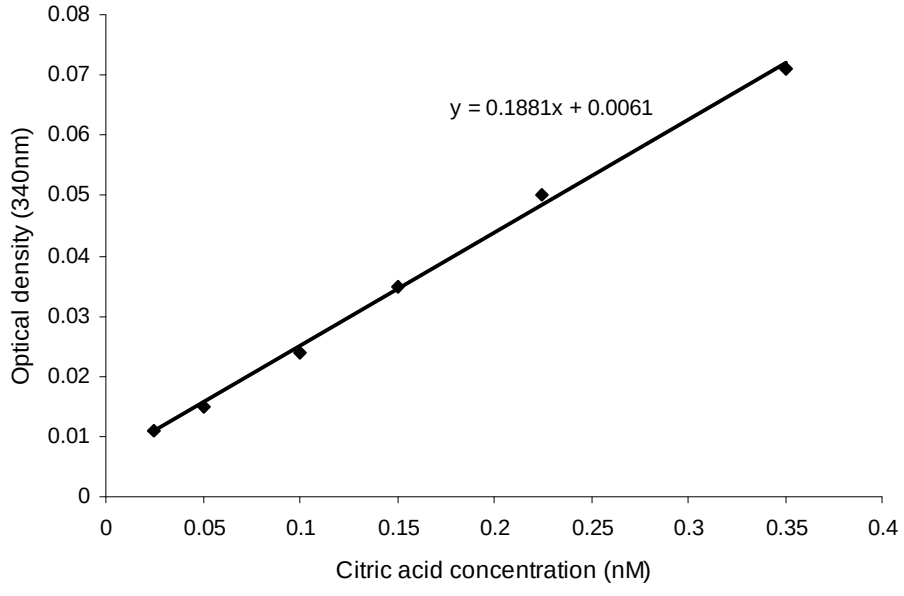


Figure 3.16: Standard curve for citric acid uptake assay. OD_{340} was measured over a range of citric acid concentrations (0.025, 0.05, 0.1, 0.15, 0.225 and 0.35 nM citric acid). Plotting a line of best fit provided an equation for calculating citric acid concentration.

ACO1 encodes the enzyme aconitase involved in the Kreb's cycle. Aconitase catalyses the reversible isomerization of the tricarboxylic acids (TCA's) citrate and isocitrate (Gangloff *et al*., 1990). Δ*aco1* was used as a positive control for the assay; its deletion disables the catalysis of citrate. An initial experiment using WT and Δ*aco1* cell cultures grown in MB media, [pH 3.5] (Section 2.8.1) proved that the enzymatic reaction did record a change in OD_{340} reading. The intracellular citric acid concentration for WT and Δ*aco1* was calculated using the equation of the line from the standard curve: y = 0.1881x + 0.0061 (Table 3.6.).

Table 3.6: Citric acid assay readings for WT and Δ*aco1*. Optical density readings at 340nm were recorded for each sample (control, WT and Δ*aco1*) before and after the addition of enzyme (citrate lyase). Citric acid concentration was calculated using the equation of the line from the standard curve.

Sample	**Initial OD_{340} (A_1)**	**+ citrate lyase OD_{340} (A_2)**	**Change in OD_{340} (A_1-A_2)**	**Change in OD_{340} control**	**Citric acid concentration nM**
Control	0.859	0.863	0.004	-	-
Wt A	1.310	1.208	0.102	0.098	0.49
Wt B	1.260	1.168	0.092	0.088	0.44
Δaco1	1.228	1.114	0.114	0.110	0.55
Δaco1	1.166	1.072	0.094	0.090	0.45

The cultures diplayed similar intracellular citric acid content ranging from 0.44 - 0.55 nM. We decided that the enzymatic approach was not sensitive enough for the measurement of citric acid uptake into the cell. Instead we decided that the use of radiolabeled citric acid may provide a more accurate approach.

3.5.2. ^{14}C – Citric acid uptake assay

Studies into uptake and extrusion of benzoic acid by *S. cerevisiae* have previously been carried out using radiolabeled 7-^{14}C benzoic acid (Henriques *et al*., 1997). We adjusted the method as follows using ^{14}C-citric acid to look at uptake in the WT, Δ*hog1*, Δ*sky1* and the double mutant Δ*hog1*/Δ*sky1** (Section 2.8.2). Cultures (50 ml) were grown to mid-exponential phase in MB, [pH 3.5], harvested and washed twice with sterile, cold dH_2O and resuspended in 50 ml fresh MB minus glucose. 20 ml cultures were incubated at 30°C with shaking. Prior to the addition of 74 MBq/mmol (2 mCi/mmol) ^{14}C-citric acid, a 1 ml background sample was taken, filtered and washed twice with sterile cold dH_2O. At time 0 min, a 1 ml sample was taken, filtered and the supernatant collected to record the total scintillation count. 1 ml samples were then taken over a time course of 1 hour, initially at 1 minute intervals for the first 5 minutes then over a range of 2, 3 and 5 minute intervals. Each experiment was carried out in triplicate. Counts per minute of radioactivity (CPMA) for each sample were plotted against time (Figures 3.17 – 3.19).

Preliminary results for citric acid uptake without the presence of glucose showed insignificant ^{14}C counts for WT, Δ*hog1* and Δ*hog1*/Δ*sky1* (Figure 3.17). The data displays an increase in ^{14}C CPMA for Δ*sky1* within 2 minutes of incubation with the radio-labeled citric acid suggesting uptake into the cells. The decrease in radioactivity counts over time 2-4 min of the assay suggests the citric acid is being extruded from the cells (Figure 3.17). The assay was subsequently executed with the presence of an energy source (+ glucose). The data for WT, Δ*hog1*, Δ*sky1* and Δ*hog1*/Δ*sky1* strains incubated with ^{14}C-citric acid in the presence of glucose, revealed no significant change in CPMA over time (Figure 3.18). Comparison of both data sets for WT, Δ*sky1*, Δ*hog1* and Δ*hog1*/Δ*sky1* with and without glucose have been shown (Figure 3.19). This clearly shows little change in CPMA for

each strain, with or without glucose, with the exception of Δ*sky1* – glucose, presenting an average CPMA peak of ~6000 counts per minute. The margin of error shown for Δ*sky1* – glucose, calculated from the triplicate sets of results, substantiates this result (Figures 3.17 and 3.19).

* More detail on the rationale for creating the double deletion Δ*hog1*/Δ*sky1* strain in Chapter 5 (Section 5.2).

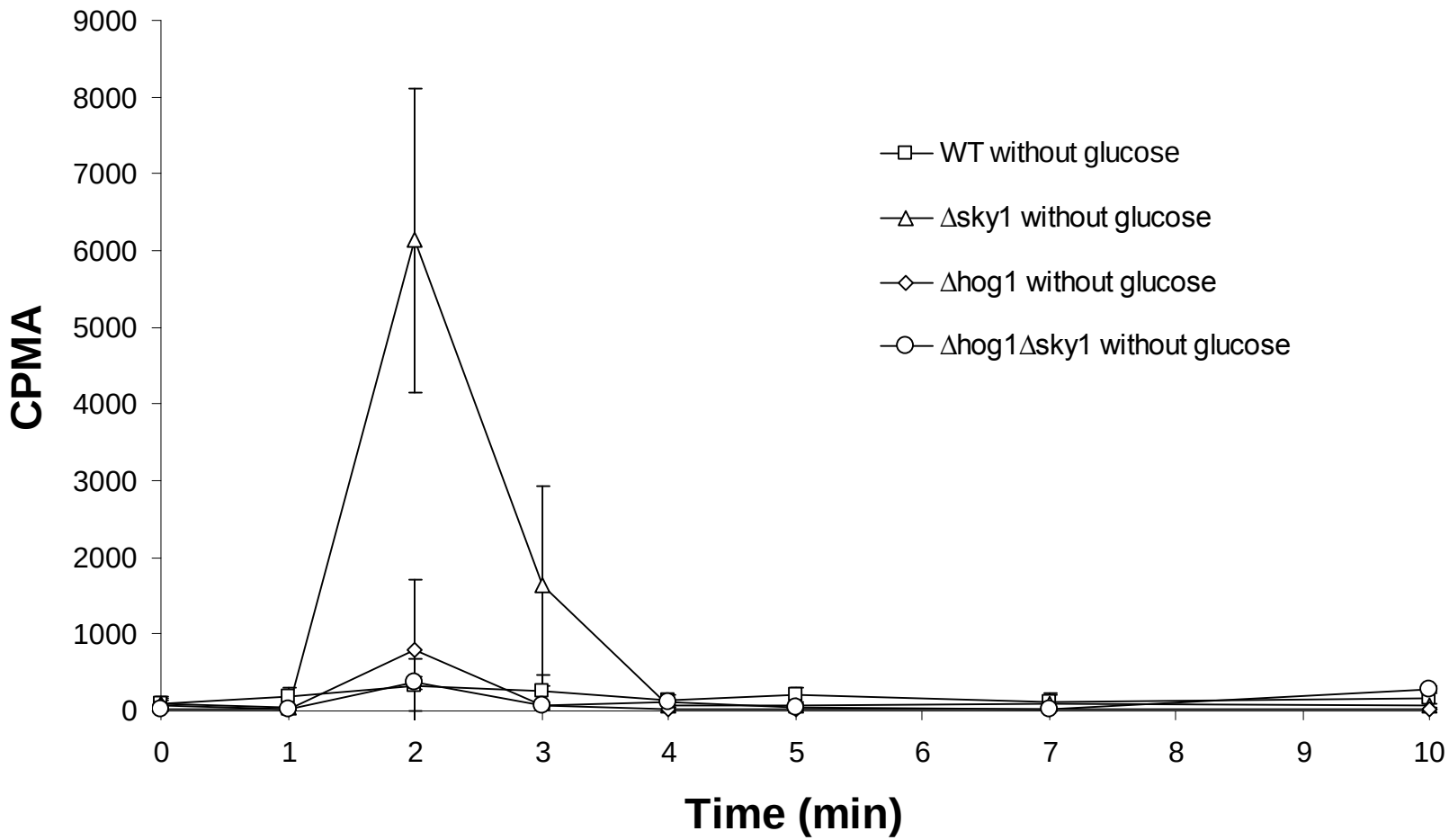


Figure 3.17: Comparison of the citric acid uptake (CPMA) of WT, Δ*sky1*, Δ*hog1* and Δ*hog1*Δ*sky1* without glucose.
Triplicate liquid cultures of *S. cerevisiae* BY4741a (WT) (□), Δ*sky1* (Δ), Δ*hog1* (◊), and Δ*hog1*Δ*sky1* (○) were grown over a 1 hour period in MB - glucose, [pH 3.5] at 30°C in the presence of 74 MBq/mmol ^{14}C-citric acid. CPMA readings were taken for 1 ml cell samples at regular intervals (1, 2, 3, 4, 5, 7, &10 minutes). The values are shown as mean ± standard deviation (error bars) of replicate experiments.

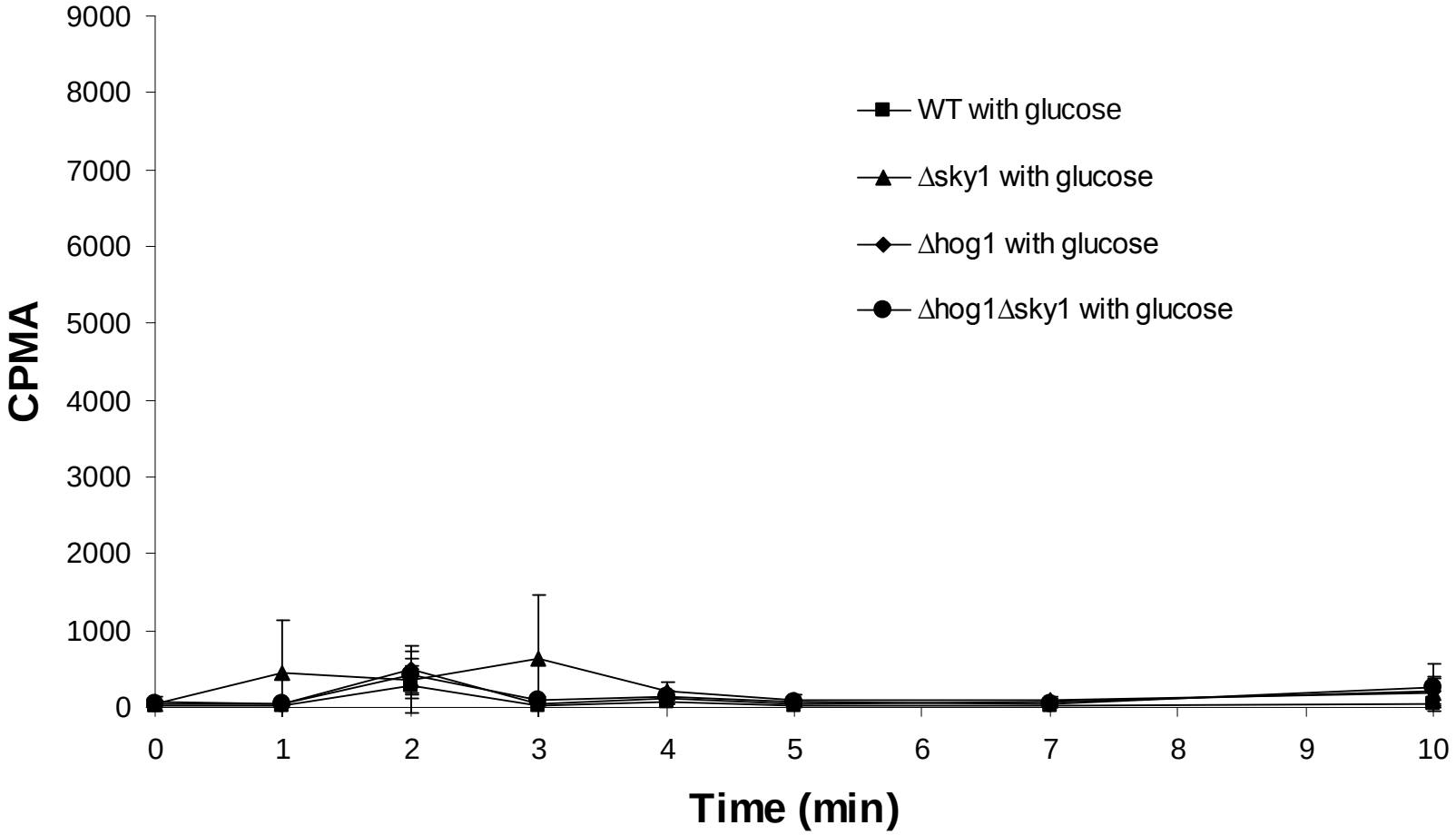


Figure 3.18: Comparison of the citric acid uptake (CPMA) of WT *Δsky1, Δhog1* and *Δhog1Δsky1* with glucose. Triplicate liquid cultures of *S. cerevisiae* BY4741a (WT) (■), Δ*sky1* (▲), Δ*hog1* (♦), and Δ*hog1*Δ*sky1* (●) were grown over a 1 hour period in MB + glucose, [pH 3.5] at 30°C in the presence of 74 MBq/mmol ^{14}C-citric acid. CPMA readings were taken for 1 ml cell samples at regular intervals (1, 2, 3, 4, 5, 7, &10 minutes). The values are shown as mean ± standard deviation (error bars) of replicate experiments.

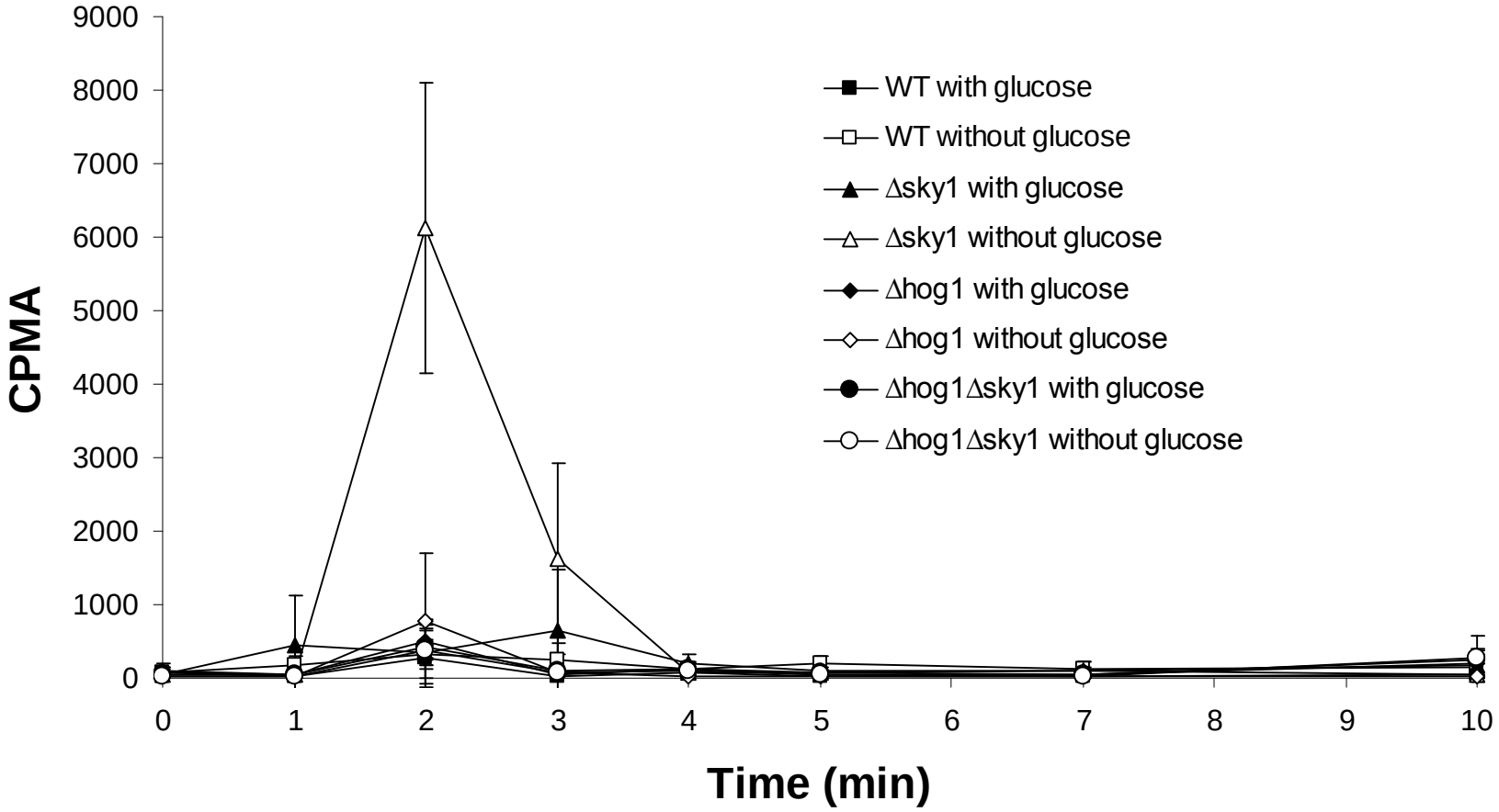


Figure 3.19: Comparison of the citric acid uptake (CPMA) of WT *Δsky1, Δhog1* and *Δhog1Δsky1* with and without glucose. BY4741a (WT) (■,□), Δ*sky1* (▲,Δ), Δ*hog1* (♦,◊), and Δ*hog1*Δ*sky1* (●,○) were grown over a 1 hour period in MB - glucose, [pH 3.5], and MB + glucose, [pH 3.5] respectively, at 30°C in the presence of 74 MBq/mmol ^{14}C-citric acid. CPMA readings were taken for 1 ml cell samples at regular intervals (1, 2, 3, 4, 5, 7, & 10 minutes). The values are shown as mean ± standard deviation (error bars) of triplicate experiments.

3.6. 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 citric acid stress recovery (Lawrence *et al*., 2004). Lawrence and co workers identified a number of gene deletions that were hypersensitive to citric acid, including Δ*hog1*, Δ*msn4*, and Δ*ssk1*. Their work identified an important new role for the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway in the regulation of adaptation to citric acid stress. Their screen also identified two gene deletions Δ*sky1* and Δ*ptk2* as being hypersensitive to citric acid. These genes encode two protein kinases involved in polyamine metabolism and regulate ion transport across the plasma membrane (Erez and Kahana, 2002). The reversible protein phosphorylation catalysed by protein kinases is critically involved in the regulation of nearly all cellular processes and signaling pathways (Rubenstein and Schmidt, 2007), making these two genes of particular interest as candidates for further study for citric acid stress recovery.

Of the 52 deletion strains involved in polyamine metabolism or those encoding proteins shown to interact with Sky1p, 16 exhibited hypersensitivity to citric acid; Δ*bim1*, Δ*cbc2*, Δ*hal3*, Δ*hmt1*, Δ*lys14*, Δ*met2*, Δ*mud1*, Δ*nam8*, Δ*npl3*, Δ*ptk2*, Δ*pub1*, Δ*ric1*, Δ*sky1*, Δ*trk1*, Δ*YHR087w*, and Δ*ypt6.*

Δ*sky1*, Δ*ptk2*, Δ*trk1* and Δ*hal3* are involved in polyamine and ion homeostasis. Trk1p is a component of the Trk1p-Trk2p potassium transport system. Deletion of either *SKY1* or *PTK2* in Δ*trk1*/Δ*trk2* cells increases spermine tolerance, implying Trk1,2p independent activity. However, deletion of *SKY1* in Δ*trk1*/Δ*trk2* cells increased LiCl sensitivity and

improved growth on low K^+ and low pH media in a Trk1,2p dependent manner (Erez and Kahana, 2002). It has been suggested that disruption of *SKY1* results in increased Trk-mediated K^+ uptake, causing a decrease in membrane potential, resulting in a decrease in the uptake of toxic cations. Decreasing membrane electrical potential and cation uptake is associated with mutations in the genes of; the Pma1 plasma membrane H^+-ATPase; the Trk high-affinity K^+ transporter; and the regulators of these systems. Deletion of *SKY1* has no effect on the ''in vivo'' activity of the H^+-ATPase, but it increases the activity of the Trk system (Forment *et al*., 2002). However, deletion of *PTK2* does affect the glucose-induced change in *Km* of the plasma membrane H^+-ATPase. Previous studies have shown that upon exposure to weak acids such as sorbic acid, the plasma membrane H^+-ATPase that regulates intracellular pH 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).

Loss of the high affinity K^+ uptake system Trk1p, has been shown to confer sensitivity to growth in preservatives (Mollapour *et al*; 2004; MacPherson *et al*., 2005). This suggests that cation accumulation is an important factor in adaptation to weak-acid preservatives by spoilage yeasts. A mutant screen carried out by Mollapour *et al*. (2004) showed that the Δ*trk1* mutant grows poorly in sorbic acid. Long-term preservative exposure leads to K^+ accumulation in *S. cerevisiae* (and that increased K^+ availability improves growth in preservative) suggests that it is a conserved adaptive response conferring stress-specific physiological advantage. Indeed, K^+ uptake by yeast has been linked to regulation of oxidative phosphorylation (Aiking *et al*., 1977), intracellular pH and the cell cycle (Yenush *et al*., 2002), all of which would be critical to weak acid preservative resistance. It has previously been shown that K^+ uptake by the Trk system helps alkalinize cytosolic

pH by promoting H^+ extrusion (Yenush *et al*., 2002). In response to benzoic acid the Δ*trk1* mutant fails to accumulate K^+, resulting in the inability to counteract the cytosolic acidification caused by the preservative. However, sensitivity may not be the simple consequence of reduced K^+ uptake capacity; *TRK* mutants exhibit more hyperpolarized plasma membrane voltage (Madrid *et al*., 1998) which could affect growth by disturbing other transport processes (MacPherson *et al*., 2005). Δ*hal3* also exhibited a sensitive phenotype to citric acid. *HAL3* encodes Hal3p, an inhibitor of the phosphatase Ppz1p, which in turn is involved in the regulation of K^+ transport (Yenush *et al*., 2002). Changes in membrane potential and in K^+ uptake have been highlighted as crucial factors influencing the response of *S. cerevisiae* to weak acids. The growth assay results suggest that *SKY1*, *PTK2*, *TRK1* and *HAL3* confer resistance to citric acid stress. In previous studies it has been shown that *TRK1* is involved in the adaptation of *S. cerevisiae* to the weak acids; sorbic acid and benzoic acid, the growth assay results suggest this may also be the case for citric acid.

Of the other 12 deletions strains which exhibited sensitive phenotypes in the presence of citric acid, 11 of the genes encode proteins which interact with Sky1p via primary, secondary, tertiary or quaternary interactions (Figure 3.9). *NPL3* encodes a nuclear shuttling protein Npl3p, involved in mRNA transport. Sky1p in the cytoplasm phosphorylates Npl3p. This is imperative for the viable transport of this SR protein to the nucleus where it binds to mRNA and transports it back to the cytoplasm (Aubol *et al*., 2002). Imported Npl3p is methylated by Hmt1p in the nucleus which facilitates Npl3p export (Yun and Fu, 2000). Interestingly, nuclear mRNA export via Sky1p/Npl3p pathway is inhibited during heat shock and high salt stress; whereas mRNAs for heat shock proteins are exported through a distinct pathway after stress. It has been suggested

that the balance between both pathways, perhaps modulated by Sky1p, may be important for expression of stress tolerance genes (Forment *et al*., 2002). Δ*npl3*, Δ*sky1* and Δ*hmt1* all displayed hypersensitivity to citric acid stress. A further three deletions strains encoding interacting proteins of Npl3p exhibited hypersensitivity to citric acid: Δ*pub1*, Δ*nam8* and Δ*cbc2*, all of which are involved in RNA binding.

YHR087W expresses a protein of unknown function involved in RNA metabolism. Its deletion was extremely sensitive to citric acid stress. Although little is known about the function of the protein in *S. cerevisiae*, the *YHR087W* orthologue in *S. pombe* is expressed during sporulation and environmental stress. Synthetic genetic array analysis of *YHR087W* also revealed genetic interactions with proteins involved in RNA and rRNA processing including Npl3p (Savchenko *et al*., 2005). A study on the transcriptional responses of benzoic, sorbic, acetic and propionic acid stress in *S. cerevisiae* identified 14 genes whose transcript levels were significantly up regulated in response to all four acids. *YHR087W* was one of these identified (Abbott *et al*., 2007). This suggests that *YHR087W* plays a role in the general weak organic acid stress response and our results suggest this is true for the tri-carboxylic acid citrate also.

The remaining citric acid hypersensitive deletion strains express proteins with various functions; Ric1p and the Ypt6p GTPase, function in a common pathway required for localization of trans-Golgi network membrane proteins (Bensen *et al*., 2001); Met2p catalyses the first step in the methionine biosynthetic pathway; Lys14p regulates the lysine biosynthesis pathway; Bim1p is a microtubule binding protein; and Mud1p involved in nuclear mRNA splicing. None of these proteins have been implicated in stress response previously.

A protein-protein interaction of particular interest was the secondary interaction of Sky1p with Nmd5p through Mpc54p. Nmd5p is an importin β homologue required for the import of Hog1p into the nucleus. Δ*nmd5* cells completely fail to translocate Hog1p to the nucleus following stress treatment (Ferrigno *et al*., 1998). Since the HOG MAPK pathway has been shown to be involved in the regulation of adaptation to citric acid stress (Lawrence *et al*., 2004), and *SKY1* seems to confer resistance to citric acid, then perhaps there is a possible relationship between these protein kinases linked via the importin Nmd5p? The construction of Δ*nmd5* and subsequent growth assays confirmed that deletion of *NMD5* results in hypersensitivity to citric acid stress. This may be solely due to the mislocalisation of Hog1p or perhaps due to interference with regulation of other proteins. Previous studies have shown that Nmd5p is required for the nuclear accumulation of the heat shock protein Ssa4p upon ethanol stress exposure (Quan *et al*., 2004). Others stresses including oxidative and osmotic stress do not induce the accumulation of Ssa4p to the nucleus. There is however evidence of up-regulation of Ssa4p upon exposure to sorbic acid stress, suggesting its involvement in weak acid adaptation (de Nobel *et al*., 2001). Nmd5p is also the sole importin protein responsible for shuttling Crz1p into the nucleus. Crz1p is a transcription factor which when dephosphorylated by calcineurin activates transcription of genes involved in stress response (Cyert, 2003). Interestingly Crz1p has also been found to interact with Sky1p (Ptacek *et al*. 2005). The requirement for Nmd5p for the import of Hog1p, Ssa4p and Crz1p; all of which have been previously implicated in stress response, and Δ*nmd5*'s hypersensitivity to citric acid suggest a possible role in citric acid adaptation and perhaps a link between Hog1p and Sky1p in their roles in citric acid adaptation.

In *S. cerevisiae* there has been no evidence of a transport system specifically for citric acid across the plasma membrane. In the closely related yeast *C. utilis* there is evidence of two transport systems for citric acid, a proton symport and a facilitated diffusion system for the charged and the undissociated forms of the acid, respectively however the proteins involved in these systems have not yet been identified (Cassio and Leao, 1991). Through BLAST protein sequence comparison of a known citrate transporter protein in *D. melanogaster* to *S. cerevisiae* proteins, three phosphate transporters were found to have high sequence similarity. Following growth assays in liquid media only one of the transporter deletion strains Δ*pho87*, displayed a resistant phenotype to citric acid. However, the 4.8% resistance to 300 mM citric acid exhibited by Δ*pho87* is not great enough to substantiate the theory that *PHO87* could be involved in citric acid transport. Although there is no evidence of any citric acid specific transport systems for the acid's transport across the plasma membrane, there is a citrate transport system within mitochondria involved in the tricarboxylic acid (TCA) cycle. The mitochondrial citrate transport protein Ctp1p catalyses the exchange of citrate plus a proton across the mitochondrial inner membrane in exchange for either, another tricarboxylate + H^+, a dicarboxylate, or phosphoenolpyruvate (Kaplan *et al*., 1995). The possible involvement of the TCA cycle in citric acid adaptation has been previously suggested (Lawrence *et al*., 2004). Lawrence identified the up-regulation of two enzymes involved in the TCA cycle upon exposure to citric acid, Mdh1p (mitochondrial malate dehydrogenase) and Idh1p (iso-citate dehydrogenase 1 alpha-4-beta-4 subunit). Lawrence suggested that the up-regulation of these enzymes indicates that the cells may be attempting to remove excess citric acid by increasing the rate of metabolism through the TCA cycle.

Employment of a citric acid uptake assay could be used to test deletions of possible transporters, determine levels of uptake into the cell, if any, and study if deletion of *SKY1*, *HOG1* and their double deletion would affect uptake. An enzymatic approach, determining citric acid levels by measuring oxidized NADH was initially attempted. This method did not prove to be sensitive enough for the cell sample size. Subsequently, an uptake assay using radiolabelled citric acid, devised from a similar experiment used to measure benzoic acid uptake was employed (Henriques *et al*., 1997). The results showed little or no citric acid uptake in WT, Δ*sky1*, Δ*hog1* or Δ*hog1*/Δ*sky1* strains in the presence of glucose and similarly without the presence of glucose with the exception of Δ*sky1*. There was a definitive peak in radioactive counts (CPMA) for Δ*sky1* without glucose at the 2 minute incubation time point, suggesting citric acid uptake, followed by a decrease in CPMA to little or no counts between 2-4 minutes of incubation, suggesting extrusion of citric acid from the cell. Since the WT, Δ*hog1* and Δ*hog1*/Δ*sky1* strains showed no uptake in either assay (with or without glucose) and Δ*sky1* grown with glucose did not show any uptake of the radiolabelled citric acid, this suggests citric acid uptake is dependant on the *SKY1* deletion and the absence of glucose. *S. cerevisiae* will oxidatively metabolize glucose to some extent but generally prefer to metabolize glucose using the high-flux fermentative Embden-Meyerhof pathway. When the glucose is exhausted, cells undergo a "diauxic shift", in which they switch to fully respiratory metabolism, catabolising carbon compounds via the TCA cycle and oxidative phosphorylation in the mitochondria (Brauer *et al*., 2005). Switching to respiratory metabolism has been shown to initiate the "oxidative stress response", changing the expression of many hundereds of genes (Gasch *et al*., 2000). Following "diauxic shift" it has been found that expression patterns change to favour the high-affinity transporters and kinases. These changes in expression in addition as a direct response of the change in carbon source also occur from changes in

metabolic response and also include genes with a stress response element (Brauer *et al*., 2005). Perhaps under respiratory metabolism, the deletion of *SKY1* results in the interference with regulation of a transporter allowing citric acid uptake, or interference with cell wall components or channels to allow diffusion? The uptake assay results also showed the extrusion of the citric acid from the cell suggesting there must have been some sort of active transport?

This study has postulated a novel role for Sky1p and a number of its interacting proteins in citric acid adaptation via an as yet unknown mechanism. The importin Nmd5p also sensitive to citric acid upon deletion, and its connections with both Sky1p and Hog1p make the study of possible linkage between theses two protein kinases and importin in the adaptation to citric acid interesting.

Chapter 4

4. Citric acid induced changes in the proteome and phosphoproteome of *S. cerevisiae* BY4741a, Δ*sky1* and Δ*hog1* strains.

4.1. Introduction

The term 'Proteomics' was first coined in 1995 and was defined as the large scale characterisation of the entire protein complement of a cell line, tissue or organism (Wasinger *et al.,* 1995; Wilkins *et al.,* 1996). Proteomics has often been considered the next step in the study of biological systems, after genomics. Proteomic analysis is much more complicated than genomic analysis mostly because while a genome is rather constant, a proteome differs from cell to cell and constantly changes through its biochemical interactions with the genome and its environment. Many types of information cannot be obtained from the study of genes alone; it is the proteins, not genes, responsible for the phenotype of cells (Graves and Haystead, 2002).

In this study we used proteomic analysis in the form of 2-dimensional electrophoresis (2-DE) to analyse changes in protein expression in response to citric acid stress in *S. cerevisiae* control (WT) and Δ*sky1* strains. We measured changes in the proteome of WT and Δ*sky1* deletion strain occurring in response to growth in MB [pH 3.5] +/- 300 mM citric acid. Changes in protein expression were also compared to those identified in Δ*hog1* proteomes and overlaps between proteomes were identified. Protein phosphorylation changes in WT and Δ*sky1* strains in the presence and absence of citric acid (phosphoproteome analysis) were also investigated.

The separation and analysis of membrane proteins still remains a difficult task, especially in context of proteomic research. Classical 2-D electrophoresis (2-DE) of integral membrane proteins generally suffers from significant precipitation effects during sample application, isoelectric focusing and sample transfer from 1^{st} to 2^{nd} dimension. Liquid-phase isoelectric focusing (IEF) by free-flow electrophoresis (FFE) circumvents the sample precipitation phenomena because the sample buffer and separation buffer are very similar. Membrane proteins crucial to cell homeostasis include transporters, channels, and receptors. Classical 2-DE has failed to enable proteomic analysis of these poorly soluble and hydrophobic proteins, leaving a large range of proteins whose response to citric acid stress remains unknown. In this study we performed free-flow isoelectric focusing separation using a ProTeam™ FFE instrument (Tecan Ltd., Reading, UK) on microsomal preparations prepared from cultures grown in the presence and absence of citric acid stress. The resultant protein fractions were then separated by molecular weight using SDS-PAGE and visualized with silver stain. Proteins were excised from gels and identified by Mass Spectrometry.

4.2. Comparative analysis of the proteome of citric acid-stressed Δ*sky1* mutant and control BY4741a strains.

Proteomic techniques were used to identify changes in protein expression between control BY4741a (WT) and Δ*sky1* strains. Analysis of WT and Δ*sky1* proteomes was performed using 2D-PAGE over two pH ranges (pH 4-7 and pH 6-11) in the presence and absence of 300 mM citric acid. Changes in protein expression levels were only considered significant if observed in the duplicate set of experiments. Mass Spectrometry techniques were used to determine the identity of these proteins.

4.2.1. Comparison of protein expression changes induced by 300 mM citric acid in control and Δ*sky1* strains over pI 4-7.

A four-way analysis was performed comparing protein changes between duplicate cultures of WT and Δ*sky1* strains in the absence and presence of 300 mM citric acid over pI range 4-7 (Figure 4.1 - 4.7). Mass spectrometry positively identified 18 of the 33 protein spots sent for analysis (Table 4.1).

In the BY4741a wild type strain, over pH range 4-7, six proteins were upregulated due to growth in the presence of 300 mM citric acid. The proteins were identified as: glycerol-3-phosphatase (Rhr2p), heat shock protein 70 (Ssz1p), heat shock protein (Ssa1p), heat shock protein (Ssb1p), pyruvate decarboxylase isozyme 1 (Pdc1p), and enolase (Eno2p). No proteins were found to be down regulated due to growth in the presence of citric acid (Figure 4.1 & 4.2).

The 2-D map of the Δ*sky1* deletion strain exhibited upregulation of six proteins and downregulation of three proteins in response to citric acid. The upregulated proteins were; Rhr2p, arginase (Car1p), aldehyde dehydrogenase (Ald3p), pyruvate dehydrogenase (Pdb1p) and a protein mixture consisting of the 6-phosphogluconate dehydrogenase (Gnd1p) and enolase (Eno1p). The downregulated proteins were identified as; glyceraldehyde 3-phosphate dehydrogenase 3 (Tdh3p), transaldolase (Tal1p) and the dihydootate dehydrogenase (Ura1p) (Figure 4.3 & 4.4).

Comparisons of the wild-type and Δ*sky1* 2-D maps displayed changes in expression of a number of proteins in response to exposure to citric acid and in response to deletion of *SKY1*. Ado1p, an adenosine kinase was down regulated in response to citric acid in Δ*sky1* relative to the wild type strain. Rps0Bp, a 40 S ribosomal protein, exhibited upregulation upon deletion of *SKY1* relative to the wild type. The vacuolar ATP synthase subunit, Vma4p was down regulated relative to the wild type in Δ*sky1* and upregulated relative to the wild type upon exposure to citric acid. Finally, the translationally controlled tumour protein Tma19p was downregulated in both Δ*sky1* and Δ*sky1* in the presence of citric acid maps relative to the wild type (Figure 4.5).

A number of the proteins identified were heat shock proteins or known to be involved in stress response: Ssb1p, Ssz1p, Ssa1p, Ald3p and Rhr2p (Table 4.1). Many proteins involved in metabolic processes such as glycolysis were identified such as Eno2p, Tdh3p and Eno1p. Vma4p, a vacuolar ATP synthase subunit involved in maintenance of intracellular pH was also identified (Figure 4.6 & 4.7).

Comparison of the experimental to predicted molecular weight, pI and percentage of peptide coverage of the identified proteins can be found in Table 4.1.

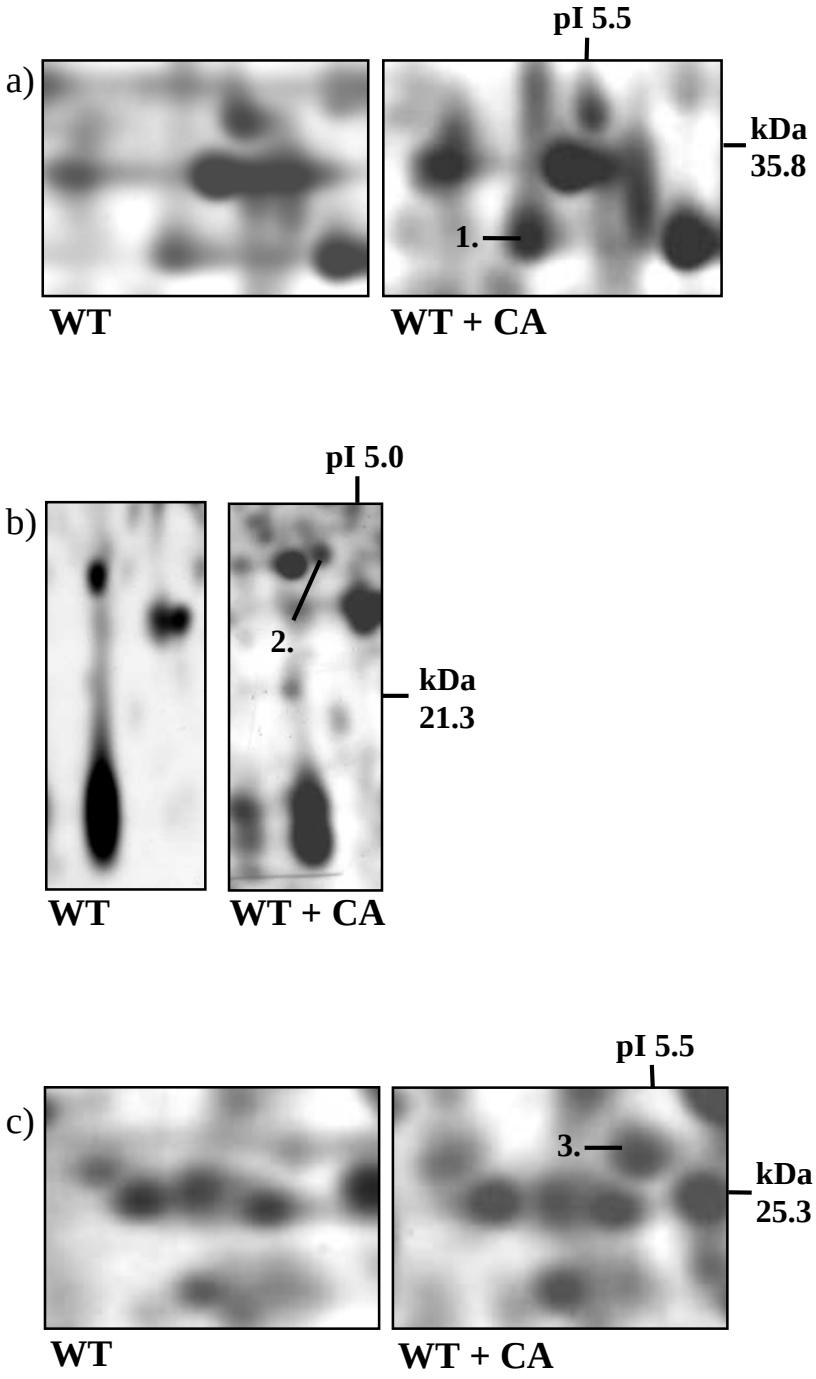


Figure 4.1: Detection of up- and down-regulated proteins from pI 4-7 2DE gels for WT with and without 300 mM citric acid (CA). Proteins 1-3 were citric acid-induced and identified via Mass Spectrometry: a) protein 1: Rhr2p, b) protein 2: Mixture; Ssz1p, Ssa1p, c) protein 3: Mixture; Ssb1p, Ccl1p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.1).

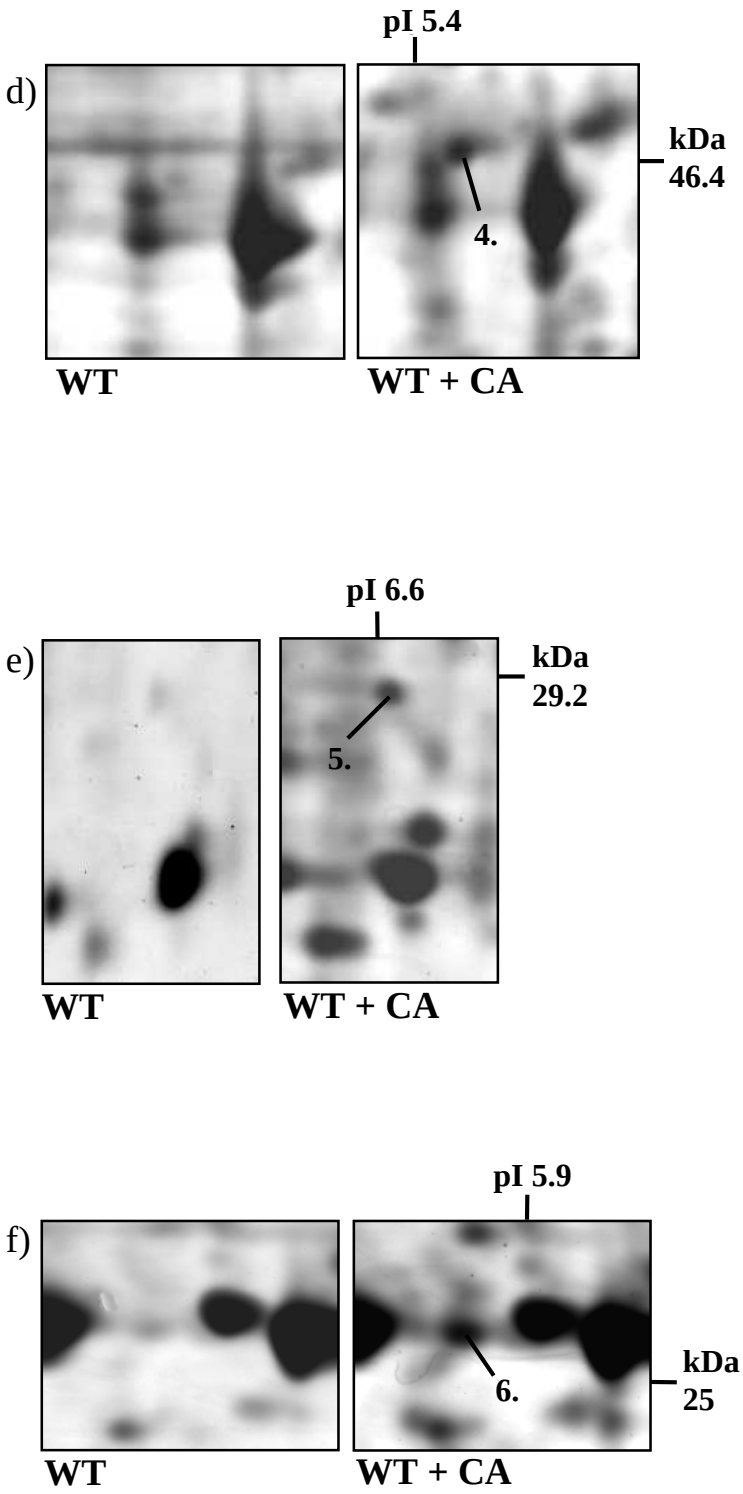


Figure 4.2: Detection of up- and down-regulated proteins from pI 4-7 2DE gels for WT with and without 300 mM citric acid (CA). Proteins 4, 5 and 6 were citric acid-induced and identified via Mass Spectrometry: d) protein 4: Ssb1p, e) protein 5: Pdc1p, f) protein 6: Eno2p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.1).

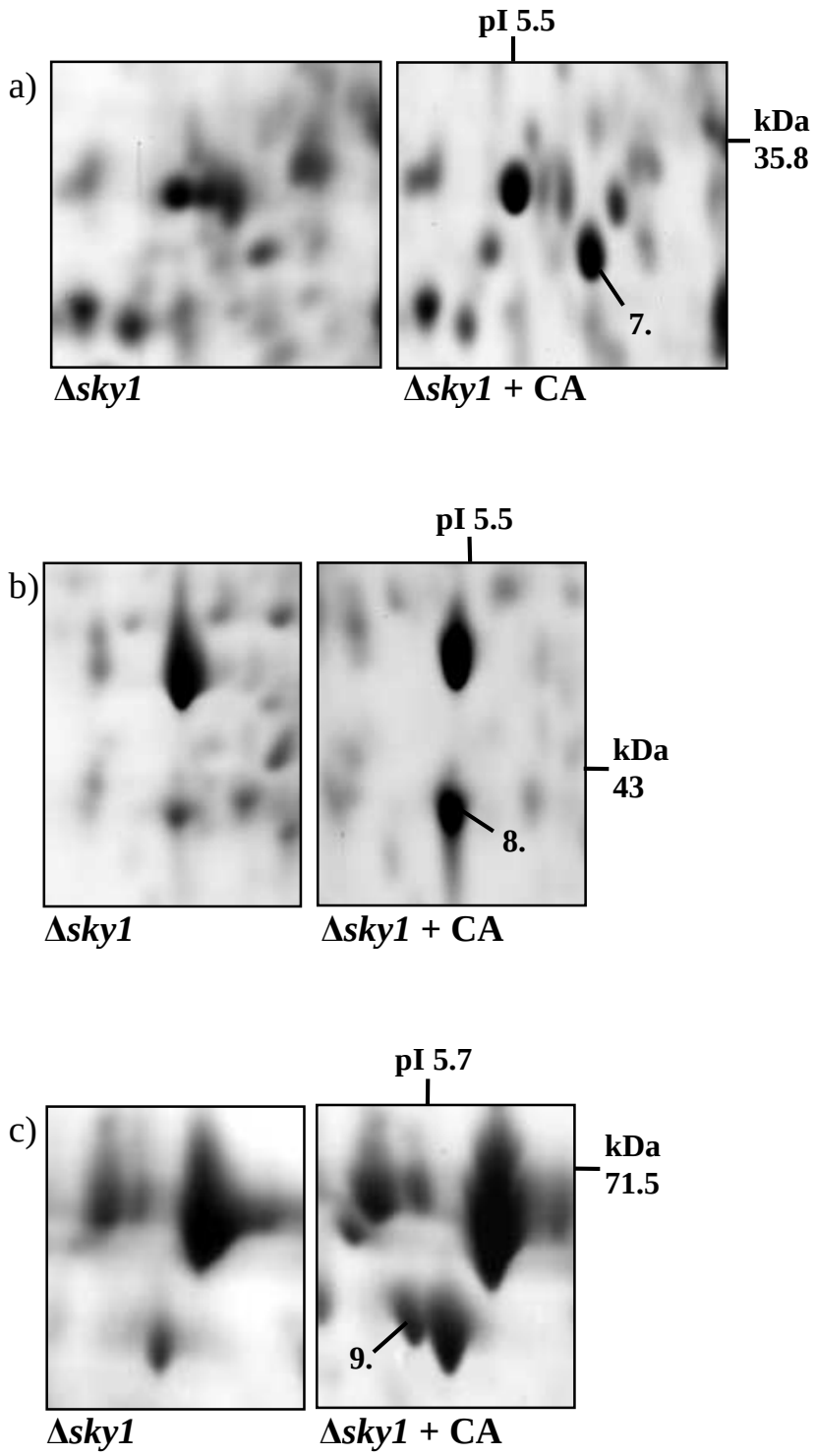


Figure 4.3: Detection of up- and down-regulated proteins from pI 4-7 2DE gels for Δ*sky1* with and without 300 mM citric acid (CA). Proteins 7, 8 and 9 were citric acid-induced and identified via mass spectrometry: a) protein 7: Rhr2p, b) protein 8: Car1p, c) protein 9: Ald3p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.1).

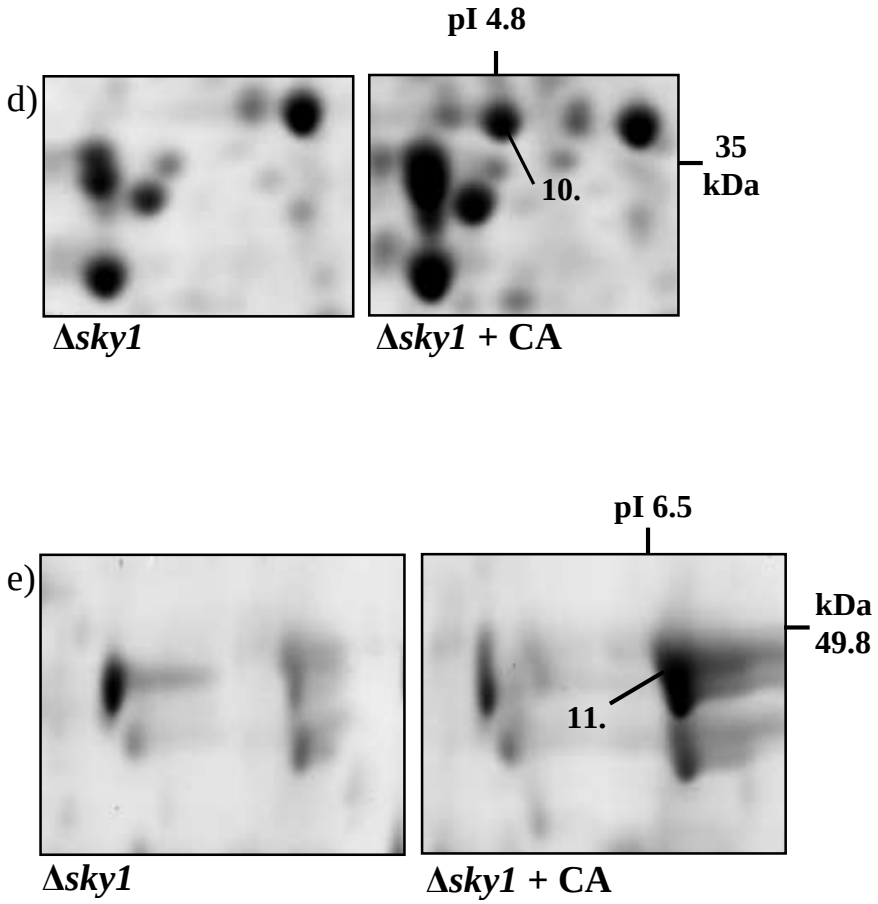
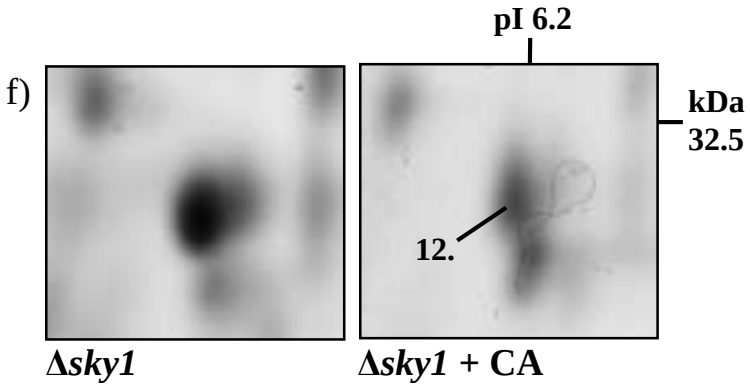


Figure 4.4: Detection of up- and down-regulated proteins from pI 4-7 2DE gels for Δ*sky1* with and without 300 mM citric acid (CA). Protein 10 and 11 were both citric-acid induced in Δ*sky1* and were identified via Mass Spectrometry: d) protein 10: Pdb1p, e) protein 11: Mixture; Gnd1p, Eno1p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.1).



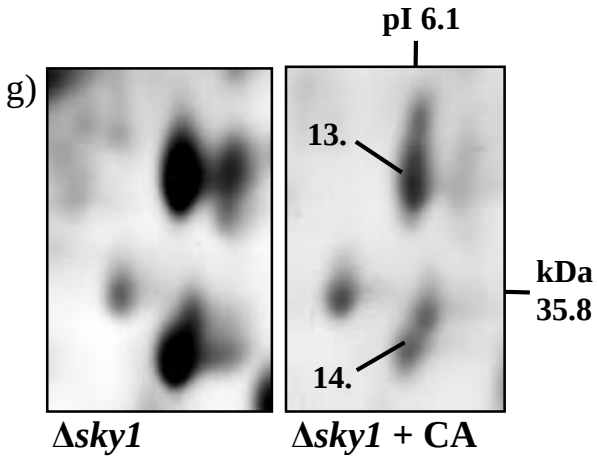


Figure 4.5: Detection of up- and down-regulated proteins from pI 4-7 2DE gels for Δ*sky1* with and without 300 mM citric acid (CA). Proteins 12, 13 and 14 were all shown to be down-regulated in response to citric acid in Δ*sky1*. The proteins were identified via Mass Spectrometry: f) protein 12: Tdh3p, g) protein 13: Tal1p, protein 14: Ura1p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.1).

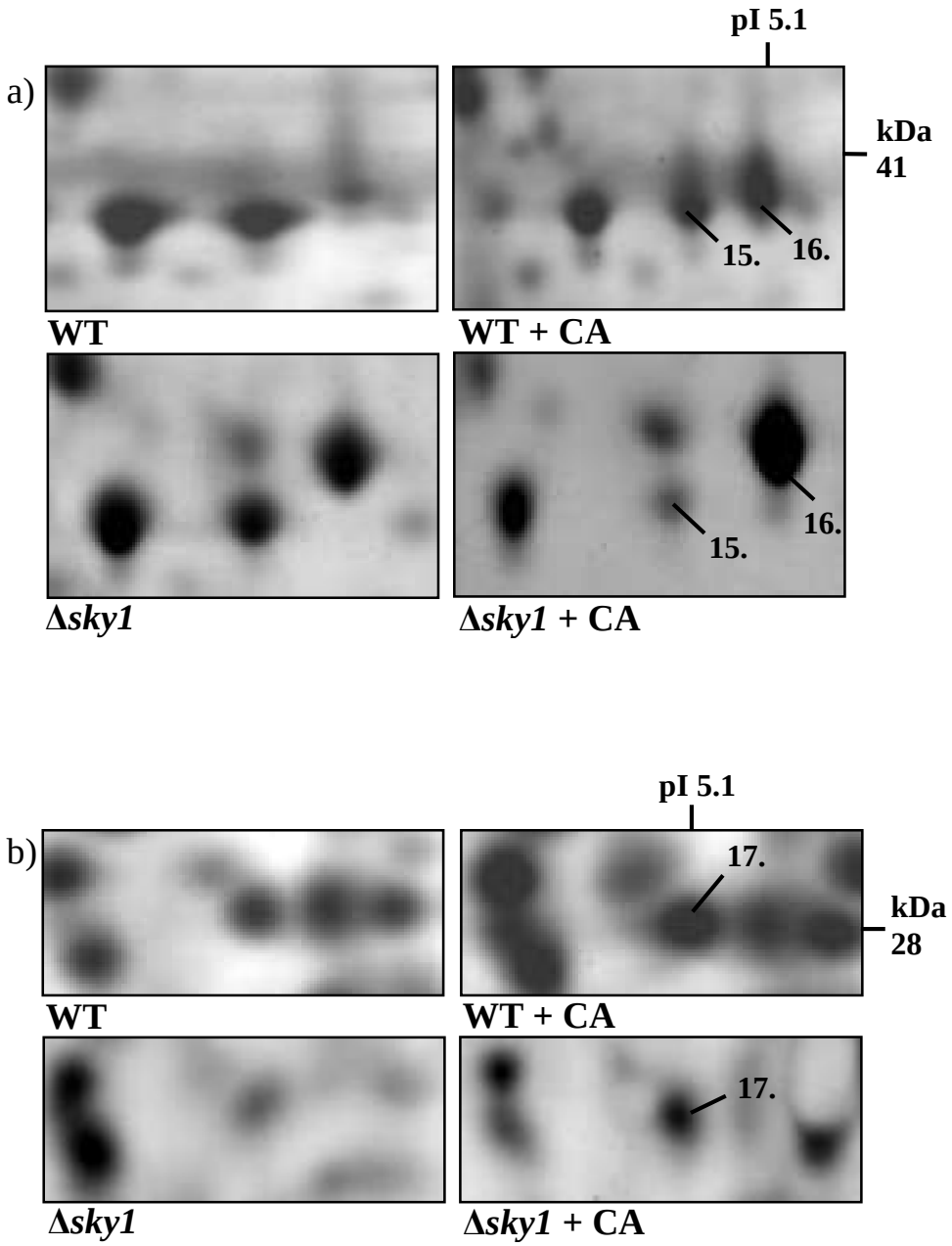


Figure 4.6: Detection of up- and down-regulated proteins from pI 4-7 2DE gels for WT with and without 300 mM citric acid (CA) and Δ*sky1* with and without 300 mM citric acid (CA). Protein 15 was down regulated in response to citric acid in Δsky1, protein 16 was up-regulated in response to loss of *SKY1*, and protein 17 was down-regulated in response to loss of *SKY1* but up-regulated in response to citric acid in Δ*sky1*. Proteins were identified via Mass Spectrometry: a) protein15: Ado1p, protein 16: Rps0Bp, b) protein 17: Vma4p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.1).

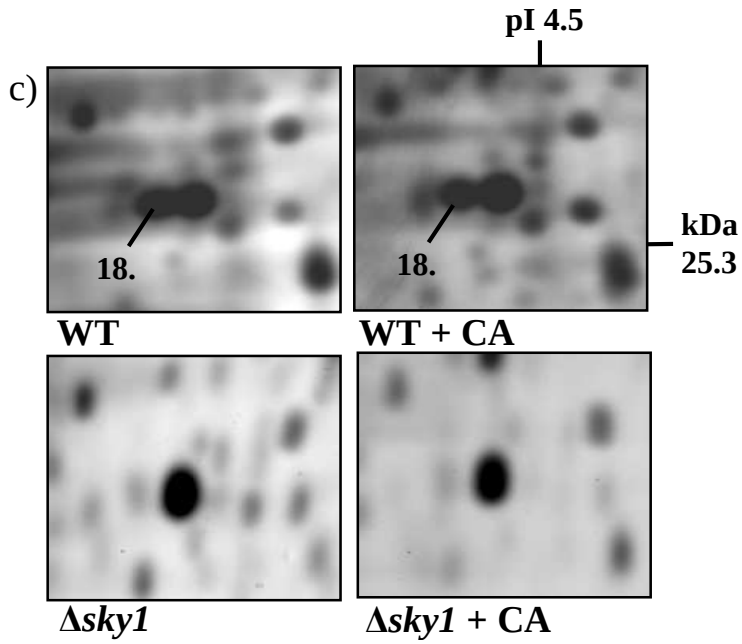


Figure 4.7: Detection of up- and down-regulated proteins from pI 4-7 2DE gels for WT with and without 300 mM citric acid (CA) and Δ*sky1* with and without 300 mM citric acid (CA). Protein 18 was down-regulated in response to loss of *SKY1* and was identified via Mass Spectrometry**:** c) protein 18: Tma19p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.1).

Table 4.1: Identification of *S. cerevisiae* proteins by Mass Spectrometry over pI 4-7.

Spot No.	% matched peptides [a]	% matched peptide coverage [b]	Molecular Mass (kDa) Exp	Molecular Mass (kDa) Pred [c]	pI Exp	pI Pred [c]	Top ranking protein in MS-Fit search [d]	SwissProt Accession No.
1	38	45	35.0	28.1	5.50	5.35	Rhr2 (glycerol-3-phosphatase)	P41277
2	33	23	26.5	62.3	4.90	5.10	Mixture: Ssz1 (heat shock protein 70)	P38788
	23	19		69.9		5.00	Ssa1 (heat shock protein)	P10591
3	23	9	27.0	66.7	5.30	5.32	Mixture: Ssb1 (heat shock protein)	P11484
	23	15		45.5		6.45	Ccl1p (cyclin)	P37366
4	42	14	47.0	66.7	5.45	5.32	Ssb1(heat shock protein)	P11484
5	38	6	29.5	61.7	6.70	5.80	Pdc1 (pyruvate decarboxylase isozyme 1)	P06169
6	32	16	21.0	46.9	5.80	5.67	Eno2 (enolase 2)	P00925
7	25	46	33.5	28.1	5.60	5.35	Rhr2 (glycerol-3-phosphatase)	P41277
8	26	27	41.0	36.0	5.50	5.38	Car1 (arginase)	P00812
9	53	21	67.0	55.7	5.70	5.56	Ald3 (aldehyde dehydrogenase)	P54114
10	22	34	37.0	40.0	4.80	5.23	Pdb1 (pyruvate dehydrogenase)	P32473
11	33	27	50.5	53.9	6.60	6.19	Mixture: Gnd1 (6-phosphogluconate dehydrogenase)	P38720
	29	32		46.7		6.04	Eno1 (enolase 1)	P00924
12	22	19	32.0	35.8	6.2	6.46	Tdh3 (glyceraldehyde 3-phosphate dehydrogenase 3)	P00359
13	38	28	39.0	37.1	6.10	6.09	Tal1 (transaldolase)	P15019
14	40	22	36.0	35.0	6.10	5.80	Ura1 (dihydroorotate dehydrogenase)	P28272
15	38	32	40.0	36.5	5.00	4.99	Ado1 (adenosine kinase)	P47143
16	18	26	41.0	28.0	5.10	4.69	Rps0B (40S ribosomal protein S0-B)	P46654
17	15	25	21.0	26.5	5.10	5.33	Vma4 (vacuolar ATP synthase subunit E)	P22203
18	20	37	27.0	18.8	4.45	4.41	Tma19 (translationally controlled tumour protein)	P35691

[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 peptide divided by the total number of amino acids in the predicted protein sequence expressed as a percentage.

[c] Theoretical pI and molecular weights obtained from the protein entry in the SGD database (URL: http://www.yeastgenome.org).

[d] The program MS-Fit (URL: http://prospector.ucsf.edu/uscfhtml4.0/msfithtm) was used to search the SwissProt database for proteins with calculated tryptic peptide masses that matched the measured experimentally derived masses (mass accuracy +/- 0.15 Da).

4.2.2. Comparison of protein expression changes induced by 300 mM citric acid in control and Δ*sky1* strains over pI 6-11.

2-D-PAGE analysis for WT, Δ*sky1* and Δ*hog1* with and without 300 mM citric acid over pI range 6-11 was also carried out. Analysis of the pH 6-11 proteomes displays lower abundance of proteins and suggestion of fewer changes in protein regulation between comparative gels.

There were several protein 'spots' showing significant changes in expression which could not be identified due to their low masses < 20 kDa. Of those which were excised, 14 proteins were identified using mass spectrometry.

Analysis of changes in the wild type protein expression in response to growth in 300 mM citric acid, over pH range 6-11 has previously been studied, as has changes in the Δ*hog1* proteome pI 4-7 (Lawrence *et al.*, 2004). However, previous analysis has not studied the changes in Δ*hog1* over pI 6-11 in response to citric acid. Here we have highlighted protein expression changes over pI range 6-11 for Δ*hog1* and Δ*sky1* in citric acid treated and untreated cells.

The 2-D map of the Δ*sky1* deletion strain exhibited downregulation of six proteins and upregulation of four proteins in response to citric acid. The downregulated proteins were; pyruvate decarboxylase isozyme 1 (Pdc1p), a 40 S ribosomal protein (Rps5p), glyceraldehyde 3-phosphate dehydrogenase 3 (Tdh3p), phosphoglycerate mutase 1 (Gpm1p), a pyruvate kinase (Cdc19p), and the mitochondrial porin (Por1p). The upregulated proteins were: glyceraldehyde 3-phosphate dehydrogenase (Tdh1p),

phosphoglycerate mutase (Gpm1) and the translational elongation factor EF-1 alpha (Tef2p) (Figure 4.8).

Exposure of Δ*hog1* cells to 300 mM citric acid resulted in the upregulation of four proteins and downregulation of one protein. The upregulated proteins were identified as; a component of the 20 S proteasome (Pre5p), translation elongation factor EF-1 alpha (Tef2p), and a protein mixture containing glyceraldehyde 3-phosphate dehydrogenase (Tdh1p) and the mitochondrial porin (Por1p) (Figure 4.9).

Comparison of the experimental to predicted molecular weight, pI and percentage of peptide coverage of the identified proteins can be found in Table 4.2.

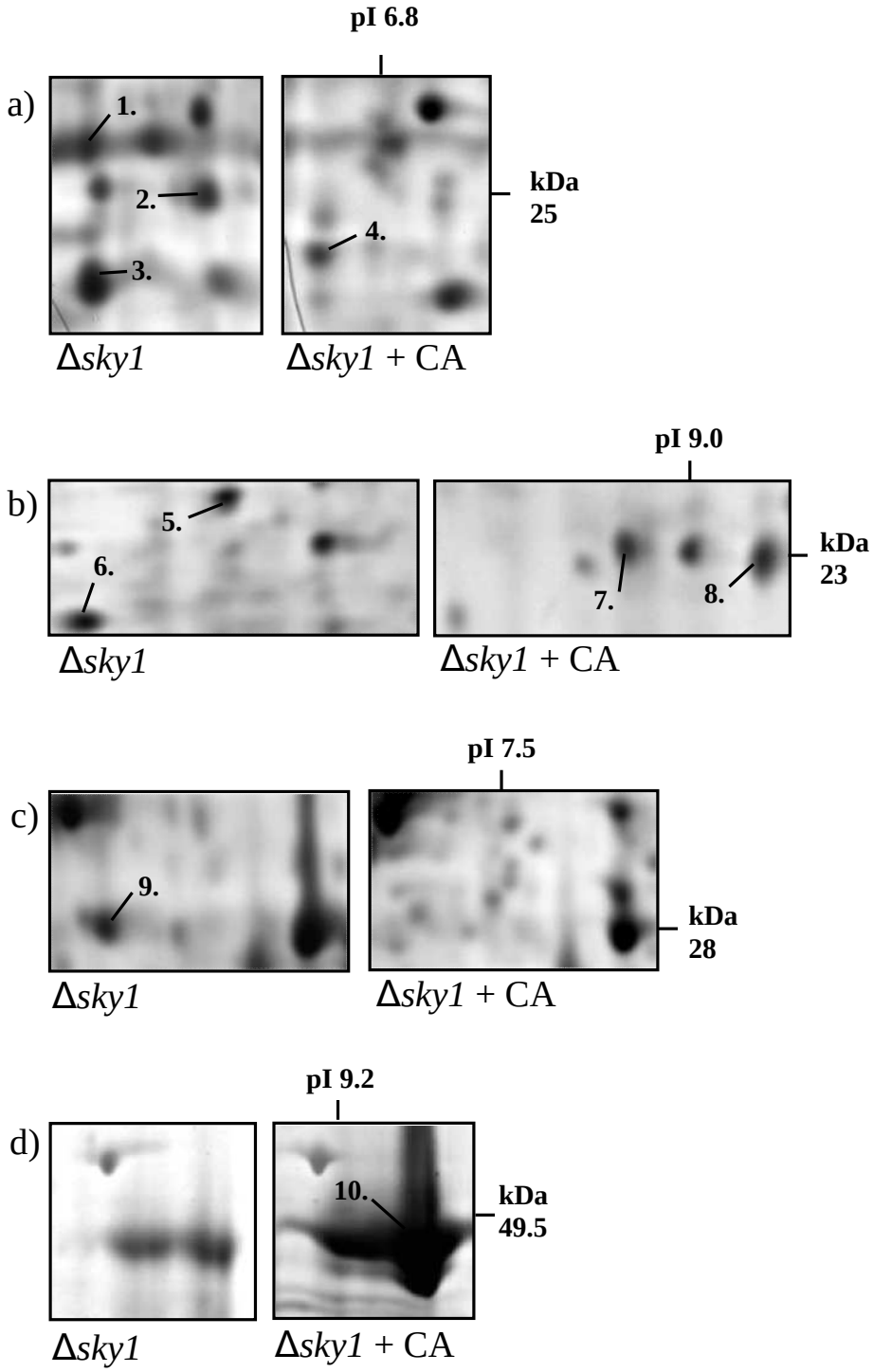


Figure 4.8: Detection of up- and down-regulated proteins from pI 6-11 2DE gels for Δ*sky1* with and without 300 mM citric acid (CA). Proteins 1, 2, 3, 5, 6 & 9 were down-regulated in Δ*sky1* in response to citric acid. Proteins 4, 7, 8 & 10 were up-regulated in response to citric acid in Δ*sky1*. Proteins were identified via Mass Spectrometry: a) protein 1: Pdc1p, protein 2: Rps5p, protein 3:Tdh3p, protein 4: Tdh1p. b) protein 5: Gpm1p, protein 6: Cdc19p, protein 7: Gpm1p, protein 8: Gpm1p. c) protein 9: Por1p. d) protein 10: Tef2p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.2).

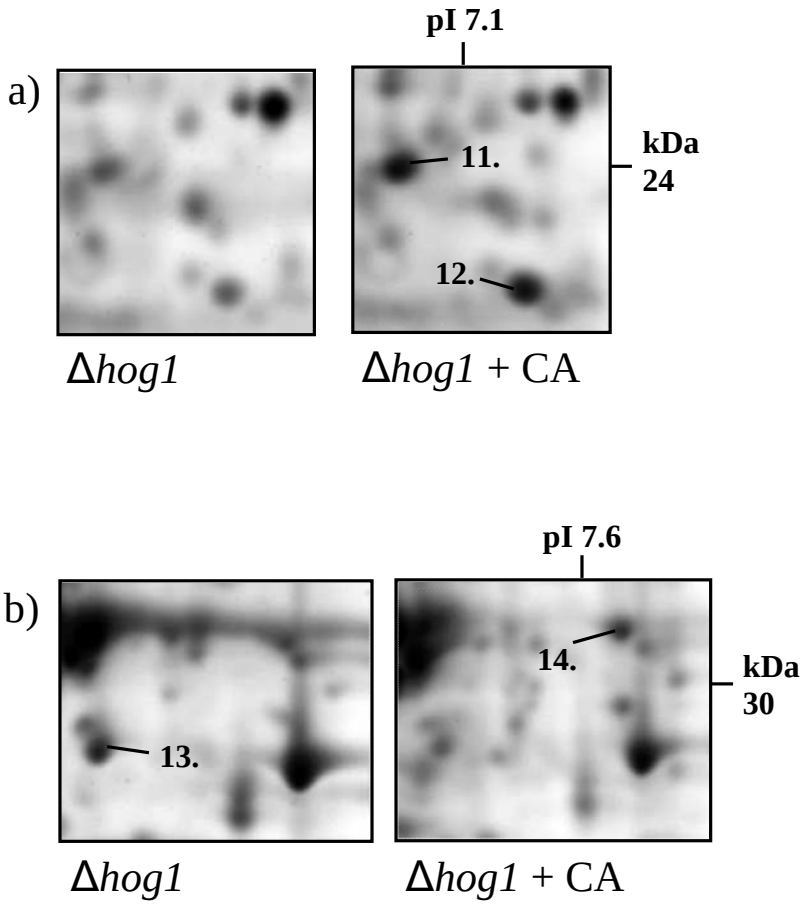


Figure 4.9: Detection of up- and down-regulated proteins from pI 6-11 2DE gels for Δ*hog1* with and without 300 mM citric acid (CA). Proteins 11, 12, & 14 were up-regulated in response to citric acid in Δ*hog1*. Protein 13 was down-regulated in response to citric acid in Δ*hog1*. Proteins were identified via Mass Spectrometry: a) protein 11: Pre5p, protein 12: Tef2p. b) protein 13: Tdh1p, protein 14: Mixture; Tdh1p, Por1p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.2).

Table 4.2: Identification of *S. cerevisiae* proteins by Mass Spectrometry over pI 6-11.

Spot No.	% matched Peptides [a]	% matched peptide coverage [b]	Molecular Mass (kDa) Exp	Molecular Mass (kDa) Pred [c]	pI Exp	pI Pred [c]	Top ranking protein in MS-Fit search [d]	SwissProt Accession No.
1	23	14	26.0 (frg)	59.8	6.50	5.62	Pdc1 (pyruvate decarboxylase)	P06169
2	20	40	25.0	24.9	6.90	8.62	Rps5 (40s ribosomal protein)	P26783
3	17	37	23.0	35.7	6.70	6.96	Tdh3 (glyceraldehyde 3-phosphate dehydrogenase 3)	P00359
4	15	32	24.0	35.8	6.7	8.29	Tdh1 (glyceraldehyde 3-phosphate dehydrogenase)	P00360
5	29	44	25.0	26.4	8.70	8.27	Gpm1 (phosphoglycerate mutase 1)	P00950
6	38	17	21.0 (frg)	53.6	8.20	7.55	Cdc19 (pyruvate kinase)	P00549
7	25	44	23.0	26.4	8.80	8.27	Gpm1 (phosphoglycerate mutase 1)	P00950
8	21	38	23.0	26.4	9.20	8.27	Gpm1 (phosphoglycerate mutase 1)	P00950
9	30	50	28.0	30.5	7.30	7.70	Por1 (Mitochondrial porin)	P04840
10	23	35	50.0	50.4	9.50	9.14	Tef2 (Translational elongation factor EF-1 alpha)	P02994
11	22	64	24.0	25.6	7.00	7.08	Pre5 (20s proteasome)	P40302
12	11	12	21.0 (frg)	50.4	7.2	9.14	Tef2 (Translational elongation factor EF-1 alpha)	P02994
13	19	33	29.0	35.8	7.20	8.29	Tdh1 (glyceraldehyde 3-phosphate dehydrogenase)	P00360
14							Mixture:	
	17	40	31.0	35.8	8.7	8.29	Tdh1 (glyceraldehyde 3-phosphate dehydrogenase)	P00360
	14	47	31.0	30.5	8.7	7.70	Por1 (Mitochondrial porin)	P04840

[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 peptide divided by the total number of amino acids in the predicted protein sequence expressed as a percentage.

[c] Theoretical pI and molecular weights obtained from the protein entry in the SGD database (URL: http://www.yeastgenome.org).

[d] The program MS-Fit (URL: http://prospector.ucsf.edu/uscfhtml4.0/msfithtm) was used to search the SwissProt database for proteins with calculated tryptic peptide masses that matched the measured experimentally derived masses (mass accuracy +/- 0.15 Da).

(frg) Indicates that the protein detected was a proteoytic cleavage fragment.

4.2.3. Overlapping proteins both up and down regulated in Δ*sky1* and Δ*hog1* proteomes

The 2-D analysis of protein expression changes in wild type, Δ*sky1* and Δ*hog1* proteomes in response to citric acid showed similar expression responses elicited by both Δ*sky1* and Δ*hog1* deletion strains. The knowledge that Hog1p is involved with citric acid adaptation couple with the discovery of altered expression of these proteins in both Δ*sky1* and Δ*hog1* proteomes provide further evidence for the involvement of *SKY1* in citric acid adaptation.

Eno2p was upregulated in response to citric acid in wild type cells, but no change in expression was seen in either Δ*hog1* or Δ*sky1* 2-D maps. Both Car1p and Pdb1p were up regulated upon exposure to citric acid in both deletion strains but no change was exhibited for the wild type strain (Figure 4.10).

Up-regulation of Pdb1p and Car1p in both proteomes suggest that the two protein kinases may either regulate similar proteins in response to citric acid or that they are both on a related pathway.

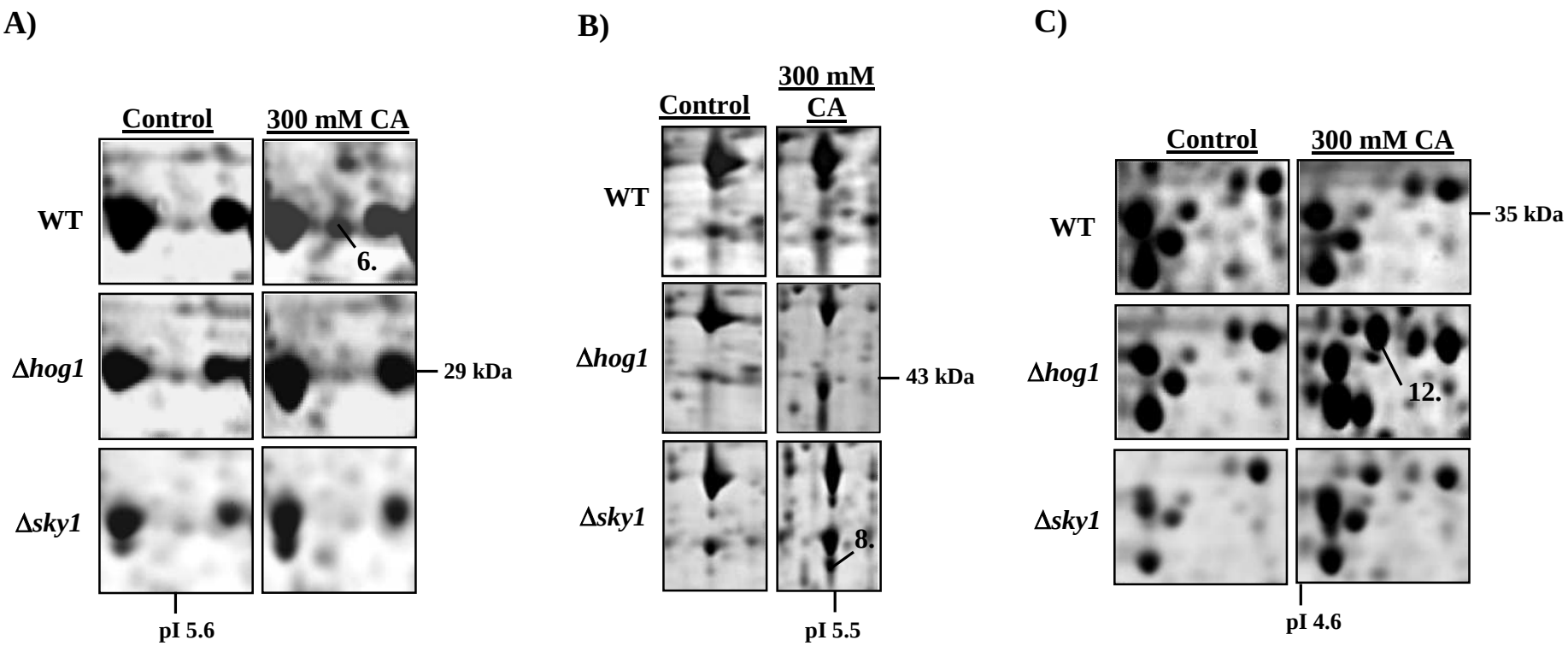


Figure 4.10: Identification of citric acid-induced protein expression overlap in WT, Δ*hog1*, and Δ*sky1* proteomes. Protein expression in wild-type (WT), Δ*hog1*, and Δ*sky1* in the presence and absence of 300 mM citric acid (CA), pH 3.5, over pI range 4-7, is shown . A) Eno2p (protein 6) is citric acid-induced in WT but not in either Δ*hog1* or Δ*sky1*. B) Car1p (protein 8) is citric acid-induced in both Δ*hog1* and Δ*sky1* but not in WT. C) Pdb1p (protein 12) is citric acid-induced in both Δ*hog1* and Δ*sky1* but not in WT. Molecular masses (in kilodaltons) are shown on the y axis and pI values are shown on the x axis. A representative result of two replicate experiments is shown.
(Mass spectrometry data: Table 4.1)

4.3. Combination of Pro Team™ FFE with SDS-PAGE for the two-dimensional separation of membrane proteins from microsomal protein preparations.

4.3.1. Liquid-phase isoelectric focusing of microsomal protein samples by free-flow electrophoresis (FFE).

To study changes in expression levels of membrane proteins under citric acid stress, an approach using a combination of the Pro Team™ FFE unit and SDS-PAGE was used.

Microsomal protein samples (6 mg) for WT cultures and WT grown in the presence of 300 mM citric acid were separated using free-flow electrophoresis, fractionating the microsomal protein into 96 samples (Section 2.9). The resultant fractions were pH-probed and their protein concentrations calculated. Initially protein concentration was determined using a Bradford reagent (Coomassie Plus-The Better Bradford Assay) purchased from Pierce (Perbio Science, Northumberland, UK). This proved inaccurate and OD_{280} readings for each sample were used as a comparison of protein concentration for each sample instead. The pI and OD_{280} data for duplicate WT and WT + 300 mM citric acid microsomal samples was plotted graphically (Figure 4.11) and a level of high reproducibility was evident.

Concurrent with previous data attained using 2-DE gel analysis where few proteins were visualized at the pH extremities, analysis of the pI and OD_{280} data (Figure 4.11) suggests that there is little protein content in fractions ranging in the low pH range 0-4, and also the high pH range above pH 10. Protein levels were found to be most abundant at pH range 4-10. Thus, providing further encouragement that the Tecan FFE unit had successfully fractionated the microsomal protein preparation.

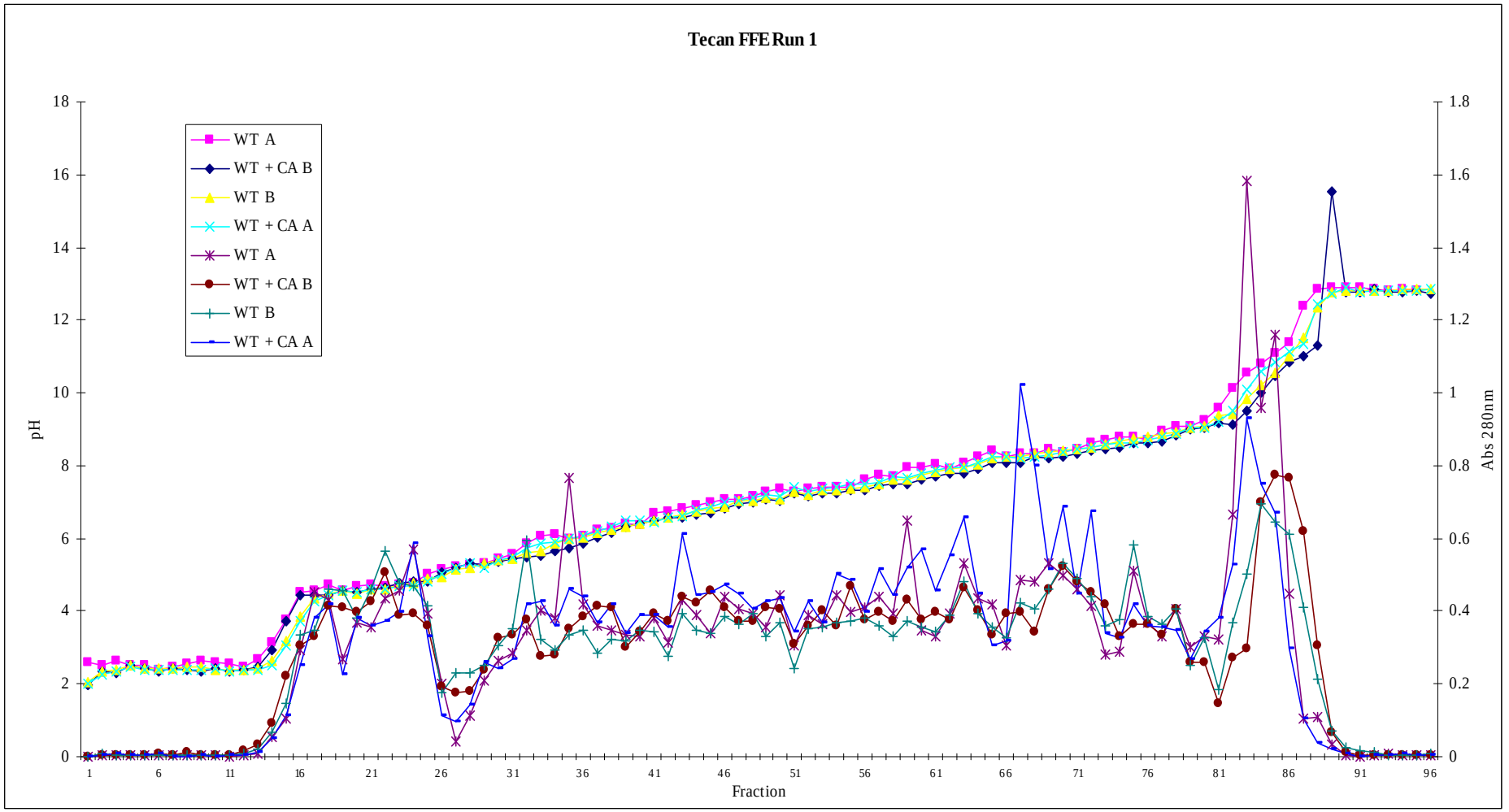


Figure 4.11: OD_{280} and pI data for microsomal protein preparation from WT and WT in the presence of 300 mM citric acid, separated by free-flow electrophoresis. The Pro Team™ FFE unit was loaded with 6mg of microsomal protein preparation from WT and WT + 300 mM citric acid, where the proteins samples were separated into 96 fractions using free-flow electrophoresis. OD_{280} and pI data for each fraction and duplicate samples has been shown and a high level of reproducibility between samples was evident.

4.3.2. SDS-PAGE Electrophoretic separation of fractionated proteins and identification by Mass Spectrometry.

To determine whether the separated protein fractions were concentrated enough for protein identification via mass spectrometry and whether there was clear distinction in the banding pattern for proteome comparison, a selection of fractions were run on a 1D SDS PAGE and silver stained (Figure 4.12). It was evident that the protein bands were distinguishable; however the protein, particularly the WT + CA samples had to be concentrated to ensure identification.

Prior to concentration of the samples, an assortment of duplicate WT fractions with pIs ranging from 5.57-8.94 were run on a 1D SDS PAGE and four distinct band excised and sent off for identification to determine whether or not they were membrane proteins (Figure 4.13).

Three proteins were identified Hsc82p (heat shock protein), Eno2p (enolase II), and Adh1p (alcohol dehydrogenase) (Figure 4.13). All three are located solely in the cytoplasm with the exception of Hsc82p which can also be found in mitochondrion. This was not an encouraging result; however contaminants were to be expected using the crude microsomal preparation method. One theory is that membrane proteins may be less abundant than the contaminants and only identifiable following concentration of the fractions.

A duplicate set of 15 fractions 44-58 from WT samples, were pulled (600µl each) and concentrated using a protein concentration kit (Calbiochem, Merck Chemicals Ltd., Nottingham, UK). The samples were further concentrated using acetone precipitation

twice, which removed the high salt content caused by the concentration kit. The protein was concentrated 10-fold. Concentrated duplicate protein samples at varying concentrations were run on 1D SDS PAGE. Ten bands were excised and sent off for identification (Figure 4.14).

Only 4 proteins were identified and were all identified as being ribosomal: Rpl2Ap, Rpl3p, Rpl4Ap, and Rpl5p. The other six bands were not identified due to low protein concentration. The presence of ribosomal protein contaminants in membrane preparations has been observed previously and is a problem encountered regularly (Navarre *et al.*, 2002). This does not necessarily mean that the membrane protein fractionation was unsuccessful; it may mean higher protein levels are required for membrane protein identification.

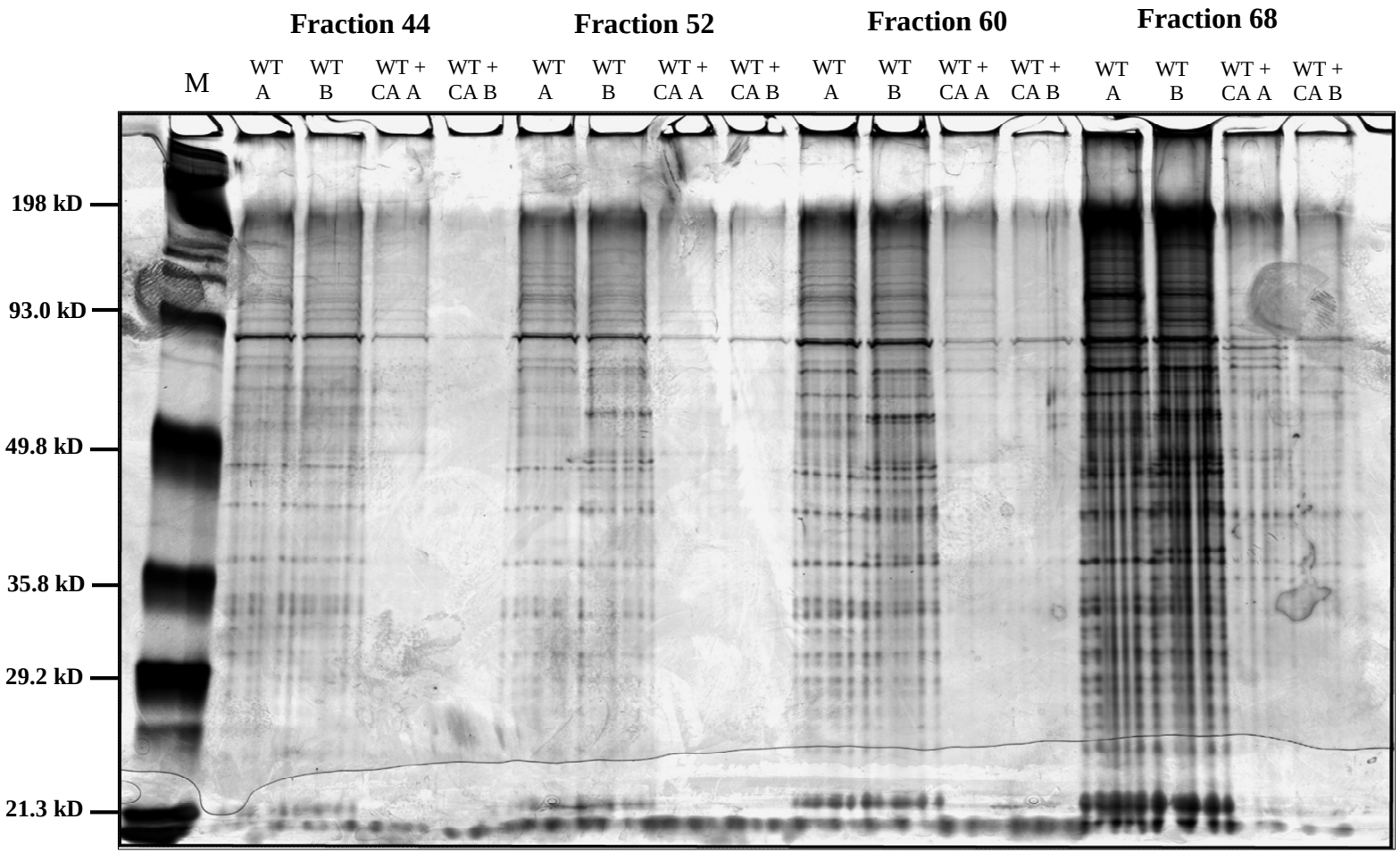


Figure 4.12: Silver stained 1D SDS PAGE of fraction no 44, 52, 60 and 68 from duplicate sets of WT and WT + 300 mM citric acid fractionated on the TECAN FFE unit. Visualisation of the microsomal protein fractions showed distinguishable banding patterns for each sample. However, the WT + 300 mM citric acid fractions had a considerably lower protein concentration.

Table 4.3: Identification of *S. cerevisiae* proteins separated by FFE and SDS-PAGE:

Spot No.	% matched Peptides [a]	% matched peptide coverage [b]	Molecular Mass		pI		Top ranking protein in MS-Fit search [d]	SwissProt Accession No.
			(kDa) Exp	(kDa) Pred [c]	Exp	Pred [c]		
1	40	32	93.0	80.8	7.3	4.76	Hsc82 (cytoplasmic chaperone of the Hsp90 family)	P15108
2	16	32	47.0	46.8	8.4	5.67	Eno2 (enolase 2)	P00925
3	15	27	43.0	40.0	9.0	10.64	Adh1 (alcohol dehydrogenase 1)	P00330

[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 peptide divided by the total number of amino acids in the predicted protein sequence expressed as a percentage.
[c] Theoretical pI and molecular weights obtained from the protein entry in the SGD database (URL: http://www.yeastgenome.org).
[d] The program MS-Fit (URL: http://prospector.ucsf.edu/uscfhtml4.0/msfithtm) was used to search the SwissProt database for proteins with calculated tryptic peptide masses that matched the measured experimentally derived masses (mass accuracy +/- 0.15 Da).

Table 4.4: Identification of *S. cerevisiae* proteins separated by FFE and SDS-PAGE:

Spot No.	% matched peptides [a]	% matched peptide coverage [b]	Molecular Mass		pI		Top ranking protein in MS-Fit search [d]	SwissProt Accession No.
			(kDa) Exp	(kDa) Pred [c]	Exp	Pred [c]		
5	29	23	43.0	39.1	6.30	10.64	Rpl4A (60s ribosomal protein L4-A)	P10664
7	18	27	38.0	27.4	6.30	11.10	Rpl2A (60s ribosomal protein L2-A)	P05736
8	37	36	33.0	33.7	6.30	6.36	Rpl5 (60s ribosomal protein L5)	P26321
9	17	32	32.0	43.8	6.30	10.29	Rpl3 (60s ribosomal protein L3)	P14126

[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 peptide divided by the total number of amino acids in the predicted protein sequence expressed as a percentage.
[c] Theoretical pI and molecular weights obtained from the protein entry in the SGD database (URL: http://www.yeastgenome.org).
[d] The program MS-Fit (URL: http://prospector.ucsf.edu/uscfhtml4.0/msfithtm) was used to search the SwissProt database for proteins with calculated tryptic peptide masses that matched the measured experimentally derived masses (mass accuracy +/- 0.15 Da).

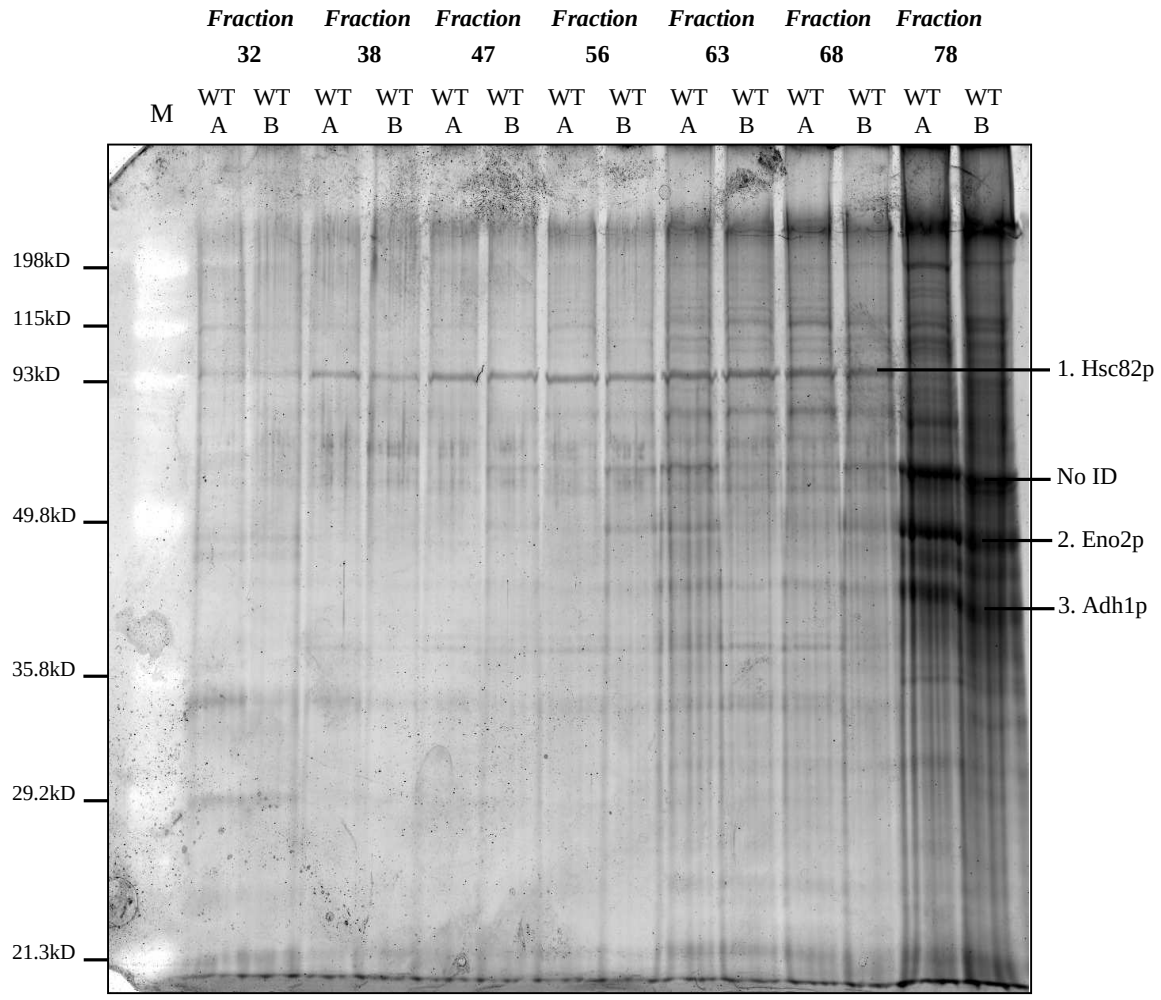


Figure 4.13: Identification of protein bands excised from SDS PAGE of fractions 32, 38, 47, 56, 63, 68 and 78 from duplicate sets of WT samples fractionated on TECAN FFE. The SDS-PAGE gel was stained with Sypro Ruby and four protein bands were excised and sent off for identification using Mass Spectrometry. Three proteins were identified; Hsc82p, Eno2p and Adh1p.

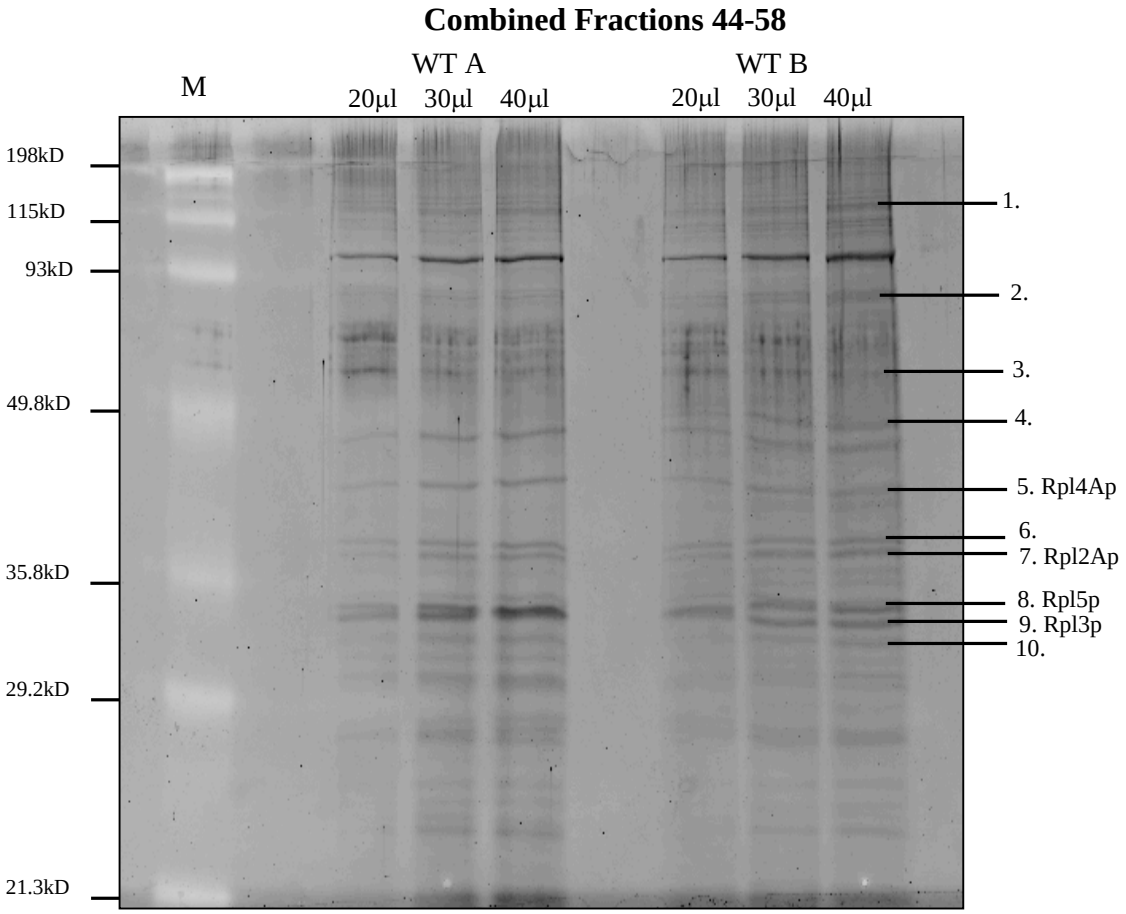


Figure 4.14: Identification of protein bands excised from SDS-PAGE of WT microsomal protein samples fractionated on TECAN FFE. Fractions 44-58 from duplicate sets of WT microsomal protein samples were combined and concentrated 10-fold and separated by SDS-PAGE. The gel was stained with Sypro Ruby and 10 bands were excised and sent for identification. Only four protein bands were identified; RplAp, Rpl2Ap, Rpl5p and Rpl3p.

4.3.3. Repeat experimentation for FFE and SDS-PAGE protein separation using higher input protein concentration.

Free flow elecrophoresis (FFE) was performed for a second time, this time with 10-fold more protein sample loaded onto the FFE unit (60 mg). The OD_{280} and pH of each fraction were recorded as before and plotted graphically for comparison (Figure 4.15). The OD_{280} and pI readings for both WT and WT in the presence of 300 mM citric acid were reproducible once more, with the exception of the WT + 300 mM citric acid fractions 13-26. Precipitation of protein during the run caused abnormal fractionation and readings for these fractions. To determine whether the protein was concentrated enough and whether the banding patterns were still distinguishable; 1D SDS PAGE of every second WT fraction was run (Figure 4.16).

The SDS-PAGE (Figure 4.16) showed the fractions to contain high protein concentration, suggesting protein identification would be more successful. The distinction between bands was less clear with the higher protein concentration and may prove difficult for sample comparison. The banding pattern across the fractions seemed very similar with the strongest bands being present in each fraction.

Due to time constraints and high expense in running the Pro Team™ FFE unit, this study was abandoned to allow trouble shooting and optimization of the Pro Team™ FFE unit's operation.

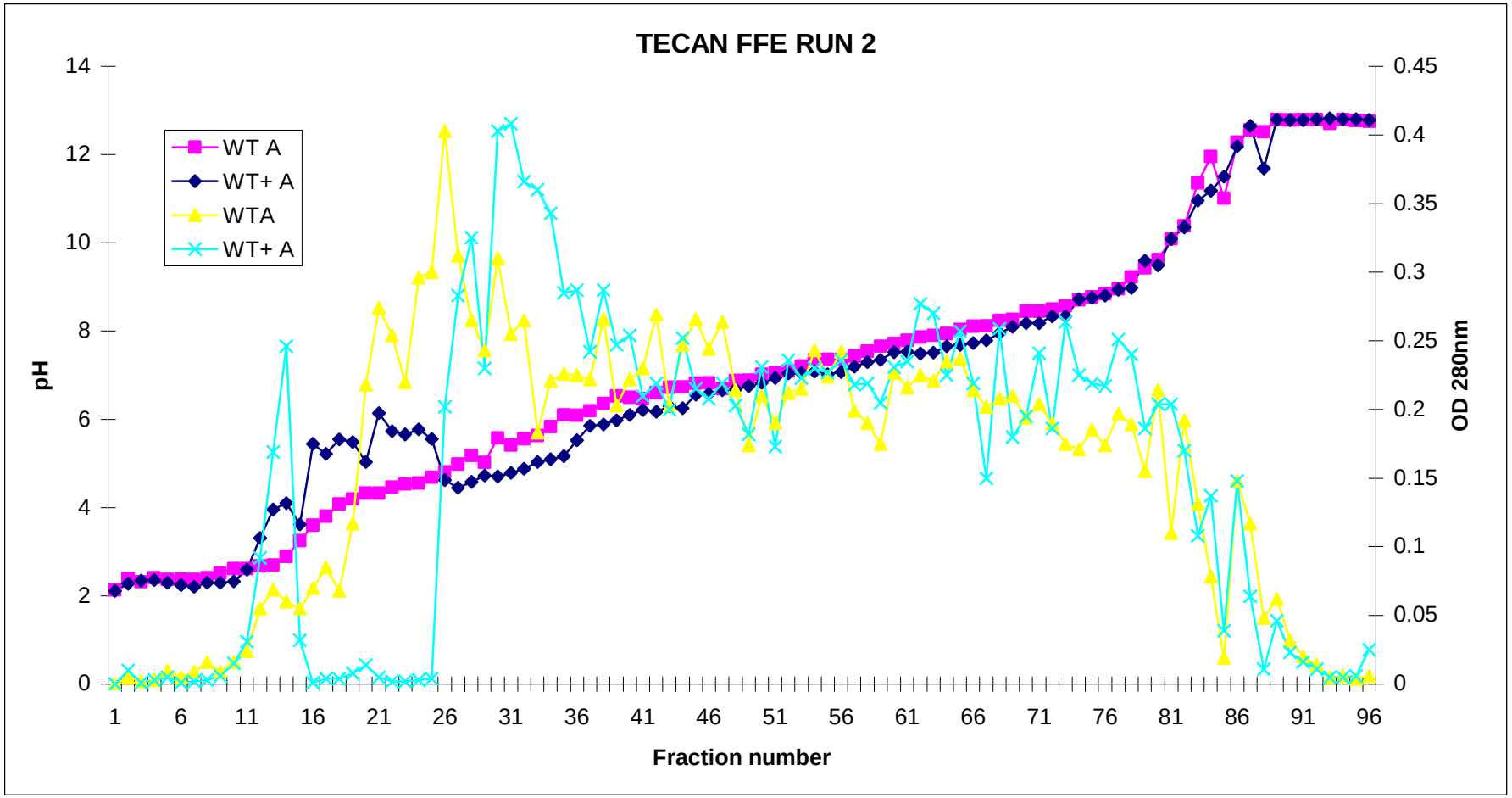


Figure 4.15: OD_{280} and pI data for microsomal protein preparation from WT and WT in the presence of 300 mM citric acid, separated by free-flow electrophoresis (Run 2). The Pro Team™ FFE unit was loaded with 60 mg of microsomal protein preparation from WT and WT + 300 mM citric acid, where the proteins samples were separated into 96 fractions using free-flow electrophoresis. OD_{280} and pI data for each fraction and duplicate samples has been shown and a high level of reproducibility between samples was evident. Slight precipitation of protein caused fractions 13-26 to have abnormal pH and OD_{280} reading in the WT + 300 mM citric acid sample. (OD_{280} readings were taken for 1:10 dilution of sample).

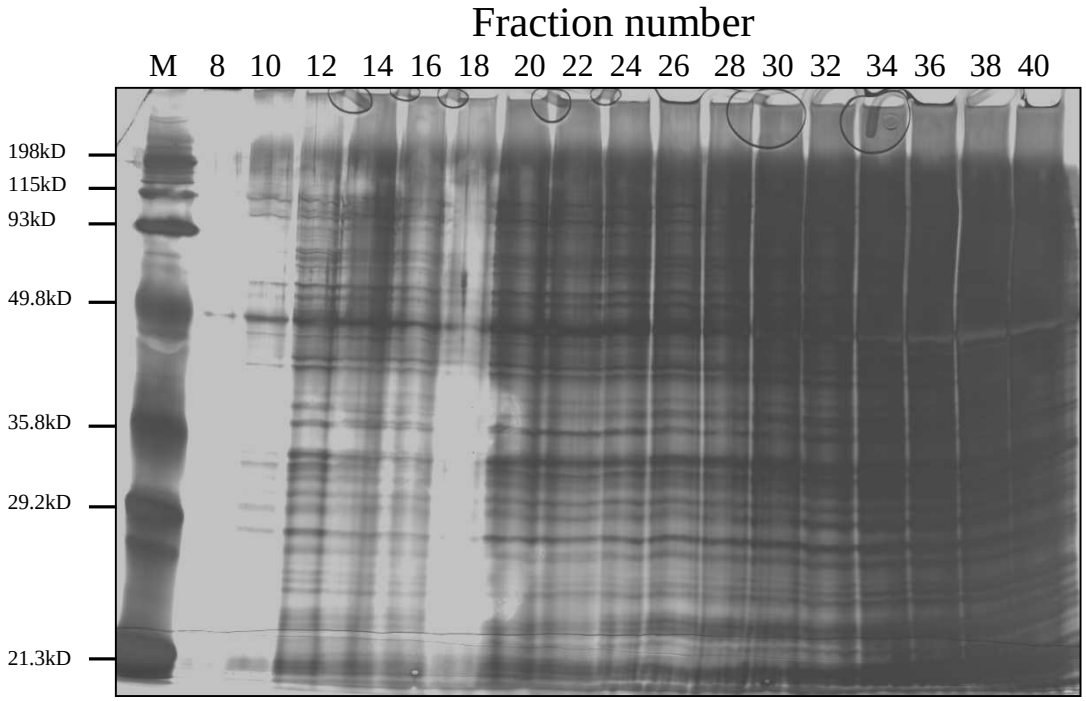


Figure 4.16: Silver stained 1D SDS PAGE of WT sample (2nd run) fractionated on TECAN FFE. Every second protein fraction from 8-40 of the WT sample is shown. The fractions obtained from FFE were a result of 60mg microsomal protein loaded onto the unit (10-fold greater than 1st run). Banding pattern difficult to distinguish and appears identical across the fractions.

4.4. Phosphoproteome analysis of WT

4.4.1. Citric acid treatment results in changes in the yeast phosphoproteome of WT strain.

In this study an affinity purification column developed by QIAGEN (PhosphoProtein Purification Kit, QIAGEN Ltd., West Sussex, UK) and optimized for the use with yeast (Makrantoni *et al.*, 2005) to enable capture of yeast phosphoproteins, was couple with 2-D PAGE to allow phosphoproteomic analysis for BY4741a in response to citric acid stress.

The 2-D phosphoprotein gels identified nine proteins with changes in phosphorylation state. Seven putative phosphoproteins showed increased phosphorylation in response to citric acid. Eno1p and Eno2p displayed citric acid-induced phosphorylation, as did the heat shock protein Ssz1p, the ribosomal protein Rpp0p, and the translation elongation factor Yef3p (Figure 4.17). Two proteins exhibited reduced phosphorylation in response to citric acid exposure; Pup1p, a putative 20 S proteasome subunit, and Lsp1p, a protein kinase inhibitor (Figure 4.18).

All of these proteins with the exception of Pup1p had database (SwissProt: http://us.expasy.org/sprot/), and/or published literature evidence, indicating phosphorylation, or ATP binding activity (Table 4.6).

Comparison of the experimental to predicted molecular weight, pI and percentage of peptide coverage of the identified proteins can be found in Table 4.5.

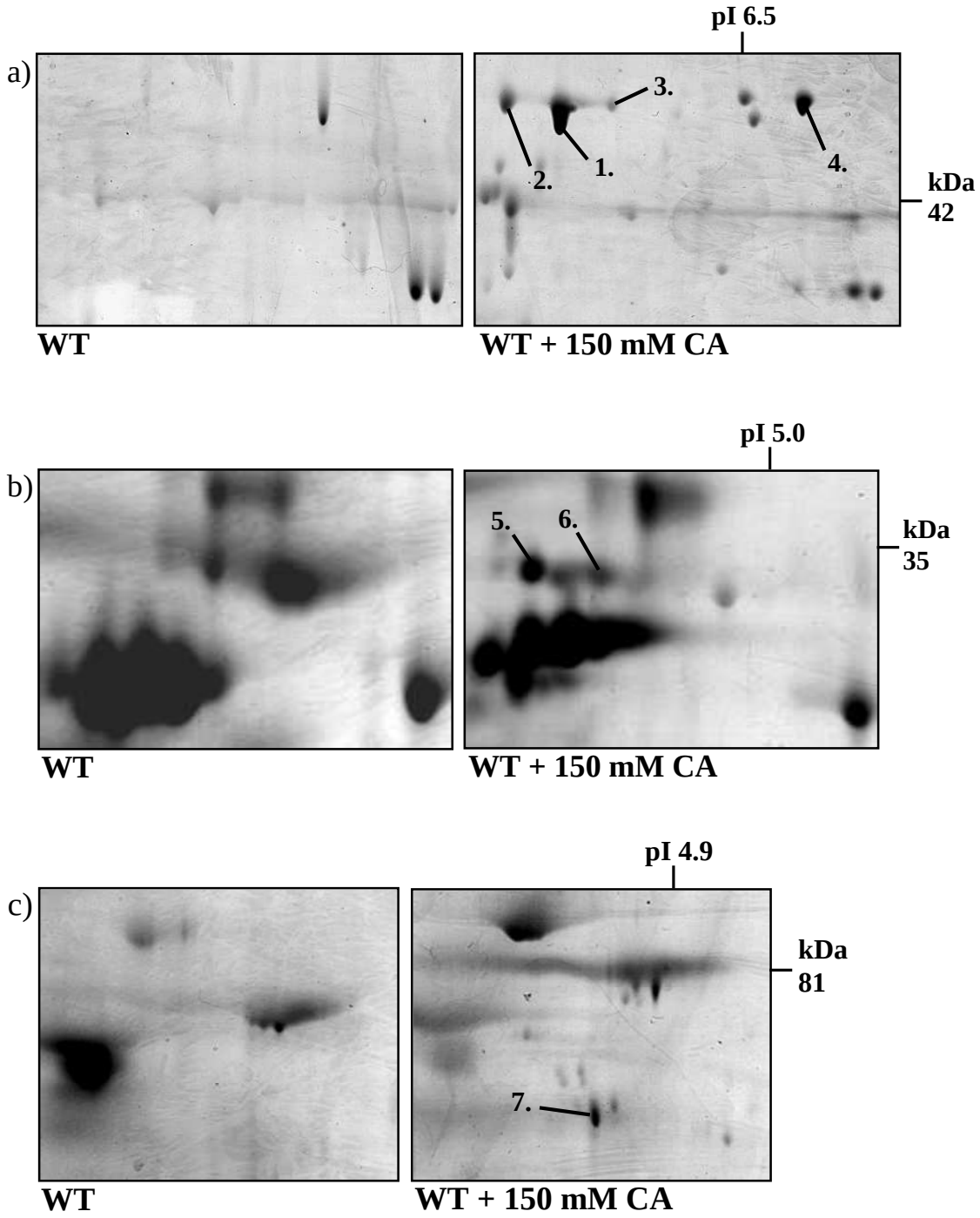


Figure 4.17: Detection of up- and down-regulated proteins from pI 4-7 2DE phosphoproteome gels for WT with and without 150 mM citric acid (CA). Proteins 1, 2, 3, 4, 5, 6 and 7 were citric acid-induced and identified via Mass Spectrometry: a) protein 1, 2, & 3: Eno2p, protein 4: Eno1p b) protein 5: Ssz1p, protein 6: Rpp0p c) protein 7: Yef3p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.5).

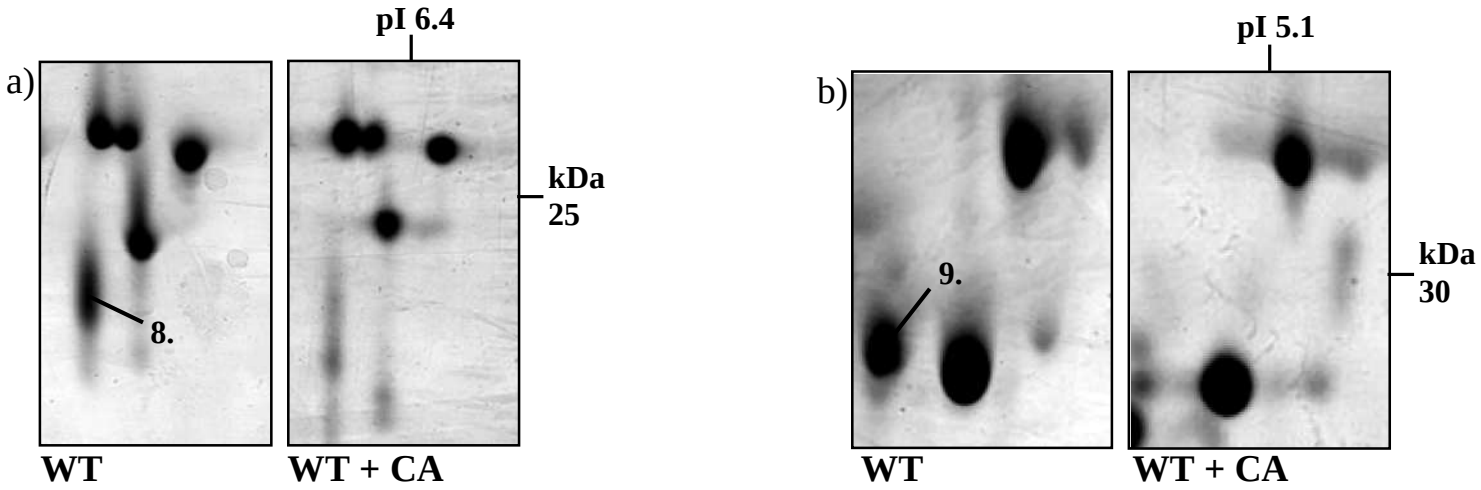


Figure 4.18: Detection of up- and down-regulated proteins from pI 4-7 2DE phosphoproteome gels for WT with and without 150 mM citric acid (CA). Proteins 8 and 9 were down regulated under citric acid treatment and identified via Mass Spectrometry: a) protein 8: Pup1p, b) protein 9: Lsp1p. Molecular masses (in kilodaltons) are shown on the *y* axis and pI values are shown on the *x* axis. A representative result of two replicate experiments is shown (Mass spectrometry data can be found in Table 4.5).

Table 4.5: Identification of *S. cerevisiae* phosphoproteins by Mass Spectrometry.

Spot No.	% matched peptides [a]	% matched peptide coverage [b]	Molecular Mass		pI		Top ranking protein in MS-Fit search [d]	SwissProt Accession No.
			(kDa) Exp	(kDa) Pred [c]	Exp	Pred [c]		
1	22	14	47.5	46.8	5.90	5.67	Eno2 (enolase 2)	P00925
2	16	26	48.0	46.8	5.80	5.67	Eno2 (enolase 2)	P00925
3	9	28	48.0	46.8	6.10	5.67	Eno2 (enolase 2)	P00925
4	15	32	49.0	46.7	6.60	6.60	Eno1 (enolase 1)	P00924
5	27	44	34.0 (frg)	62.2	4.60	5.10	Ssz1 (heat shock protein 70)	P38788
6	30	33	34.0	33.7	4.70	4.75	Rpp0 (conserved ribosomal protein PO)	P05317
7	24	17	55.5 (frg)	116.8	4.80	5.95	Yef3 (translational elongation factor eEF3)	P16521
8	29	38	22.0	28.5	6.30	6.60	Pup1 (putative proteasome subunit)	P25043
9	27	41	28.0	38.0	4.90	4.44	Lsp1 (protein kinase inhibitor)	Q12230

[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 peptide divided by the total number of amino acids in the predicted protein sequence expressed as a percentage.

[c] Theoretical pI and molecular weights obtained from the protein entry in the SGD database (URL: http://www.yeastgenome.org).

[d] The program MS-Fit (URL: http://prospector.ucsf.edu/uscfhtml4.0/msfithtm) was used to search the SwissProt database for proteins with calculated tryptic peptide masses that matched the measured experimentally derived masses (mass accuracy +/- 0.15 Da).

(frg) Indicates that the protein detected was a proteoytic cleavage fragment.

Table 4.6: Evidence for protein phosphorylation

Spot No.	Protein [a]	Functional evidence [b]	Database [c]	Reference [d]
1 2 3	Eno2p	Converts 2-phospho-D-glycerate to phosphoenol pyruvate + H_2O	SwissProt	(Ficarro *et al.*, 2002) (Gruhler *et al.*, 2005) (Albuquerque *et al.*, 2008)
4	Eno1p	Converts 2-phospho-D-glycerate to phosphoenol pyruvate + H_2O	SwissProt	(Ficarro *et al.*, 2002) (Gruhler *et al.*, 2005) (Albuquerque *et al.*, 2008)
5	Ssz1p	ATP binding/Phosphorylation	SwissProt	(Huang *et al.*, 2005) (Li *et al.*, 2007) (Chi *et al.*, 2007)
6	Rpp0p	Phosphorylation	SwissProt	(Rodriguez-Gabriel *et al.*, 1998) (Li *et al.*, 2007) (Albuquerque *et al.*, 2008)
7	Yef3p	ATP binding/phosphorylation	SwissProt	(Futcher *et al.*, 1999) (Ficarro *et al.*, 2002) (Albuquerque *et al.*, 2008)
9	Lsp1p	Phosphorylation	SwissProt	(Zhang *et al.*, 2004) (Smolka *et al.*, 2007) (Albuquerque *et al.*, 2008)

[a] Protein names according to the SGD database (URL: http://www.yeastgenome.org).
[b] Functional evidence was obtained from the protein entry in the SGD database (URL: http://www.yeastgenome.org), or the MIPS database (URL: http://mips.gsf.de.genre/proj/yeast/index.jsp).
[c] Database entry of phosphorylation: SwissProt (http://us.expasy.org/sprot/).
[d] References indicating previous studies on phosphorylation of the protein listed.

4.5. Cell wall integrity pathway spotting

Lsp1p was identified in the phosphoproteomic screen (Section 4.4), and has been characterized as having protein kinase inhibitor activity and is known to negatively regulate the protein kinase Pkh1p and the down stream signaling pathways Pck1p and Ypk1p, both of which are involved in cell wall integrity (SGD, Zhang *et al.*, 2004).

Following the identification of this protein kinase inhibitor and knowing its involvement in cell wall integrity (CWI), we decided to undertake a 'spotting' screen to test Δ*lsp1* and deletion strains encoding proteins shown to interact with Lsp1p for changes in growth upon exposure to citric acid. With evidence of reduced phosphorylation of Lsp1p upon exposure to citric acid, we wanted to determine how growth would be affected in Δ*lsp1* in citric acid stressed conditions. We also tested various deletion strains encoding proteins known to be involved in the cell wall integrity pathway for sensitivity to citric acid to determine whether expression of any of theses genes were important for optimum growth, and also whether the cell wall integrity pathway could be involved in citric acid adaptation.

The growth of 19 deletion strains (including Δ*lsp1*, genes encoding proteins known to interact with Lsp1p and other genes involved in CWI pathway) under increasing citric acid stress conditions was assayed by spotting serial dilutions onto MB, pH 3.5 with citric acid concentrations ranging from 0 mM – 400 mM (Figure 4.19)

Deletion of *LSP1* did not alter the strain's growth. Such that it exhibited similar growth as that of the parent strain BY4741a with citric acid treatment. The deletion strains Δ*rom2* and Δ*ypl150w* exhibited slightly resistant phenotypes under citric acid stress compared to BY4741a. The deletion of two genes *APP1* and *VPS64*, who encode proteins of 'unknown function', both proved to be sensitive with increasing citric acid concentrations relative to that of the parent strain. Finally, deletion strains Δ*bck1* and Δ*slt2* exhibited very poor growth compared to the parent in each spotting assay including 0 mM citric acid (Figure 4.19).

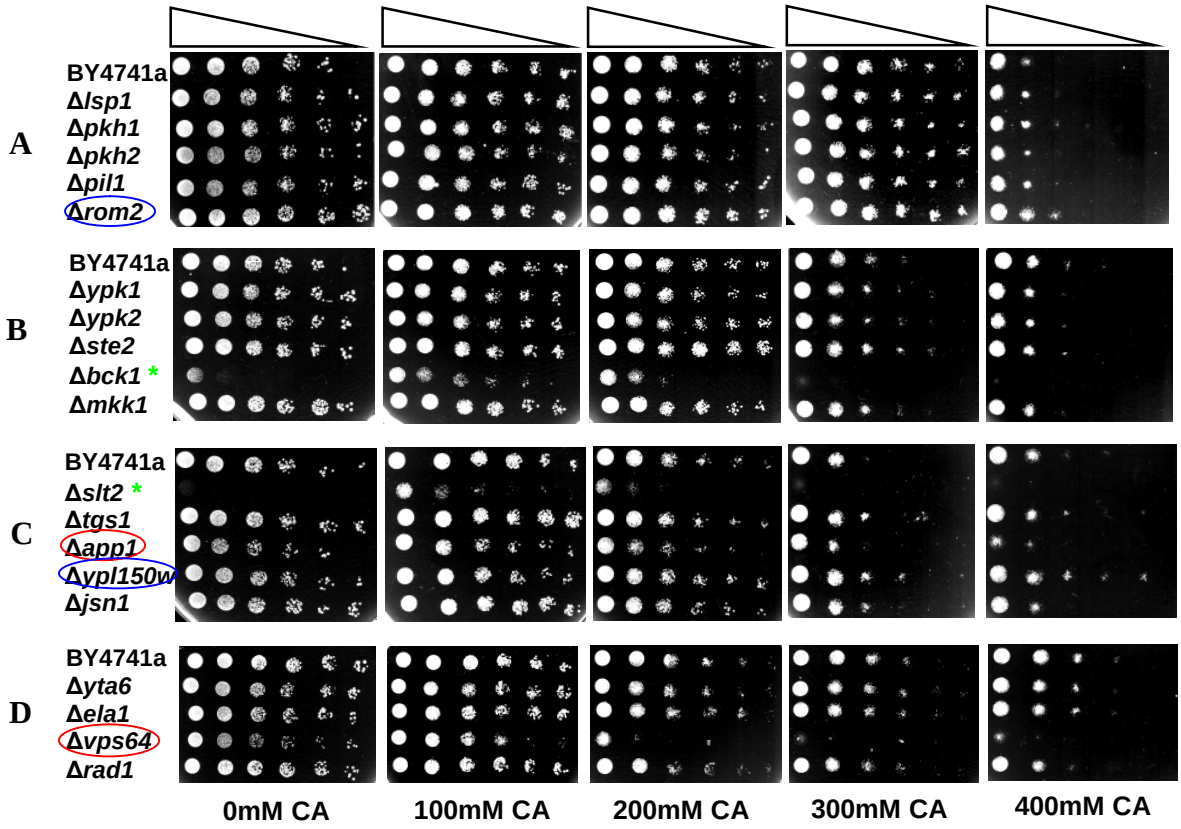


Figure 4.19: Growth of Cell Wall Integrity pathway genes and Lsp1p interacting proteins' gene deletions under citric acid stress. Serial dilutions of BY4741a (control) and various deletion strains were spotted onto MB agar plates at pH 3.5 with and without the presence of 100, 200, 300, and 400 mM citric acid. Plates were incubated for 48 h at 30°C before plates were photographed. Deletion strains circled in red denote those showing a sensitive phenotype and those circled in blue; a resistant phenotype. The deletion strains asterixed with a green star denote strains which are pH sensitive.

4.6. Discussion

4.6.1. Proteomic analysis

In this study we have carried out 2D-PAGE analysis of changes in the proteome in wild type and in Δ*sky1* during growth in the presence of citric acid over pI range 4-7. Gels of the wild type were primarily used as a control for the Δ*sky1* analysis since proteomic analysis of WT in the presence of citric acid has been studied previously (Lawrence *et al.*, 2004). Many of the changes in protein expression found in our study are consistent with Lawrence's previous analysis. Upregulation in expression levels of Rhr2p, Ssa1p, Pdc1p, and Eno2p were detected in wild type cells following exposure to citric acid in both this study and Lawrence's. Additionally, Ssz1p, Ssb1, Ccl1p and Rps0Bp were detected to be upregulated following citric acid stress in WT cells.

Many of the proteins identified as being upregulated in response to citric acid were heat shock proteins (HSP) or others known to be involved in stress response. The heat shock proteins Ssa1p, Ssb1p and Ssz1p which were upregulated upon exposure to citric acid in the wild type, are known to be induced upon exposure to a range of stress factors such as osmostress, heat shock, exposure to H_2O_2 and low pH (de Nobel *et al.*, 2001). Their up-regulation could be explained by either; an increased damage and aggregation of intracellular proteins; or due to an inhibitory effect of citric acid on protein synthesis and turnover leading to increased requirement for their chaperone function (Lawrence *et al.*, 2004; de Nobel *et al.*, 2001). None of these heat shock proteins were up-regulated in response to citric acid in Δ*sky1* suggesting *SKY1* positively regulates the expression of these proteins vital for the response to citric acid stress.

Upregulation of Rhr2p, a glycerol-3-phosphatase (alias Gpp1p), was identified in response to the presence of citric acid in both wild type and Δ*sky1* proteomes. This protein is required for glycerol biosynthesis and is known to be involved in the cellular response to various stresses including; osmotic, anerobic, and oxidative stress (Pahlman *et al.,* 2001). Rhr2p induction under hyperosmotic stress is partially dependent on the HOG pathway but is also shown to be under strong influence of protein kinase A (PKA) activity, also involved in cell stress response (Pahlman *et al.,* 2001). Rhr2p has also previously been shown to be upregulated in response to citric acid in wild type cells (Lawrence *et al.,* 2004). At high external osmolarity, yeast cells compensate for the loss of water by increased glycerol production, this process also seems to be induced in response to citric acid stress.

A number of proteins involved in glycolysis were upregulated in response to citric acid; Eno2p in wild type cells; and Eno1p, Gpm1p, and Tdh1p in Δ*sky1* cells. Tdh1p has been implicated as a stress protein, known to be expressed upon osmotic stress, and more recently sorbic acid stress (de Nobel *et al.*, 2001). The upregulation of Eno2p in response to citric acid stress is also consistent with previous proteomic analyses (Lawrence *et al.*, 2004). The enhanced expression of such proteins involved in glycolysis, supports previous observations which stress's the importance of energy-generating metabolism for weak acid adaptation (Piper *et al.,* 1997). Changes in the expression of enzymes within the glycolytic and connecting pathways serve for the production of stress protectants, compatible solutes, and reserves such as glycerol and trehalose (Hohmann, 2002). The upregulation of Eno1p, Gpm1p and Tdh1p in Δ*sky1* cells in response to citric acid exposure suggests that during citric acid stress, *SKY1* negatively regulates their expression.

Two further proteins classified as being involved in *S. cerevisiae* cellular stress response; Ald3p and Gnd1p, were upregulated upon citric acid exposure in Δ*sky1* cells, suggesting that they are also negatively regulated by *SKY1* during citric acid stress. Expression of Ald3p is positively regulated by Msn2p and Msn4p in response to various different stresses including heat shock, oxidative stress, DNA damage and sorbic acid stress (Navarro-Avino *et al.,* 1999; de Nobel *et al.,* 2001; Schuller *et al.,* 2004). Gnd1p has an important role in protecting yeast from oxidative stress (Izawa *et al.,* 1998) and Δ*gnd1* exhibits sorbic acid sensitivity (Mollapour *et al.,* 2004).

A subunit of the vacuolar membrane H^+-ATPase, Vma4p has previously been found to be upregulated in response to citric acid stress (Lawrence *et al.,* 2004). The wild type proteome in this study does exhibit slight upregulation of Vma4p but more interestingly the Δ*sky1* proteome shows Vma4p to be downregulated with loss of *SKY1* but upregulation in Δ*sky1* upon exposure to citric acid. This suggests that *SKY1* is involved in Vma4p's basal expression and also that Vma4p is important for citric acid adaptation. Previous studies have shown that deletion of *VMA4* exhibits hypersensitivity to various acids including sorbic acid, lactic acid, acetic acid and hydrochloric acid suggesting a general acid stress response (Mollapour *et al.,* 2004; Kawahata *et al.,* 2006). Although the upregulation of Vma4p in response to citric acid may be a result of a general acid stress response, this study also provides evidence that Vma4p expression is regulated by Sky1p.

Tma19p, a protein of unknown function, but which is known to associate with ribosomes (Fleischer *et al.,* 2006), was found to be repressed by deletion of *SKY1*. Tma19p is also involved in oxidative stress response (Rinnerthaler *et al.,* 2006). This suggests that *SKY1*

positively regulates the expression of this protein. A number of proteins involved in protein synthesis including ribosomal proteins and the translation initiation factor Tef2p exhibited changes in expression in response to citric acid stress. Rps0Bp was upregulated in wild type cells in response to citric acid. The Δ*sky1* proteomes also showed upregulation of Rps0Bp resulting solely from deletion of *SKY1* and further upregulation of expression in response to citric acid stress in Δ*sky1* cells. Rps5p a component of the 40s ribosomal unit was downregulated in response to citric acid in Δ*sky1* cells suggesting *SKY1* positively regulates Rps5p expression in response to citric acid stress. Various studies have observed changes in ribosomal protein expression in response to a range of stresses; osmotic stress has been shown to inhibit the expression of many ribosomal protein genes (Varela *et al.,* 1992; Gasch *et al.,* 2000). Generally genome-wide expression analyses have shown coordinate repression of ribosomal protein genes as the common response to all environmental stresses (Marion *et al.,* 2004). Contradictory to this observation, our proteomes provide evidence that some ribosomal proteins are upregulated in response to citric acid stress, suggesting that their increased expression may be important to citric acid adaptation.

A comparative analysis of the effect of citric acid on protein expression in wild-type cells and Δ*hog1* cells was previously carried out for pI 4-7 (Lawrence *et al.,* 2004). Our study's comparative analysis of the wild type and Δ*sky1* proteomes also compared the proteomes of Δ*hog1*, highlighting similarities in protein expression between the two protein kinase deletion strains and also giving further proteomic analysis of Δ*hog1* over pI 6-11.

Three similarities between the proteomes of Δ*sky1* and Δ*hog1* in comparison to the wild type were highlighted. Upon exposure to citric acid Pdb1p and Car1p were upregulated in both Δ*sky1* and Δ*hog1* proteomes with no change in expression of either protein in the wild type proteome. Eno2p was found to be upregulated upon citric acid exposure in the wild type proteome but no change in Eno2p expression was exhibited in either the Δ*sky1* or the Δ*hog1* proteomes. Not only do these results suggest Sky1p and Hog1p; negatively regulate Pdb1p and Car1p; and positively regulate Eno2p, but they also suggest some functional redundancy between Sky1p and Hog1p in their roles in citric acid adaptation.

Tef2p, a translation initiation factor, was also found to be upregulated in both Δ*sky1* and Δ*hog1* proteomes in response to citric acid exposure, but from different spots in the proteomes. In mammalian cells, phosphorylation of Tef2p results in an inhibition of protein synthesis. Studies have since linked Hog1p, and another protein kinase Rck2p, in the transient downregulation of protein synthesis in response to osmotic stress in *S. cerevisiae* (Teige *et al.*, 2001). The upregulation of Tef2p in both Δ*sky1* and Δ*hog1* in response to citric acid suggest that Tef2p is negatively regulated by Sky1p and Hog1p in response to citric acid and suggests further functional overlap in their adaptation to citric acid stress.

More recently the trend in studies looking at the effect of stresses on organisms have centered on the used of microarray techniques rather than using proteomic tools such as 2-D SDS PAGE. Comparitive studies between the proteomic studies in this chapter and microarray studies on the effects of sorbic acid, benzoic acid and acetic acid stress in *S. cerevisiae* have shown little correlation (Mollapour *et al*., 2004; de Nobel *et al.*, 2001; Schuller *et al.*, 2004; Abbott *et al*., 2007). This is confirmed in studies by de Nobel (2001)

who carried out a parallel and comparative analysis of both the proteome and transcriptome of sorbic acid-stressed *S. cerevisiae*. Only one of the protein changes detected in their study, the upregulation of Hsp26p, was matched by a corresponding change in transcription of *HSP26*. The detection of proteins induced by weak acid stresses in proteomic, but not transcription analyses could be explaned by increased translational rates, increased stability or a decrease in protein denaturation (de Nobel *et al*., 2001). In summary it seems that simultaneous analysis of changes at the level of the proteome and the transcriptome are necessary to provide an accurate evaluation of the changes incurred in *S. cerevisiae* under weak organic acid stress.

4.6.2. Utilization of FFE in the separation of membrane proteins

Classical 2-D PAGE has enabled the successful elucidation of the *S. cerevisiae* proteome and enabled visualization of the vast array of proteins up and downregulated in response to citric acid stress. However, the 2-D PAGE method is limited in so far as highly basic and hydrophobic proteins are concerned. Proteomic analysis of highly hydrophobic proteins such as membrane proteins using classical 2-D PAGE is problematic. The 2-DE lysis buffers used have a lower capacity to extract membrane proteins from their lipid environment and keep them solubilized in aqueous conditions so precipitation of hydrophobic proteins can occur. During the IEF stage proteins are least soluble at their pI, so the hydrophobicity of the membrane proteins means they tend to aggregate. The transfer from the (hydrophobic) gel matrices of the IPG strips to the SDS gels is also hindered by the hydrophobicity of membrane proteins (Braun *et al.*, 2007). Since around one third of all genes in organisms encode for membrane proteins it is imperative that new approaches be taken to enable proteomic analysis of membrane proteins (Wallin and von Heijne, 1998; Stevens and Arkin, 2000).

Various alternative techniques have been used to overcome the problems encountered by 'classical' 2-D PAGE to enable proteomic analysis of membrane proteins. The use of SDS/SDS-PAGE, 16-BAC/SDS-PAGE, CTAB/SDS-PAGE and more recently free flow electrophoresis in combination with SDS-PAGE (FFE-IEF/SDS) have enabled successful proteomic analysis of membranous samples (Braun *et al.*, 2007; Choi *et al.*, 2008).

The use of free-flow isoelectric focusing was initially introduced in 1964 by Hannig (Hannig *et al.*, 1964), but low resolution compared to gel-IEF systems meant it was

restricted to isolation of high molecular proteins (Weber *et al.,* 2004). The recent introduction of Prolytes, new detergents and the commercially available Pro Team™ FFE equipment has resulted in novel applications of free-flow isoelectric focusing including the fractionation of membrane proteins (Zischka *et al.,* 2003; Choi *et al.,* 2008).

In this study we proposed to use a combination of FFE-IEF and SDS-PAGE to analyze changes in membrane protein expression from *S. cerevisiae* microsomal extracts in response to citric acid stress. The microsomal protein samples from duplicate wild type and duplicate wild type + 300 mM citric acid cultures were obtained by differential centrifugation and fractionated using the Pro Team™ FFE system (Tecan). Initial results were promising when pI and OD_{280} readings for each fraction were plotted. All four samples showed a high level reproducibility both with pI and protein concentration across the fractions. Upon running selected fractions on SDS-PAGE it was apparent that the levels of protein from the citric acid treated sample (WT + 300 mM CA) were much lower than that of the untreated (WT). This could be due to an inaccuracy in protein sample concentration loaded onto the FFE system or could be due to the effect of citric acid on protein expression. The ability to distinguish protein band on the SDS-PAGE was also encouraging. The identification of three proteins Hsc82p, Eno2p and Adh1p excised from the SDS-PAGE of untreated cells, was disconcerting since they are all cytosolic proteins. This confirmed that the crude microsomal protein sample fractionated was contaminated with proteins from the cytosol. This did not necessarily mean that membrane proteins weren't present in the fractions; perhaps the contaminants were more abundant. A range of fractions were combined (44-58) and concentrated 10-fold in an attempt to concentrate any less abundant membrane proteins present in the fractions. The subsequent proteins identified from the combined and concentrated fractions were all

ribosomal; Rp14Ap, Rpl2Ap; Rpl5p and Rpl3p. Contaminants are common place when using crude protein preparations. The presence of ribosomal protein contaminants in membrane preparations has been observed previously and is a problem encountered regularly (Navarre *et al.*, 2002). One theory for the absence of any membrane proteins could be that during protein concentration, membrane proteins were lost through precipitation or that they could not be eluted from the column. However, the column-Protein Concentrate Kit was specifically used to prevent this.

The second run of the FFE using 10-fold more microsomal protein sample produced reproducible pI and OD_{280} readings as before however some precipitation of the WT + 300 mM citric acid occurred. This may be due to the higher concentration of protein loaded onto the FFE system. The SDS-PAGE of fractions from the second run were highly concentrated, so much so that protein bands were difficult to distinguish between. Due to time constraints and high expense of running the Pro Team™ FFE unit the study had to be abandoned however, consequent studies and literature concerning the utilization of FFE have provided valuable directives in how this method can be executed successfully in the future. The identification of contaminants in the fractionated microsomal protein samples and failure to identify any membrane proteins suggests that the microsomal protein preparation has to be optimized. More refined membrane protein isolation methods such as sucrose or ficoll gradients may be the solution to the high levels of non-membranous protein contamination. This should also reduce the complexity of the banding patterns exhibited from the crude microsomal protein preparation. Failing that, more specific membrane protein preparations could be analysed. Proteomic analysis of *S. cerevisiae* mitochondrial proteins using FFE has previously been shown to be successful (Zischka *et al.,* 2003; Zischka *et al.,* 2006).

4.6.3. Phosphoproteomic analysis

The study of the reversible phosphorylation/dephosphorylation of proteins is of great interest as this common post-translational modification, plays a key role in many signal transduction, regulatory and metabolic pathways. Analysis of the yeast phosphoproteome seemed to be the next natural step in our study of the effect of citric acid exposure on *S. cerevisiae*.

Global analysis of the yeast phosphoproteome has been executed using a number of different approaches. The measurement of incorporated radiolabelled inorganic phosphate into proteins has been one approach (Futcher *et al.*, 1999). Whilst successful, concerns over working with radioactive isotopes have encouraged new approaches. New methods employing affinity capture to enrich phosphopeptides from the proteolysis of a whole-cell protein extract have successfully provided global analysis of the yeast phosphoproteome (Ficarro *et al.*, 2002). This study detected more than 1000 phosphopeptides and consequently resulted in the identification of 216 peptide sequences defining 383 phosphorylation sites; of which only 18 had previously been identified. Since then, a simple, more economic approach using; an affinity enrichment step to isolate intact phosphoproteins; 2-D electrophoresis to visualize; and mass spectrometry techniques to identify the phosphoproteins has been applied with high success (Metodiev *et al.*, 2004; Makrantoni *et al.*, 2005).

The method employed in this study to analyse changes in the phoshoproteome upon exposure to citric acid stress is the consistent with that used in the analysis of changes in the yeast phosphoproteome in response to sorbic acid (Makrantoni *et al.*, 2005). 2-D gel analysis for BY4741a (wild type) in the presence of citric acid revealed 5 putative

phosphoproteins reproducibly showing induced phosphorylation relative to the untreated wild type sample. Furthermore, growth in citric acid resulted in 2 proteins exhibiting reduced phosphorylation relative to the untreated wild type cells. This result could indicate two possible senarios; that either the expression of the phosphorylated protein increases without any increase in phosphorylation of the protein; or the phosphorylation state of the protein is increased without any increase in expression of the protein. The affinity column approach used cannot discriminate between these two possible senarios, or their possible combination but provides putative phosphoproteins for further study (Makrantoni *et al.,* 2005).

The five putative phosphoproteins showing induced phosphorylation in response to citric acid stress were Eno2p, Eno1p, Ssz1p, Rpp0p and Yef3p. Each of these proteins has previous publication evidence, indicating phosphorylation, and/or ATP binding activity. Eno1p, Rpp0p and Yef3p were identified using both the radiolabelled isotope method (Futcher *et al.,* 1999), and previously with the same affinity capture method used in this study (Makrantoni *et al.,* 2005). Furthermore, using the publicly available NetPhos V. 2.0 database (http://www.cbs.dtu.dk/services/NetPhos/), the sequences of the 5 induced proteins were analysed and each were predicted to contain phosphorylation sites on serine, threonine and tyrosine residues substantiating the possibility that their phosphorylation state is indeed altered upon citric acid stress. To determine whether the putative phosphoproteins altered phosphorylation state in response to citric acid was a true result rather than due to an increased protein expression, data presenting citric acid inducible genes/protein expression both from this study and previous studies (Lawrence *et al.,* 2004) were compared with the altered phosphoproteins identified, to determine if the studies showed any in common. The proteomic analysis (pI 4-7) of this study has shown

expression of Eno2p and Ssz1p to be up-regulated in response to citric acid. Eno2p has also shown to be upregulated in response to citric acid previously (Lawrence *et al.,* 2004). This suggests that the apparent induced phosphorylation of Eno2p and Ssz1p in citric acid treated cells may purely be due to an increased expression of the protein rather than increased phosphorylation. There is no previous evidence of citric acid-induced changes in the proteome or transcriptome for Eno1p, Rpp0p or Yef3p. Thus, the induced phosphorylation observed cannot be attributed to an increased protein expression but more likely that the proteins are displaying induced phosphorylation in response to citric acid stress. Eno1p like Eno2p is a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis (McAlister and Holland, 1982). The induced phosphorylation shown in citric acid treated cells by the phosphoproteomic analysis may be due to a changed state in phosphorylation or perhaps like Eno2p, there is an upregulation in protein expression in response to citric acid stress.

Previous studies have shown that Rpp0p, a ribosomal protein, is phosphorylated on serine 302. The results in their study indicated that Rpp0p phosphorylation was not required for the overall ribosomal activity, but suggested that it may effect the expression of specific proteins involved in metabolic processes such as osmoregulation (Rodriguez-Gabriel *et al.,* 1998). The induced phosphorylation of Rpp0p seen in this study upon exposure to citric acid stress suggests Rpp0p phosphorylation may affect protein expression specific to citric acid adaptation also.

The translation elongation factor Yef3p is specific to yeast and other higher fungi, other eukaryotes only possessing two translation elongation factors. It still remains unclear why

S. cerevisiae requires the function of Yef3p but it is essential for cell viability (Anand *et al.,* 2003). As afore mentioned Yef3p has previous evidence for phosphorylation (Futcher *et al.,* Makrantoni *et al.,* 2005). There is no previous evidence for changes in expression of Yef3p with citric acid stress. However, transcriptome analysis of *S. cerevisiae* in response to sorbic acid stress did exhibit a 1.4-fold repression of *YEF3* (de Nobel *et al.,* 2001). Although it is clear that different weak organic acids elicit a unique response from the cell, there are also some general responses expressed to all weak acids. If Yef3p is regulated by a general response to weak organic acids, then the repression of *YEF3* shown in the transcriptome would verify that the induced phosphorylation shown by the phosphoproteome is not due to any upregulated protein expression. However, the induced phosphorylation seen may also be a unique response specific to citric acid stress and further study of Yef3p could prove interesting.

The two proteins exhibiting reduced phosphorylation upon exposure to citric acid were Pup1p and Lsp1p. Lsp1p has previous evidence of phosphorylation (Zhang *et al.,* 2004; Smolka *et al.,* 2007; Albuquerque *et al.,* 2008), but Pup1p does not. However both, when analysed using NetPhos, were predicted to contain phosphorylation sites on serine, threonine and tyrosine residues suggesting that even though there is no previous evidence of Pup1p phosphorylation, the results of this study are plausible. Analysis of previous proteomic studies (Lawrence *et al.,* 2004) and data from the proteomes in this study showed that neither Pup1p nor Lsp1p has evidence of any changes in expression upon exposure to citric acid. This would imply that the reduced phosphorylation exhibited for both proteins was a direct effect of a citric acid stress response.

Pup1p is a component of the 26 S proteasome. Specifically it is one of seven β subunits which form two identical inner β-rings. These are flanked by two α-rings, and together compose the 20 S proteolytic core of the proteasome. Pup1/β2 is one of only three proteolytic active β-subunits (Jager *et al.*, 1999). Post-translational modifications such as phosphorylation have previously been suggested to affect the function of the proteasome especially in view of the fact that the 20 S proteasome has many potential Tyr and Ser/Thr phosphorylation sites. So far phosphorylation regions have only been confirmed in the α2, α4, and α7 subunits of the 20 S proteasome (Iwafune *et al.*, 2002). The 'reduced phosphorylation' of Pup1p in this study provides evidence that the β2- subunit is also a potential phosphoprotein. The proteasome plays an essential role in the response to stress conditions; degrading proteins of pathways necessary for normal growth but harmful under stress conditions; as well as proteins mis-folded or damaged by stress (Hilt and Wolf, 1992). Thus, the change in phosphorylation state of Pup1p, an essential component of the 20 S proteasome, under citric acid stress conditions is highly feasible.

Interestingly the other protein to exhibit reduced phosphorylation was Lsp1p, a protein with evidence of protein kinase inhibitory activity. The molecular function of Lsp1p was only fairly recently characterized following a large-scale analysis of protein complexes which showed Lsp1p and another related protein kinase inhibitor Pil1p in a complex with one of two protein kinases Pkh1p or Pkh2p (Ho *et al.*, 2002). This led to a study which implicated Lsp1p as a negative regulator of Pkh1p and the downstream signaling pathways Pkc1p and Ypk1p (Zhang *et al.*, 2004). This study looked specifically at the action of Lsp1p and Pil1p in response to heat stress and concluded that both proteins were negative regulators of heat stress resistance. The Pkc1p and Ypk1p signaling pathways involved in cell wall integrity have been implicated to be involved in response to stress;

Ypk1p in heat shock; and Pkc1p to various stresses including hypo-osmotic stress, heat shock, and oxidative stress (Levin, 2005). Deletion of *BCK1* which encodes Bck1p, a MAPKKK activated by Pck1p, has previously been shown to be sensitive to citric acid stress (Lawrence *et al.*, 2004). As a negative regulator of these pathways, reduced phosphorylation of Lsp1p, as shown in our study, suggests that citric acid stress results in the dephosphorylation of Lsp1p, consequently activating the Pck1p and/or Ypk1p pathways which upregulate various stress response proteins. Since its characterization as a protein kinase inhibitor in 2004, Lsp1p has been shown to co-localize with Pil1p in the 'eisosome', and further research has linked Lsp1p phosphorylation by Pkh1p to eisosome organization (Walther *et al.,* 2006; Luo *et al.,* 2008). Organisastion of the plasma membrane and endocytosis are influenced by eisosomes and the reduced phosphorylation of Lsp1p seen following citric acid stress suggests that potentially the composition of the plasma membrane may be altered in response (Walther *et al.,* 2007).

We have presented initial evidence that Lsp1p is dephosphorylated upon exposure to citric acid stress. With previous evidence identifying Lsp1p as a negative regulator of cell wall integrity pathway components Pck1p and Ypk1p, it seemed feasible to explore the possibility that the CWI pathway could be involved in citric acid adaptation.

4.6.4. Cell wall integrity pathway screened for citric acid sensitivity

Following, the detection of citric acid-induced dephosphorylation of Lsp1p and its connection to the cell wall integrity pathway we decided to test Δ*lsp1* and deletion strains of genes encoding proteins known to interact with Lsp1p, and those encoding other proteins involved in the CWI pathway, for altered growth upon exposure to citric acid. Δ*lsp1* did not exhibit any change compared to wild-type indicating that while citric acid stress may affect the protein's phosphorylation state, loss of Lsp1p is not essential for citric acid adaptation.

Simplified, the MAPK cascade for the CWI pathway consists of Pck1p, a MAPKKK (Bck1p), a pair of redundant MAPKK (MKK1/2p), and a MAPK (Slt2/Mpk1p) (Levin, 2005). Both Δ*bck1* and Δ*slt2* exhibited extremely poor growth upon citric acid exposure and in the control suggesting that the sensitive phenotype displayed was due to low pH and not citric acid sensitivity. Previous studies have shown that deletion of *BCK1* and *SLT2* result in a sensitive phenotype to low pH (Dudley *et al.*, 2005; Motizuki *et al.*, 2008). This does not necessarily mean that the expression of neither gene is involved in citric acid adaptation but makes testing it very difficult. Since the low pH level accompanying the presence of citric acid results in hampered growth of both deletion strains, it is impossible to differentiate between the pH effect and any effect the citric acid may have on Δ*bck1* and Δ*slt2*. It is interesting to note that recent studies have shown that the activation of a CWI response to cell wall damage caused by zymolyase, requires both the HOG and SLT2 pathways (Bermejo *et al.*, 2008). Their results described how these different MAPK pathways interact in novel ways to regulate stress in yeast. With previous evidence of the HOG pathway's involvement in the cell's citric acid stress response, it would not be absurd to study the possibility that the CWI pathway may be

involved in citric acid adaptation, perhaps through interaction between the two MAPK pathways.

Our study also revealed two deletion strains Δ*rom2* and Δ*ypl150w* to exhibit resistant phenotypes upon exposure to increasing citric acid concentrations. One encodes a protein involved in the CWI; Rom2p is a guanine exchange factor protein of the GTP-binding protein Rho1p. Upon cell wall stress, sensors on the cell surface transmit signals to Rom2p, stimulating nucleotide exchange of Rho1p, which in turn activates the Pkc1p signaling pathway (Vilella *et al.,* 2005). Originally this was the main pathway in which Rom2p was described to mediate stress responses (Ozaki *et al.,* 1996). Since then studies have shown that Rom2p can also mediate stress responses and cell growth by negatively regulating the Ras-cAMP pathway (Park *et al.,* 2005). In general, previous studies have all shown Δ*rom2* strains to display sensitive phenotypes to various stresses; including oxidative stress (Vilella *et al.,* 2005), freeze-thaw stress and hyperosmotic stress (Park *et al.,* 2005). Our data, however, suggests that deletion of *ROM2* results in resistance to citric acid. Perhaps the Ras-cAMP pathway, since shown to be negatively regulated by Rom2p, is involved in citric acid adaptation?

The other deletion strain to exhibit resistance to citric acid was Δ*ypl150w*. *YPL150W* encodes for a putative protein kinase of unknown cellular role. This alleged protein kinase has been shown to interact with Lsp1p (Ho *et al.,* 2002). Our data suggests that this ORF of 'unknown function' may possibly be involved in the negative regulation of some as yet 'unknown' citric acid stress response mechanism.

Two other genes encoding known interacting proteins of Lsp1p (Ho *et al.*, 2002), exhibited a change in growth upon deletion in response to citric acid compared to wild type. Δ*app1* and Δ*vps64* both displayed sensitive phenotypes to citric acid. App1p is also a protein of 'unknown function'; however, it has been suggested as having a possible role in actin filament organization (Bon *et al.*, 2000; Samanta and Liang, 2003). The hypersensitivity of Δ*app1* to citric acid suggests that App1p is required for adaptation to citric acid stress. Likewise Vps64p, a cytoplasmic protein required for cytoplasm to vacuole targeting of proteins and also involved in pheromone-induced cell cycle arrest (Huh *et al.*, 2003; Kemp and Sprague, 2003), also seems to facilitate citric acid adaptation.

Our results show that although exposure to citric acid causes a reduction in phosphorylation of Lsp1p, the expression of Lsp1p is not essential for the adaptive response to citric acid. Our study also uncovered a few genes whose deletion resulted in changes in growth in response to citric acid. It is not clear whether either; Lsp1p and its interacting proteins; or perhaps the CWI pathway are involved in citric acid adaptation. Far greater studies would have to be carried out to determine either way; however it is interesting to note that there are well-established precedents for the shared use of MAP kinase cascade components among separate signaling pathways (Hohmann, 2002). The evidence for interactions between the HOG and the CWI pathways in response to cell wall stress upon exposure to zymolyase, poses the question of whether the response to citric acid stress also involves the interactions of separate MAP kinase cascade components or signaling pathways?

Chapter 5

5. Investigating functional interactions between Sky1p, Nmd5p and Hog1p

5.1. Introduction

Our study has shown *HOG1*, *SKY1* and *NMD5* confer resistance to citric acid stress (Lawrence *et al.,* 2004; Section 3.2.2 & 3.3). Protein-protein interaction studies have also shown Nmd5p to have possible functional linkage with Sky1p through secondary protein-protein interaction through Mpc54p. Previous studies have also established that the importin Nmd5p recognises a wide range of cargos most interestingly Hog1p (Caesar *et al*., 2006). Studies have also shown that *NMD5* null mutant strains exhibit mis-localisation of Hog1p (Ferrigno *et al*., 1998). This information suggests the possiblity that Hog1p, Sky1p and Nmd5p could retain some sort of functional linkage. This coupled with our data confirming Sky1p, Hog1p and Nmd5p confer resistance to citric acid, led us to study whether Nmd5p could possibly link Sky1p and Hog1p and their involvement in citric acid adaptation.

In this chapter we have performed a series of biochemical experiments to investigate the possible link between the protein kinases Hog1p and Sky1p, the importin Nmd5p and their possible interaction in the same signaling pathway mediating citric acid resistance.

5.2. *SKY1* and *HOG1* double deletion epistasis experiment

Both Δ*sky1* and Δ*hog1* show sensitivity to citric acid. In order to determine if the genes have an epistatic interaction and whether Sky1p and Hog1p could be present on the same pathway, a double mutant Δ*hog1*Δ*sky1* was constructed. We first attempted the double deletion by employing yeast mating techniques; mating Δ*sky1*α x Δ*hog1*a and Δ*hog1*α x Δ*sky1*a. Tetrads were attained however, micromanipulation proved unsuccessful. Subsequently a molecular approach using the Cre-*loxP* gene distruption system (Guldener *et al*., 1996; P. Dennison, unpublished) was employed.

The Cre-loxP system targets and specifically removes the gene of interest, allowing for the positive selection of clones that contain the loxP-LEU2-loxP cassette. In addition the use of this system allows the removal of the LEU2 marker, so that the double mutant has the same phenotypic markers as its parent and does not have an additional metabolic marker that could give a misrepresentation of phenotypic testing in the double mutant. The double mutant strain Δ*hog1*Δ*sky1* was constructed and supplied by P. Dennison and tested for citric acid sensitivity in this study.

The double mutant Δ*hog1*Δ*sky1* was tested in liquid culture and compared to WT, Δ*sky1* and Δ*hog1* (Figure 5.1). The Δ*hog1*Δ*sky1* strain displayed a slow growth phenotype in liquid media making it difficult to gauge if the citric acid sensitivity shown was in fact additive or due to the slow growth phenotype resulting from the double deletion. However, when grown on solid media, the growth of Δ*hog1*Δ*sky1* was consistent with that of WT (Figure 5.2).

WT, Δ*hog1*, Δ*sky1* and Δ*hog1*Δ*sky1* were spotted on solid media (MB, pH 3.5) with 0, 150, and 300 mM citric acid at 10-fold dilutions. It was clear from comparison of Δ*hog1*, Δ*sky1* and Δ*hog1*Δ*sky1*, that the double deletion exhibited additive sensitivity suggesting no epistasis and that the genes do not function directly up/down stream of each other and are more likely functioning on different pathways.

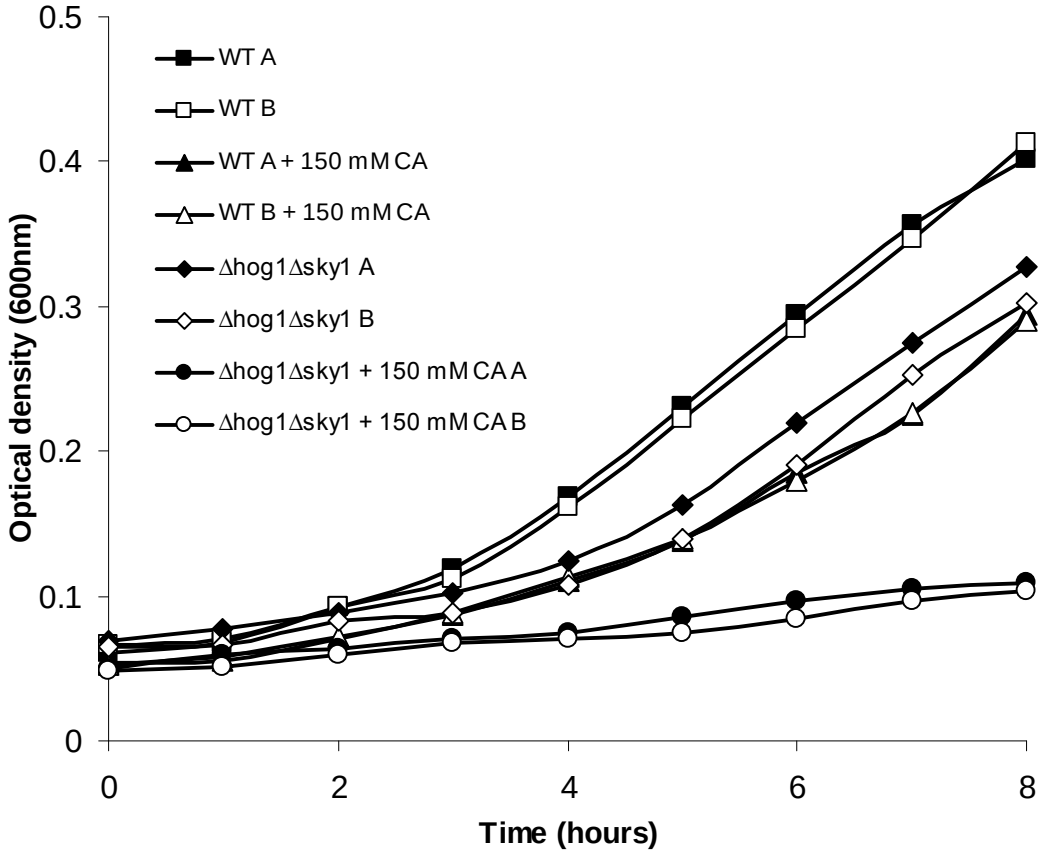


Figure 5.1: Comparison of growth of WT and Δ*hog1*Δ*sky1*, with and without 150 mM Citric acid. Duplicate liquid cultures of *S. cerevisiae* BY4741a (WT) (■,□) and deletion strain Δ*hog1*Δ*sky1* (♦,◊) were grown over a 8 hour period in MB, [pH 3.5] at 30°C. Duplicate cultures of WT (▲,Δ) and Δ*hog1*Δ*sky* (●,○) were grown along side for 7 hours at 30°C in MB, [pH 3.5] in the presence of 150 mM citric acid (CA). OD_{600} readings were recorded every hour for each culture.

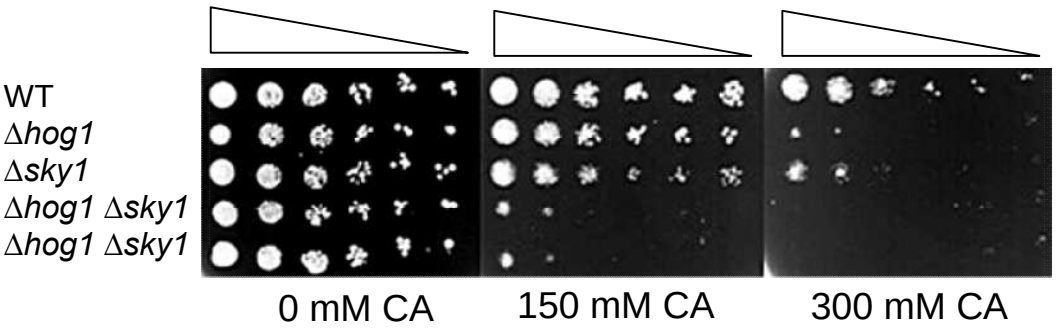


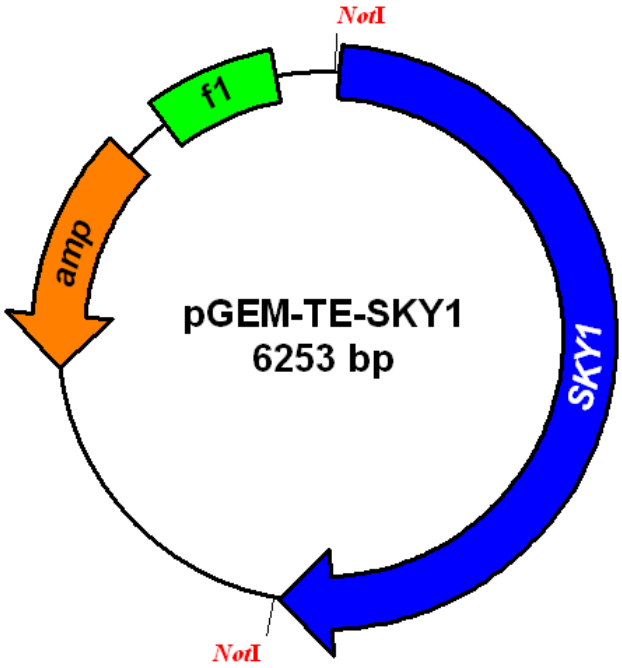
Figure 5.2 Growth of Δ*hog1*Δ*sky1* under citric acid stress on solid media. Serial dilutions of mid-exponential phase cultures of BY4741a (WT), Δ*hog1*, Δ*sky1* and Δ*hog1*Δ*sky1* were spotted onto MB agar plates [pH 3.5] with and without the presence of 150 and 300 mM citric acid. Plates were incubated for 48 h at 30°C before images were acquired.

5.3. Overexpression of *SKY1* and *HOG1* in their opposite deletion backgrounds

Since Sky1p and Hog1p seem to be important for citric acid adaptation, we decided to test whether overexpression of Sky1p and Hog1p in their opposite mutant backgrounds rescued citric acid sensitivity. To achieve this both *SKY1* and *HOG1* were PCR amplified using primers SKY1fwd and SKY1rev (Table 2.2), and HOG1fwd and HOG1rev (Table 2.2) respectively, which were then cloned into the pGEM®-T Easy vector (Promega Ltd., Hampshire, UK). This created the plasmids pGEM-TE-SKY1 and pGEM-TE-HOG1 (Figure 5.3) which were transformed into *E. coli* DH5α competent cells and selected for on LB + ampicillin solid media. Subsequently *SKY1* and *HOG1* were cleaved from these plasmids, via *Not*I digestion, and cloned separately into the *Not*I site of the overexpression vector pRS423 (Sikorski and Hieter, 1989) to create the plasmids pRS423-SKY1 and pRS423-HOG1 (Figure 5.4). These plasmids were transformed into their opposing deletion backgrounds and selected on SC-his and tested for citric acid sensitivity on solid media (Figure 5.5).

Overexpression of Hog1p in Δ*sky1* showed no rescue (Figure 5.5, A). However, overexpression of Sky1p in a Δ*hog1* mutant background partially rescued the Δ*hog1* phenotype when compared to Δ*hog1* mutant transformed with the empty vector (Figure 5.5, B). This result provides additional evidence that there is a functional link between Sky1p and Hog1p.

A.



B.

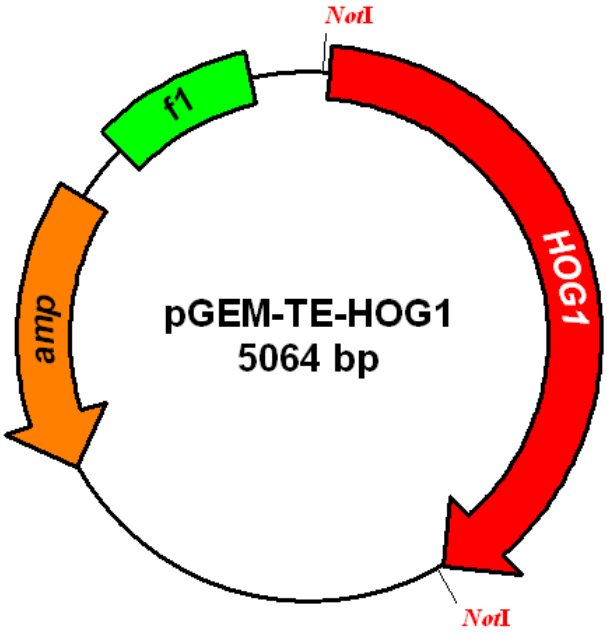
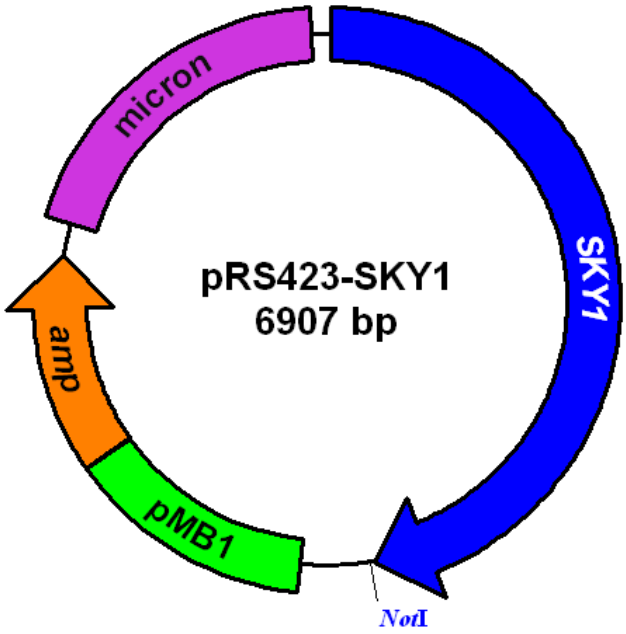


Figure 5.3: pGEM®-TE Plasmid maps incorporating *SKY1* and *HOG1*: *SKY1* and *HOG1* were PCR amplified and cloned into the pGEM®-T Easy vector creating the plasmids pGEM-TE-SKY1 (A.) and pGEM-TE-HOG1 (B.). The plasmids were transformed in DH5α competent cells and selected on LB + ampicillin solid media. Flanking the multiple cloning region are the recognition sites for *Not*I restriction digest enzyme. A *Not*I digest was used to release the SKY1 and HOG1 inserts.

A.



B.

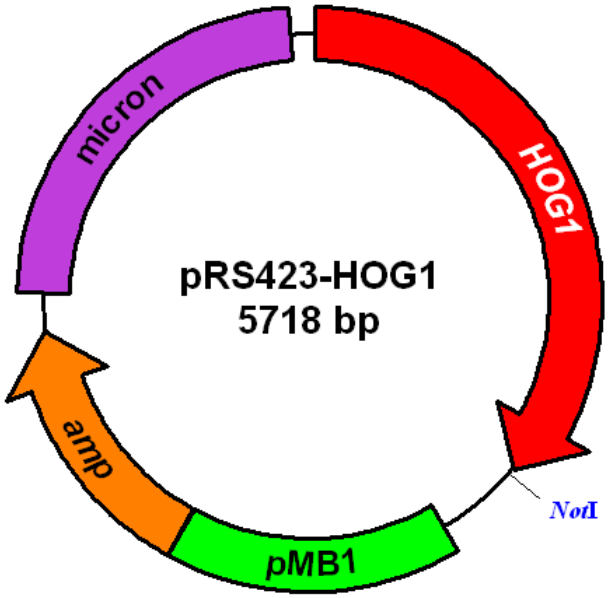


Figure 5.4: pRS423 overexpression plasmids incorporating *SKY1* and *HOG1*: *SKY1* and *HOG1* were cloned into the NotI digestion site of the overexpression vector pRS423 creating the plasmids pRS423-SKY1 (A.) and pRS423-HOG1 (B.). The plasmids were subsequently transformed into their opposing deletion strain background and tested for citric acid sensitivity on SC-his solid media.

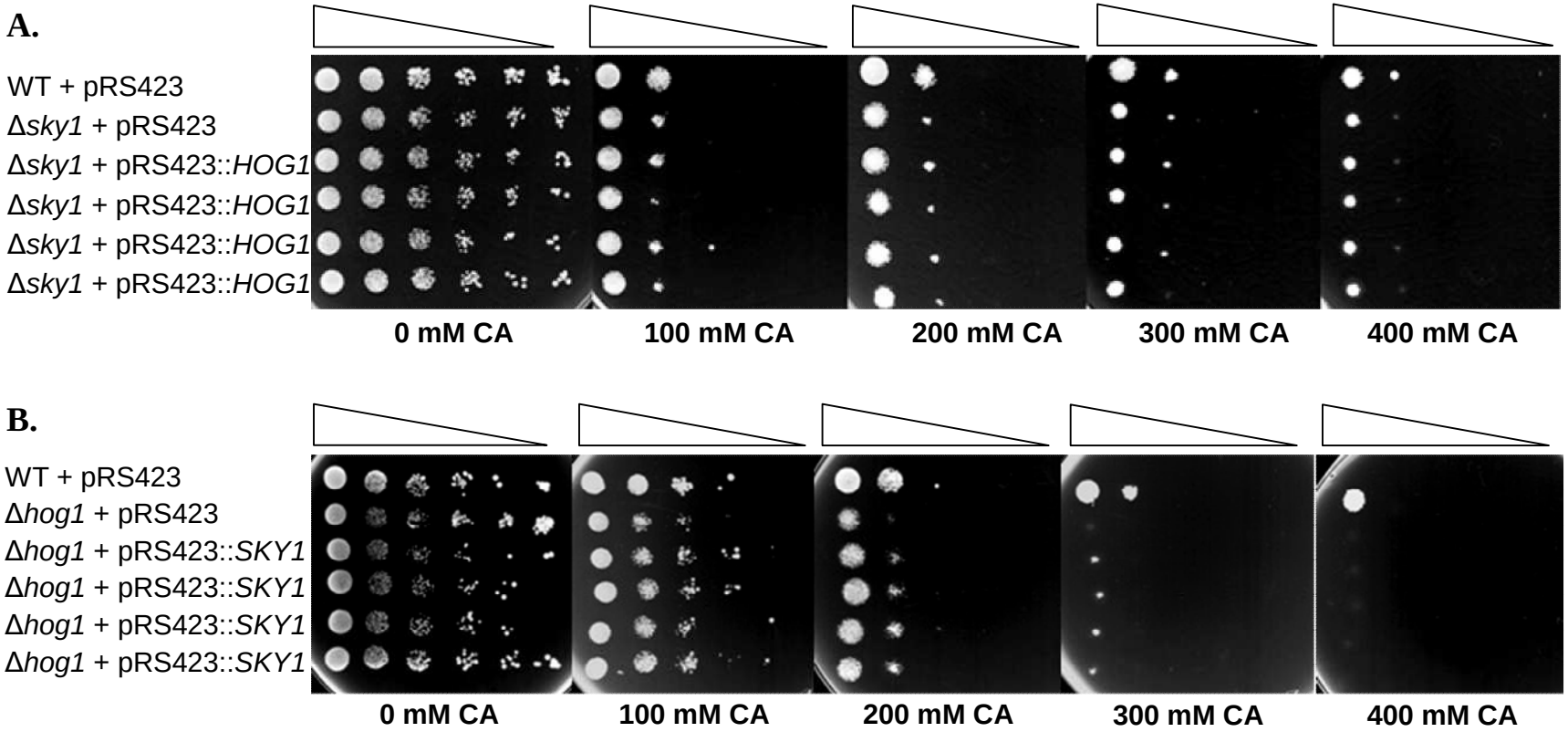


Figure 5.5 Overexpression studies of *SKY1* and *HOG1*. Growth comparison on selective agar (SC-his, [pH 3.5]) with 0, 100, 200, 300, and 400 mM citric acid (CA). BY4741a (WT) transformed with an empty multicopy plasmid (pRS423) in duplicate, Δ*sky1* and Δ*hog1* transformed with an empty multicopy plasmid (pRS423), and Δ*sky1* and Δ*hog1* transformed with pRS423 carrying the *HOG1* gene (A.) and *SKY1* gene (B.), respectively. Overexpression of *HOG1* in Δ*sky1* had no effect on citric acid sensitivity. However, *SKY1* overexpression partially rescued citric acid sensitivity in the Δ*hog1* deletion strain.

5.4. Effect of citric acid stress on Hog1p phosphorylation in Δ*sky1*

Activation of Hog1p via the dual phosphorylation of threonine-174 and tyrosine-176 occurs in response to various stresses including citric acid stress (Lawrence *et al*., 2004). If Sky1p was an important upstream component acting upon Hog1p we would expect that deletion of *SKY1* would prevent the dual phosphorylation and thus activation of Hog1p. Since our epistasis experiment suggests that they are not on the same pathway we would expect Δ*sky1* to show no change from the control experiments. In order to determine whether the SR protein kinase Sky1p has any effect upon Hog1p phosphorylation in response to citric acid stress western blotting techniques were used; Hog1p phosphorylation in BY4741a and Δ*sky1* under control and citric acid stressed conditions was examined.

Dual phosphorylation of Hog1p on residues Thr174 and Tyr176 was probed using a Phospho-p38 MAP Kinase primary antibody (Section 2.6.6; Table 2.3). Hog1p concentration control was detected using an anti-C-terminal Hog1p antibody (Hog1 (yC-20)). Actin (Act1p), a constitutively expressed protein, was used as a loading control and dectected using a beta Actin antibody (mAbcam 8224) (Figure 5.6). Appropriate horseradish peoxidase-conjugated secondary antibodies (Table 2.3) and enhanced chemiluminescence were used to visualize proteins.

Dual phosphorylation of Hog1p was evident under osmotic stress (+ 0.4 M NaCl) and citric acid stress (+ 300 mM citric acid) (Figure 5.7). Interestingly deletion of *SKY1* resulted in dual phosphorylation of Hog1p under control conditions [pH 3.5]. The parent strain (WT) [pH 3.5] did not show dual phosphorylation suggesting that deletion of *SKY1* elicits a response that results in Hog1p phosphorylation upon *SKY1* deletion alone. This suggested further evidence for linkage between Hog1p and Sky1p.

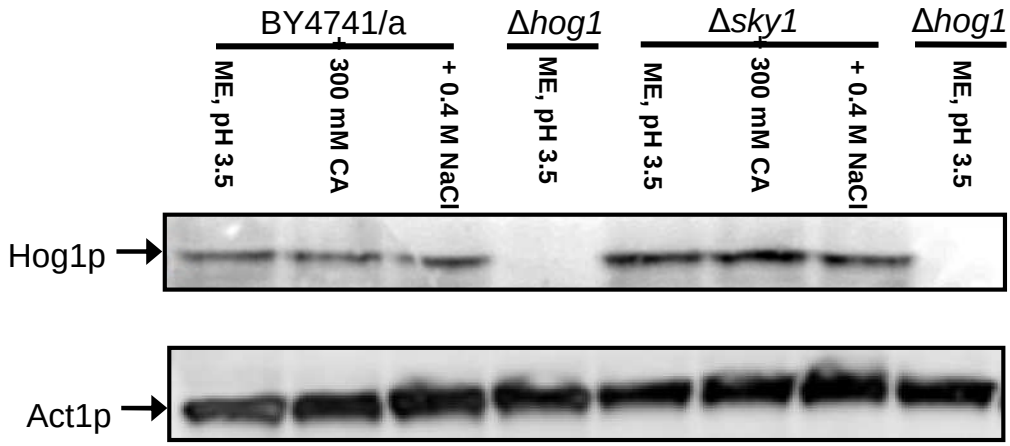


Figure 5.6 Control Western blot for Hog1p levels. WT, Δ*sky1* and Δ*hog1* strains were grown in MB [pH 3.5], under salt stress (0.4 M NaCl, [pH 4.5]), and under citric acid stress (300 mM CA, [pH 3.5]). Protein was extracted and probed with primary antibodies Hog1 (yC-20) and ACT1 (C4) to check that equal concentrations of protein were loaded in the lanes. Hog1p and Act1p levels were used as loading controls.

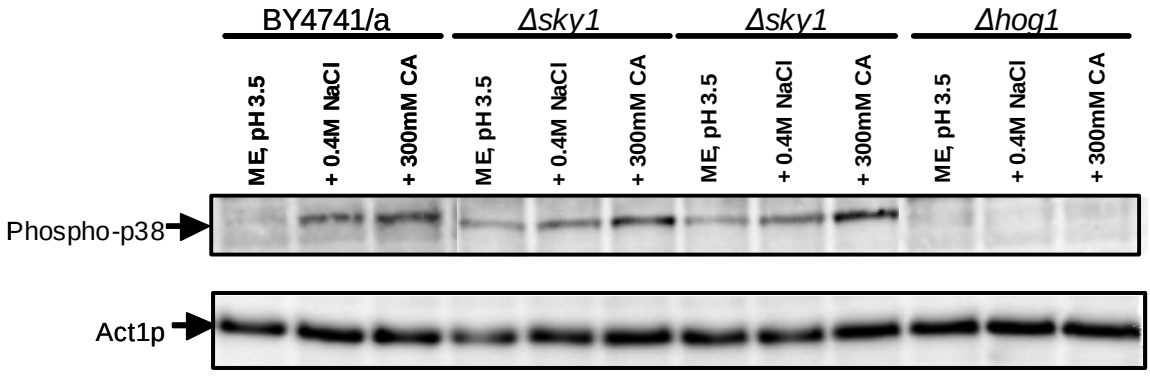


Figure 5.7 Western blot demonstrating the dual phosphorylation of Hog1p upon exposure to citric acid and also upon deletion of *SKY1*. WT, Δ*sky1* and Δ*hog1* strains were grown in MB [pH 3.5], under salt stress (0.4 M NaCl, [pH4.5]), and under citric acid stress (300 mM CA, [pH 3.5]). Protein was extracted and probed with a phospho-p38 MAP Kinase (Thr180/Tyr182) antibody; bands indicate dual phosphorylation of Hog1p. Act1p was used as a loading control.

5.5. Hog1p translocation ± 300 mM Citric acid in WT and Δ*sky1* using *HOG1-GFP* construct.

The previous set of results (Section 5.4) provided evidence that dual phosphorylation of Hog1p occurs under citric acid stress when *SKY1* is deleted. Dual phosphorylation of Hog1p has been shown to result in its translocation to the nucleus (Hohmann, 2002). To determine whether this occurred under citric acid stress conditions and in Δ*sky1*, fluorescence microscopy using GFP tagged Hog1p strains was used.

The localisation HOG1-GFP was analysed in WT and Δ*sky1* strains grown under various control and stress conditions; MB [pH 3.5], MB + 300 mM citric acid [pH 3.5], MB [pH 5.5], and MB + 0.4 M NaCl [pH 5.5] using a commercially available HOG1-GFP construct (Invitrogen Ltd., Paisley, UK) and a Δ*sky1*-HOG1-GFP constructed and supplied by a co-worker (P. Dennison). Dennison used the Cre-*loxP* gene distruption system to delete *SKY1* from the HOG1-GFP construct (Invitrogen Ltd., Paisley, UK). Hog1p has previously been shown to translocate to the nucleus upon osmotic stress (Ferrigno *et al*., 1998) and similar stress conditions were used as a comparative control to citric acid stress by using 0.4 M NaCl [pH 5.5].

The fluorescence microscopy images provided evidence that in WT cells, citric acid stress, like osmotic stress, results in the translocation of Hog1p to the nucleus. Also, this nuclear localisation of Hog1p was purely due to the addition of 300 mM citric acid and not due to the low pH, since Hog1p is localised throughout the cell at pH 3.5 (Figure 5.8).

HOG1-GFP localisation in Δ*sky1* cells did not seem to be clearly concentrated to the nucleus. Hog1p was detected to be distributed both in the nucleus and cytoplasm. Some cells did seem to have slightly more fluorescence concentrated to the nucleus. However, the levels were not significant enough to provide clear evidence of translocation when compared to the WT cells (Figure 5.9).

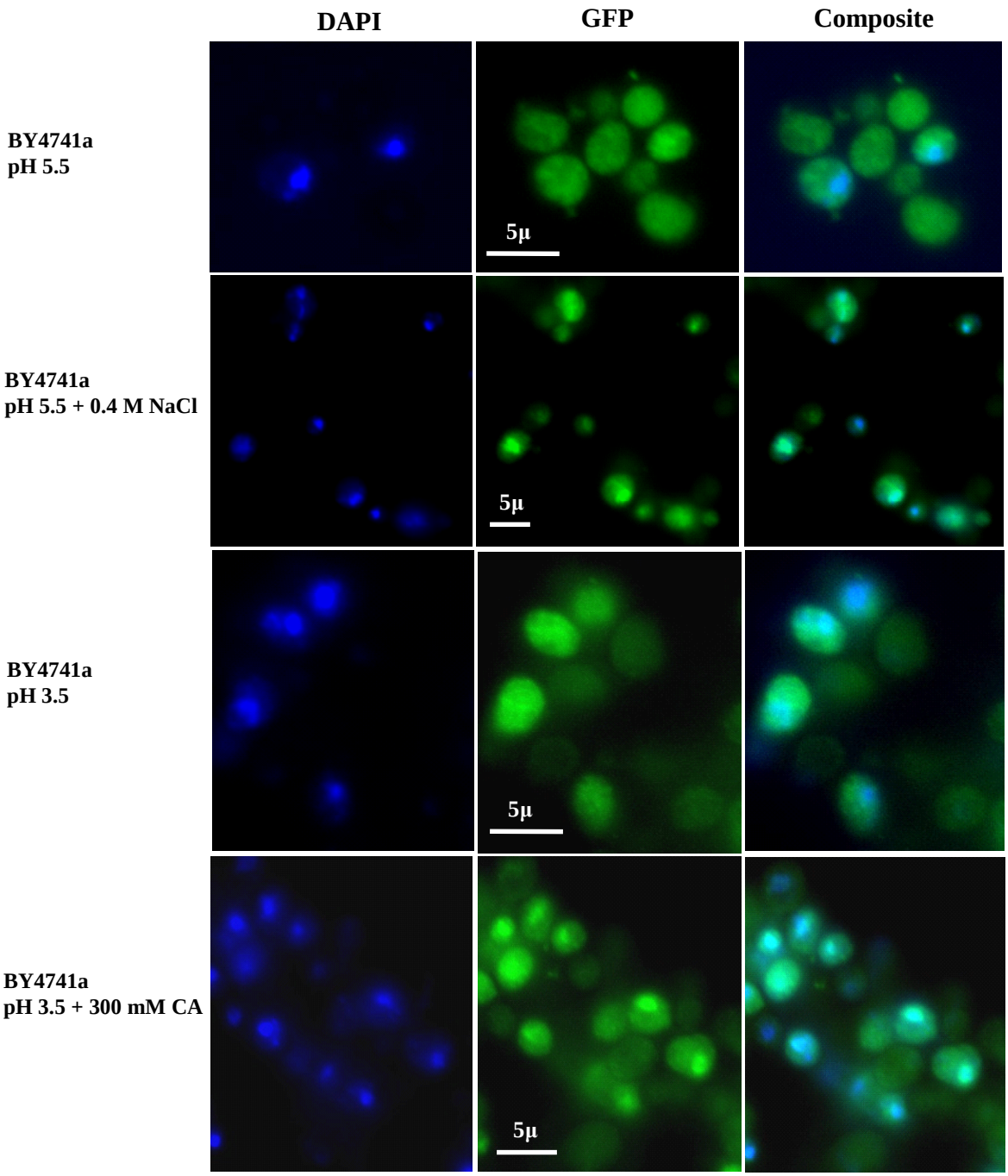


Figure 5.8 Hog1p-GFP localisation under citric acid stress. BY4741a (WT) cells showing DAPI staining of the nucleus and Hog1p localisation with GFP fluorescence. Far right shows the composite image of both stains. Cells were grown to mid- exponential phase (MB) and exposed to four conditions: MB [pH 5.5], MB + 0.4 M NaCl [pH 5.5], MB [pH 3.5] and MB + 300 mM Citric acid [pH 3.5]. Localisation of Hog1p to the nucleus can be seen in cells exposed to both NaCl [pH 5.5], and citric acid [pH 3.5], but in neither control. Scale bar denoted on figure.

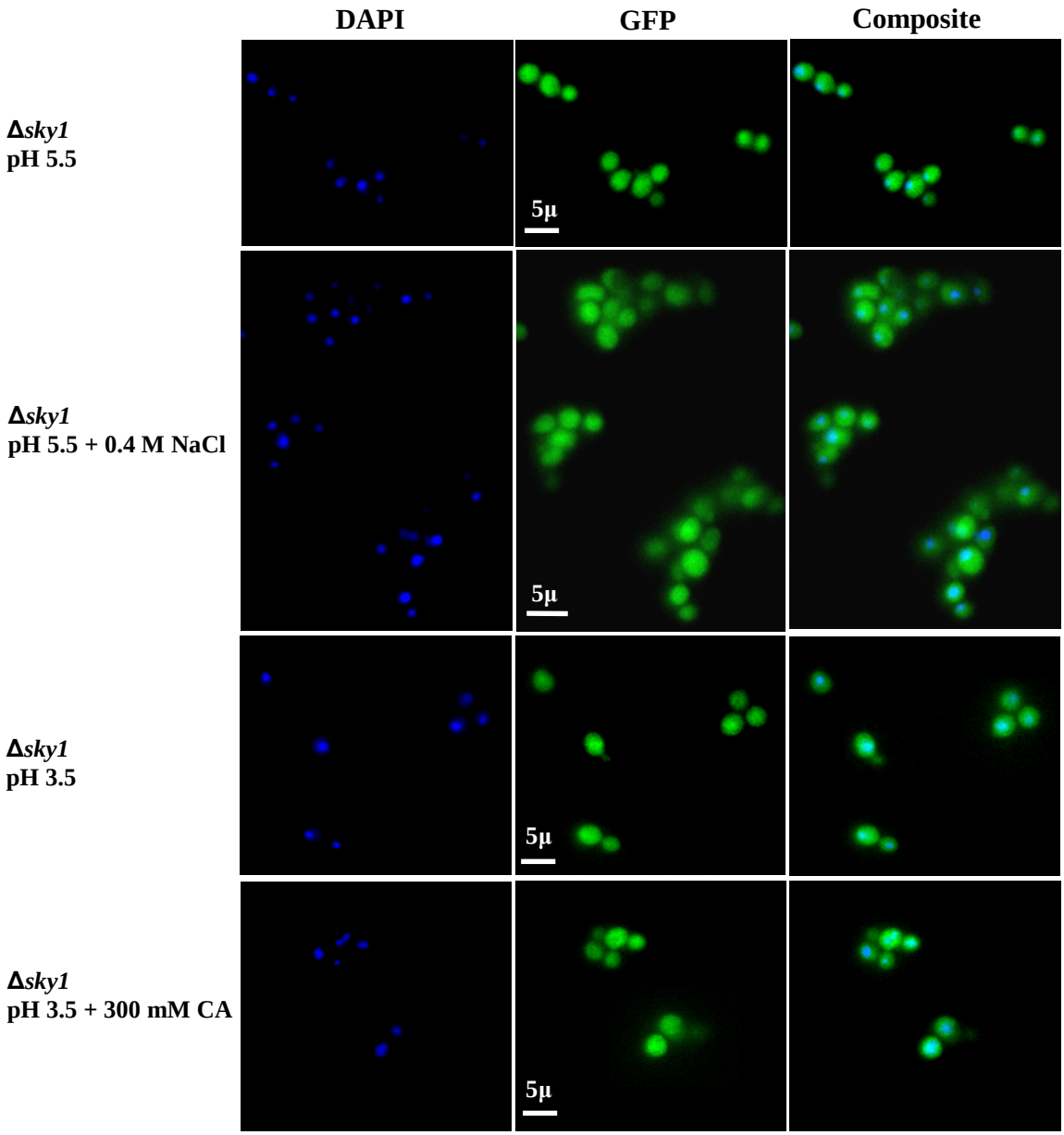


Figure 5.9 Hog1p-GFP localisation in Δ*sky1* under citric acid stress. Δ*sky1* cells showing DAPI staining of the nucleus and Hog1p localisation with GFP fluorescence. Far right shows the composite image of both stains. Cells were grown to mid- exponential phase (MB) and exposed to four conditions: MB [pH 5.5], MB + 0.4 M NaCl [pH 5.5], MB [pH 3.5] and MB + 300 mM Citric acid [pH 3.5]. Scale bar denoted on image.

5.6. Discussion

In this study a variety of biochemical approaches were used to determine whether there is any interaction between Hog1p and Sky1p and to establish if there is a direct link between these two proteins or a possible involvement in the same signalling cascade that mediates citric acid resistance.

Growth assays for the double deletion Δ*hog1*Δ*sky1* showed an additive level of sensitivity compared to the single deletions strains Δ*hog1* and Δ*sky1* under citric acid stress suggesting no epistasis between the two genes. The additive sensitivity shown by the double deletion suggests *HOG1* and *SKY1* are not situated on the same pathway and that their roles in citric acid adaptation are not directly linked. This result does not however rule out the possibility of some functional overlap in their involvement of citric acid adaptation.

Overexpression studies revealed that citric acid sensitivity in Δ*hog1* cells was slightly reduced with the overexpression of *SKY1*. However, this was not shown by the overexpression of *HOG1* in the Δ*sky1* strain. The partial rescue of growth with the overexpression of Sky1p, suggests some functional interaction between Hog1p and Sky1p in mediating citric acid resistance. Previous studies have shown that overexpression of the protein kinase Rck2p in Δ*hog1* cells partially suppressed osmotic sensitivity. These results supported the notion of a link between Hog1p and Rck2p and further research led to the classification of Rck2p as being a substrate for Hog1p, acting downstream of Hog1p (Bilsland-Marchesan *et al*., 2000).

It has previously been shown that citric acid results in the dual phosphorylation, and thus activation, of Hog1p (Lawrence *et al*., 2004). This result has been replicated in this study. Our results also showed dual phosphorylation of Hog1p in Δ*sky1* cells under citric acid stress which eliminates the possibility of *SKY1* being an important upstream component of the HOG pathway since deletion of *SKY1* does not prevent expression of Hog1p. However, Δ*sky1* exhibited dual phosphorylation of Hog1p under control growth conditions (MB, pH 3.5). This implies that loss of *SKY1* results in dual phosphorylation of Hog1p by either signalling phosphorylation or perhaps by interfering with dephosphorylation of Hog1p? Hog1p is phosphorylated by the MAPKK Pbs2p.

Studies by Lawrence and co-workers have shown that in Δ*pbs2* cells, Hog1p is not phosphorylated confirming the MAPKK Pbs2p is an important upstream component of the HOG pathway and is required for optimal activation of Hog1p in response to citric acid (Lawrence *et al*., 2004). The phosphatases Ptc1,2&3p and Ptp2p & Ptp3p act as negative regulators of Pbs2p and Hog1p respectively, and all affect the level and duration of Hog1p phosphorylation. Single deletion *PTP2*, *PTP3*, and *PTC2* mutants are each sensitive to citric acid in liquid culture (Lawrence *et al*., 2004). Δ*ptc1* and Δ*ptc3* were also hypersensitive to citric acid stress (Lawrence, unpublished). Lawrence and co-workers theorised that these results may indicate that the activity of these phosphatases is required to down-regulate the HOG pathway to prevent the growth defect induced by hyperactivation of Hog1p after stimulation by citric acid (Lawrence *et al*., 2004). Extension of this theory, linking a role for *SKY1* in the regulation of Hog1p phosphorylation and activation, is that *SKY1* is involved in the regulation of one or more of these phosphatases. This could account for the dual phosphorylation of Hog1p in Δ*sky1* cells when no external stress is present. Using protein-protein and genetic interaction data

it is possible to show possible links between the components of the HOG pathway and the protein kinase Sky1p (Figure 5.10).

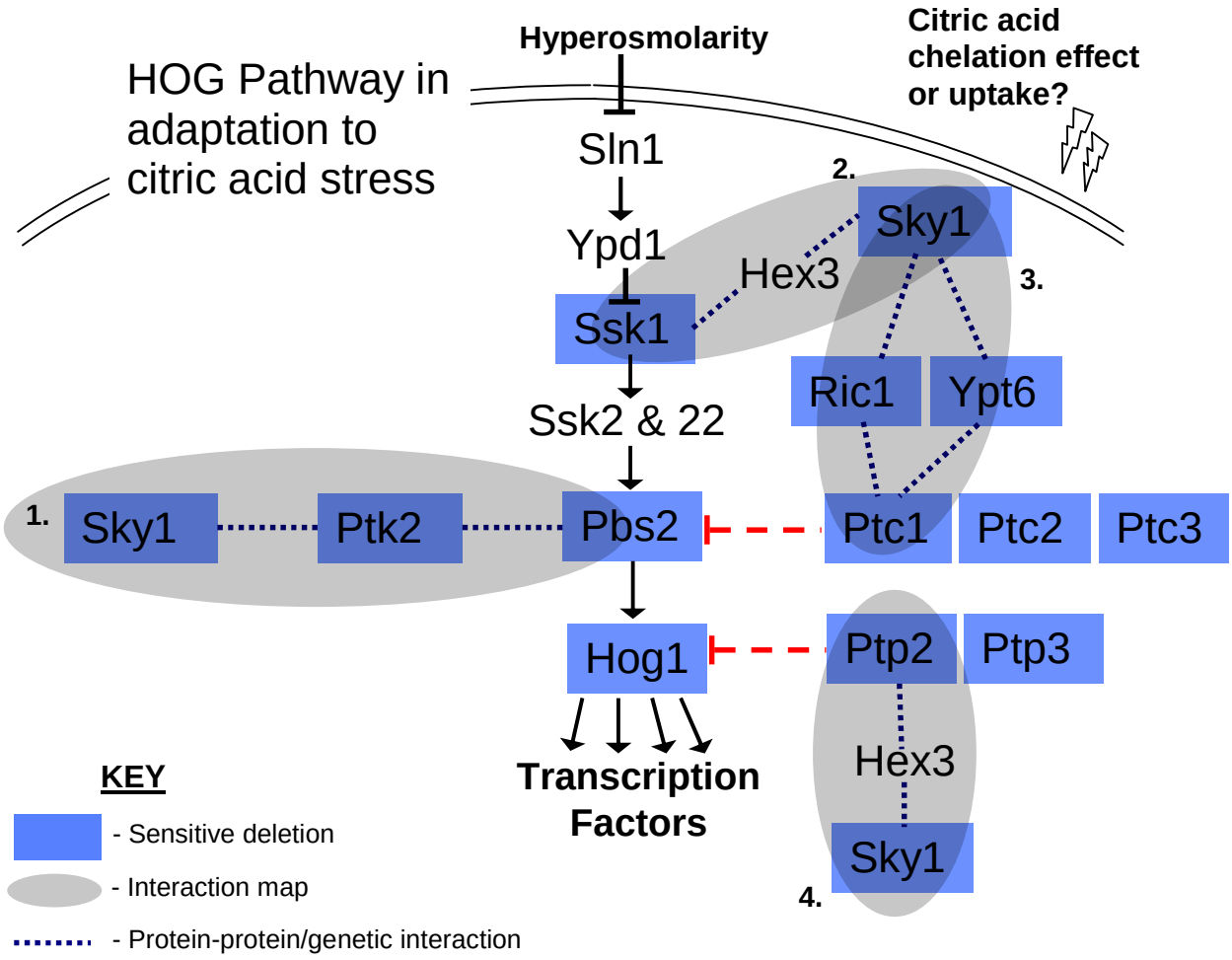


Figure 5.10: Summary of role of HOG pathway in adaptation to citric acid stress and possible interactions which may link Sky1p. Proteins whose encoding genes have been found to be sensitive to citric acid upon deletion have been indicated. Ssk1, Pbs2, Hog1 and the phosphatases Ptc1, 2 & 3, and Ptp2 & 3 are all components involved in the activation and regulation of the HOG pathway. Highlighted are four possible ways in which Sky1p may be linked to the HOG pathway in its role in citric acid adaptation. The four highlighted interaction maps linking Sky1p to components of the HOG pathway indicate proteins which interact via protein-protein or through genetic interaction (BioGRID; URL: http://www.thebiogrid.org and BioPIXIE; URL: http://pixie.princeton.edu/pixie/index.php). The effect of citric acid whether externally or intracellulary is unclear activation of adaptation pathways may be a result of citric acid uptake or as a result of metal ion chelation by citric acid.

The first theory for linkage of Sky1p to the HOG pathway is to the MAPKK Pbs2p through the protein kinase Ptk2p (Figure 5.10). Ptk2p is involved in the regulation of ion transport across the plasma membrane and both Ptk2p and Sky1p have been linked by their involvement in polyamine transport and also in regulation of salt homeostasis (Erez and Kahana, 2001). Microarray data for protein phosphorylation indicated a link between Ptk2p and Pbs2p, in fact Pbs2p was shown to phosphorylate Ptk2p (Ptacek *et al*., 2005). Since Pbs2p is acting upon Ptk2p, this renders the theory that Sky1p may influence Pbs2p activity through Ptk2p unlikely. However, the phosphorylome for Ptk2p did reveal that Ptk2p phosphorylates a cAMP-dependent protein kinase catalytic subunit Tpk3p, which in turn has been shown to phosphorylate Hog1p (Ptacek *et al*., 2005). A second possible link between Sky1p and Hog1p is through Hex3p and the osmosensing component Ssk1p. Sky1p phosphorylates Hex3p (Ptacek *et al*., 2005) and Hex3p is known to interact with Ssk1p (Ho *et al*., 2002).

These theories of how Sky1p could be linked to Hog1p in the adaptation of *S. cerevisiae* to citric acid (Figure 5.10), however, cannot readily explain why upon deletion of *SKY1*, Hog1p is consequently dually phosphorylated when no external stress is present. Theories 3 & 4 however, linking Sky1p to the phosphatases which negatively regulate Pbs2p and Hog1p would correlate with the Hog1p phosphorylation result. Sky1p can be linked to the phosphatase Ptc1p through both Ric1p and/or Ypt6p. Each of these genes, when deleted in combination, result in synthetic lethality (Tong *et al*., 2004). Also each of these genes exhibit a citric acid sensitive phenotype when deleted, implying an involvement in citric acid adaptation (Figure 3.7; Lawrence, unpublished data). Sky1p can also be linked to another phosphatase Ptp2p. Sky1p phosphorylates Hex3p, which is known to interact with Ptp2p (Ho *et al*., 2002; Ptacek *et al*., 2005). If deletion of *SKY1* results in the down

regulation of the phosphatases we would expect lesser de-phosphorylation of Pbs2p and Hog1p, which could explain the incidence of dually phosphorylated Hog1p without any external stress stimulus.

Hog1p localisation under osmotic stress has been well documented. Using green fluorescent protein (GFP) fusions of the protein kinase, Hog1p has been found to localise in the cytoplasm of unstressed cells. However, following osmotic stress, Hog1p translocates to the nucleus. This translocation occurs very rapidly, is transient, and correlates with the phosphorylation and activation of Hog1p by Pbs2p (Ferrigno *et al*., 1998). Using a similar experimental protocol we used GFP-tagged Hog1p to visualise the effect of citric acid stress on Hog1p translocation. Mirroring the effect of osmotic stress, Hog1p also translocated to the nucleus in the presence of 300 mM citric acid. The effect was due to the presence of the weak acid and not a result of the low pH (pH 3.5) as a control experiment proved the low pH alone did not result in nuclear localisation of Hog1p. Since our western blot results seemed to show *SKY1* had some connection to Hog1p, its deletion affecting Hog1p phosphorylation, we were interested to determine whether Δ*sky1* would also affect the translocation of Hog1p. The western blot suggested that Hog1p, since dually phosphorylated in Δ*sky1*, may also undergo translocation in the absence of any external stress and purely as the result of *SKY1*'s deletion. The fluorescence microscopy images for Δ*sky1* did not clearly indicate Hog1p translocation in neither the osmotic stress control experiments, under citric acid stress, or in the absence of stress (pH 3.5). There does seem to be some nuclear localisation but also localisation of Hog1p in the cytoplasm. It is not 100% clear if the experiments show true Hog1p nuclear localisation. However, there may be an explanation for this. Prior research has revealed that deletion of the phosphatase Ptp2p leads to decreased Hog1p nuclear retention

(Mattison and Ota, 2000). The phosphatases Ptp2p and Ptp3p have been found to act as a nuclear anchor for Hog1p and a cytoplasmic tether respectively. Therefore, Ptp2p and Ptp3p have more than one role in regulating the HOG pathway. One is to activate Hog1p through dephosphorylation, while the second is to regulate subcellular localisation of Hog1p through binding interactions (Mattison and Ota, 2000). A third possible role is in the adaptation of *S. cerevisiae* to citric acid stress, perhaps regulated in some way upstream by Sky1p accounting for both Hog1p's dual phosphorylation in Δ*sky1* cells and also the unclear Hog1p localisation images in Δ*sky1* cells?

It has been clearly recognised that the nuclear uptake of Hog1p following hyperosmotic stress requires the β-importin Nmd5p (Ferrigno *et al*., 1998; Westfall and Thorner, 2006). However, the nuclear translocation process of Hog1p has been shown to be more complicated than first thought. Contrary to previous reports by Ferrigno and co-workers Nmd5p does not seem to enter the nucleus following hyperosmotic stress rather it accumulates at the nuclear pore complex (NPC) (Westfall and Thorner, 2006). When cells are challenged with hyperosmotic stress, Hog1p and Nmd5p are transported to the NPC by an as-yet-unknown independent mechanism. Once there, Nmd5p somehow facilitates the passage of Hog1p through the NPC. Nmd5p is crucial in this process as nuclear entry of Hog1p does not occur when Nmd5p is absent (Westfall and Thorner, 2006). The importance of the presence of Nmd5p for the translocation of Hog1p into the nucleus may suggest another possible explanation for the apparent lack of translocation in Δ*sky1*. As afore mentioned Sky1p interacts with Nmd5p through Mpc54p. Since Nmd5p is the specific nuclear import protein required for Hog1p nuclear translocation (Ferrigno *et al.*, 1998; Westfall and Thorner, 2006), if the deletion of *SKY1* interferes with Nmd5p expression or activity then Hog1p would not translocate to the nucleus successfully.

The results of this chapter provide evidence for some level of interaction between Sky1p, Hog1p and possibly Nmd5p. They have each been shown to confer resistance to citric acid stress. The epistasis experiment suggests that *SKY1* and *HOG1* are not directly linked by the same pathway however; further results did suggest some functional redundancy. There does seem to be some plausible explanations which could account for the results shown in this chapter. The theory that Sky1p may interact with phosphatases, those involved in de-phosphorylation of Hog1p and Pbs2p, would agree with the western blot and fluorescence microscopy findings. To test these theories and determine exactly how these genes are linked in their role in adaptation to citric acid further research must be carried out.

Chapter 6

6. Discussion

6.1. Final discussion

S. cerevisiae has evolved dedicated and sophisticated mechanisms that allow adaptation to different weak organic acids. Some of these mechanisms can be classed as a generalized response to weak acid stress while others are specific to the weak acid to which the cells are exposed. The sorbic acid induced stress response has been most intensively studied and to date is the best-characterised, highlighting the involvement of the plasma membrane H+-ATPase, the efflux pump Pdr12p, and the transcription factor War1p (Piper *et al*., 1998; Kren *et al*., 2003). The adaptive mechanisms employed against the other monocarboxylic acids acetic acid and benzoic acid have also undergone extensive research characterizing similar general responses but also those specific to each acid; Spi1p in benzoic and Azr1p in acetic (Simoes *et al*., 2006; Tenreiro *et al*., 2000). Very little was known about the mechanisms employed by *S. cerevisiae* to enable adaptation to the tricarboxylic acid citrate until a recent study provided evidence for the involvement HOG pathway (Lawrence *et al.*, 2004). Also the citric acid sensitivity screen of the disruptome carried out by Lawrence *et al.*, pulled out a number of interesting genes including *SKY1*, a protein kinase involved in polyamine metabolism. We decided to determine whether this protein kinase and/or the proteins involved in cellular polyamine regulation could also play an important role in the adaptation of *S. cerevisiae* to citric acid.

One of the principle objectives of this study was to identify whether Sky1p could be an important regulatory protein involved in the recognition and response mechanism of citric acid stress. Citric acid sensitivity screening of Δ*sky1*, deletion strains of other genes

involved in polyamine metabolism, and also deletion strains of genes encoding proteins known to interact with Sky1p were tested. Δ*sky1* and many of the other deletion strains tested were sensitive to citric acid and growth assays quantified and confirmed many as being hypersensitive to citric acid. These results provide compelling evidence that Sky1p and those either involved in polyamine regulation of those shown to interact with Sky1p have a role in the citric acid stress response.

Protein-protein and genetic interactions maps for Sky1p revealed an interesting secondary interaction through Mpc54p to the importin Nmd5p. Specifically this importin is essential for the translocation of Hog1p to the nucleus (Ferrigno *et al*., 1998). With the prior evidence of the involvement of the HOG pathway in citric acid adaptation, and our data supporting the theory of the involvement of Sky1p, we wondered if there was the possibility of some link between the two protein kinases, through Nmd5p, in the response to citric acid stress. Construction of the *NMD5* deletion strain and subsequent testing identified that Nmd5p also confers resistance to citric acid stress.

Various experimental approaches were undertaken in this study to determine whether or not Hog1p and Sky1p were linked in their efforts in adaptation to citric acid stress. Growth assays for the double deletion Δ*sky1*Δ*hog1* suggested that the kinases were not situated on the same pathway and that their roles in citric acid adaptation were not directly linked. However, overexpression studies exhibited partial rescue of growth in Δ*hog1* cells overexpressing Sky1p, upon exposure to citric acid. This implied some functional interaction between Hog1p and Sky1p in mediating citric acid resistance. Further evidence for some functional redundancy between Sky1p and Hog1p was provided by the proteomic analyses. Four instances of proteins being up/down regulated

in response to citric acid occurred for both Δ*sky1* and Δ*hog1* with comparison to the wild type. The Hog1p phosphorylation studies in Δ*sky1* supported the results from the double deletion growth assay. Deletion of *SKY1* did not prevent Hog1p phosphorylation under stress conditions confirming that Sky1p is not directly upstream of Hog1p. Interestingly deletion of *SKY1* did result in dual phosphorylation of Hog1p when no external stress had been applied. From this result we proposed the theory that Sky1p regulates the expression of the phosphatases Ptc1 & Ptp2, which dephosphorylate Hog1p (Figure 6.1). Our Hog1p translocation studies for Δ*sky1* were not definitive, however results suggested deletion of *SKY1* effected the efficient translocation of Hog1p to the nucleus under citric acid stress, this could be possibly be due to an effect on the importin Nmd5p or the phosphatase Ptp2p also known to be a nuclear anchor for Hog1p (Mattison and Ota, 2000).

Aside from the evidence the study has provided for a role for Sky1p in citric acid adaptation and a possible functional interaction between Sky1p and Hog1p our study has also suggested that unlike the monocarboxylic acids, (sorbic, acetic, and benzoic acid) citric acid does not actually enter the cell. This implies that an external stress signal is relayed by plasma membrane proteins triggering citric acid adaptation mechanisms. Re-organization and altered composition of plasma membrane proteins has previously been implicated in weak organic acid stress response and our phosphoproteome analysis detected citric acid induced de-phosphorylation of Lsp1p, an important component of the plasma membrane eisosome (Walther *et al*., 2007). Lsp1p is also a negative regulator of the cell wall integrity pathway and its apparent dephosphorylation would consequently result in activation of the Pck1p and/or Ypk1p pathways which up regulate various stress response pathways (Luo *et al*., 2008). This data coupled with evidence for resistant and sensitive phenotypes for gene deletion strains encoding proteins involved in cell wall

integrity and interacting with Lsp1p, suggests it is plausible that the citric acid stress response involves the CWI pathway and induces a reorganization and/or altered composition of the plasma membrane.

In summary this thesis provides evidence for the involvement of Sky1p, interacting proteins of Sky1p, and proteins involved in polyamine metabolism in the citric acid stress response. Our study provided several results suggesting some functional redundancy and/or perhaps linkage between Sky1p and Hog1p and how they mediate citric acid resistance. And finally our results suggest citric acid does not enter the cell and suggests the possible involvement of the CWI pathway in response to the external citric acid stress (Figure 6.1). It is possible that the activation of stress response and adaptation pathways may be a result metal ion chelation by citric acid. Metal ions such as Mg^{2+}, K^{+}, Ca^{2+}, Fe^{2+} are crucial for maintaining the structural integrity of cells and organelles; for cell division and growth; for energy maintenance and as protectants under environmental stresses (Walker, 2004). The adaptive response resulting in the activation and upregulation of the genes and proteins outlined in this study may be as a consequence of metal ion limitation caused by citric acid chelation rather than an intracellular acidification caused by uptake of citric acid into the cell. Futher study into the chelation effects of citric acid on *S. cerevisiae* is essential for elucidation of how *S. cerevisiae* cells adapt to citric acid stress.

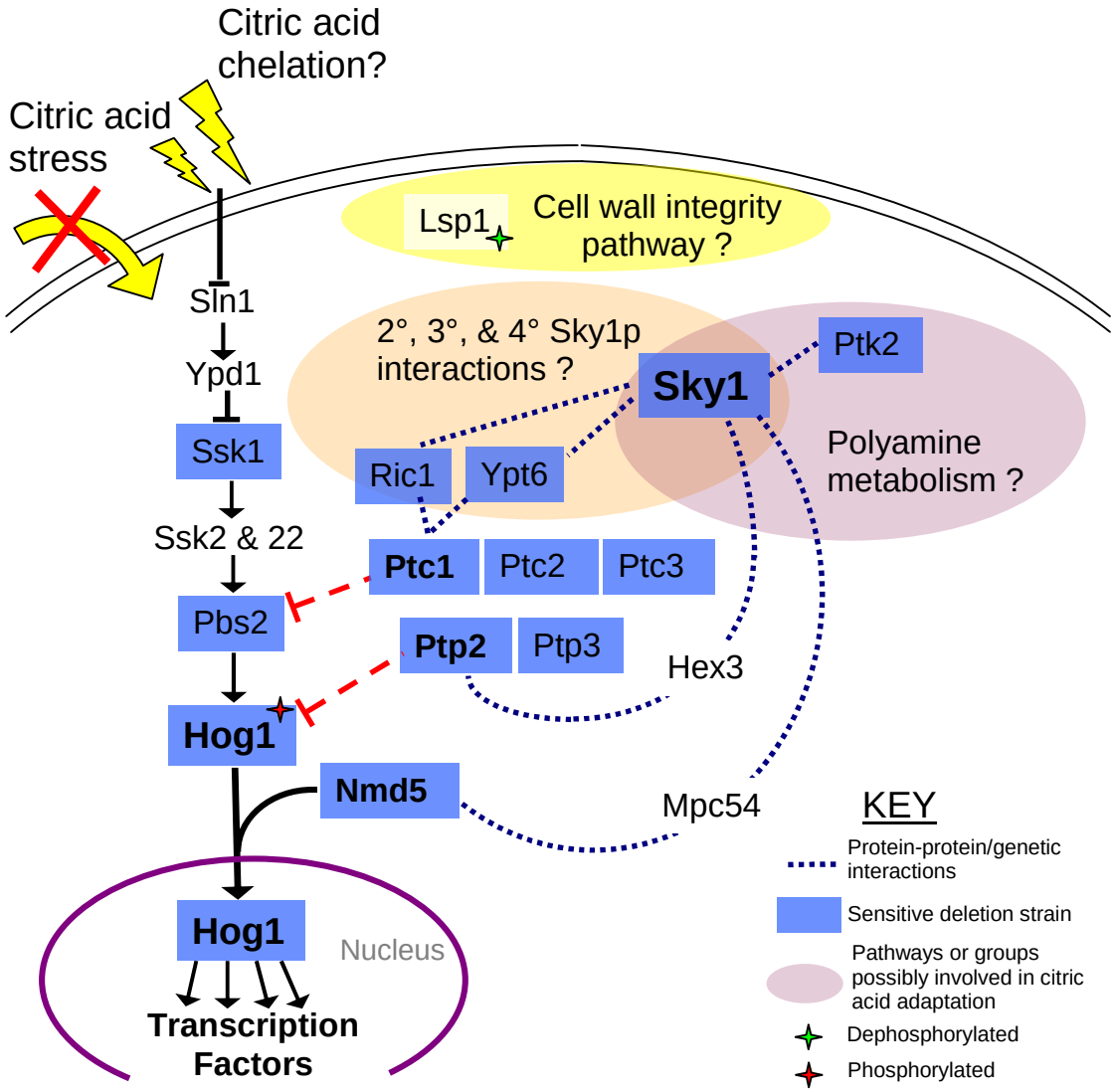


Figure 6.1: Summary of possible citric acid adaptation mechanisms in *S. cerevisiae*. Citric acid does not appear to enter the cell; implying that an external stress signal is relayed by plasma membrane proteins triggering citric acid adaptation mechanisms or perhaps as a result of chelation of crucial metal ions. Components of the HOG pathway; *SKY1* and genes encoding interacting proteins of Sky1p; and genes encoding proteins involved in polyamine metabolism are citric acid sensitive upon deletion suggesting an involvement in the citric acid adaptation. Evidence suggests some functional redundancy and/or linkage between Sky1p and Hog1p and their roles in citric acid adaptation. Perhaps through; Sky1p mediated regulation of Hog1p phosphorylation by the phosphatases Ptc1p & Ptp2p; or the possibility of Sky1p being involved in the regulation of the Hog1p importin, Nmd5p. Dephosphorylation of Lsp1p upon citric acid stress suggested the possible involvement of components of the cell wall integrity pathway in the adaptation of *S. cerevisiae* to citric acid stress.

6.2. Future work

With the growing development and success of genome and proteome wide approaches, elucidation of interactions between proteins involved in the same biological processes is becoming more and more easily achievable. Unfortunately the complexity of these interactions is often high. Many proteins function within complicated cellular pathways, interacting with other proteins more commonly as components of larger complexes. Also many of the proteins crucial in cellular processes and signalling pathways undergo post-translational modifications such as reversible phosphorylation, this modification of proteins can itself change protein-protein interactions. With two key proteins of interest in this study being protein kinases, further approaches allowing a better understanding of their citric acid induced signalling cascades would be beneficial.

Up-stream components of the Hog1p signalling cascade have been extensively studied with respect to osmotic stress. However, to identify if citric acid stress regulates a different up-stream response in activating Hog1p, a multicopy suppressor screen could be carried out. Since very little is known about the upstream effectors of Sky1p this approach would also be beneficial. Specifically a screen for multicopy suppressor gene(s) that rescue the citric acid sensitive phenotypes of both Δ*hog1* and Δ*sky1* could be informative. Similar methodologies have proven to be successful when studying deletion strains exposed to temperature stress (Ohkuni *et al.*, 2006).

An increasingly popular and successful technique used for identification of protein interactions is the tandem affinity purification (TAP) method (Puig *et al.*, 2001). It allows rapid purification under native conditions of complexes, even when expressed at their natural level. This approach could be valuable for identification of proteins interacting

with our target proteins Hog1p and Sky1p and could also be used to detect any changes in their protein interactions upon exposure to citric acid.

Our data gained from the phosphoproteomic analysis of BY4741a upon exposure to citric acid revealed changes in phosphorylation of some interesting proteins. Phosphoprotemic analysis for both Δ*sky1* and Δ*hog1* would complete the proteome/phosphoproteome analyses and could reveal phosphoproteins whose phosphorylation state is regulated by either Sky1p or Hog1p.

To determine what signalling processes occur downstream of Sky1p and Hog1p to enable citric acid adaptation, it is important to identify each of the kinases' substrates. A novel chemical genetic approach for global substrate analysis using 2D electrophoresis and an [γ-32P] ATP analog has recently successfully identified four further substrates of Hog1p phosphorylated upon osmotic shock (Kim and Shah, 2007). This technique could prove successful for substrate identification for Sky1p and Hog1p under citric acid stress.

Since our results suggest that citric acid does not actually enter the cell, this implies that the initial stress response signal is relayed from sensory proteins in the plasma membrane. Our previous attempts to perform proteomic analyses of membrane proteins with and without exposure to citric acid were unsuccessful. However, optimisation of membrane protein separation and purification methods could remedy the problems encountered. Failing that, other techniques previously shown to be successful for membrane protein analysis such as SDS/SDS-PAGE, 16-BAC/SDS-PAGE, CTAB/SDS-PAGE, and BN-PAGE could be used (Weber *et al*., 2004; Braun et al., 2007; Randelj *et al*., 2007).

The work presented in this thesis has provided significant information facilitating the elucidation of the mechanisms involved in citric acid adaptation in *S. cerevisiae*. We have substantial evidence to suggest that the SR protein kinase Sky1p is involved in citric acid tolerance and also that it shares some functional redundancy and perhaps functional linkage with Hog1p, another protein kinase involved in citric acid adaptation. Further studies to identify intermediary proteins that are involved in the signal transduction pathways mediating citric acid resistance would help clarify the exact role of Sky1p and the relationship it has with Hog1p in mediating resistance to citric acid.

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Appendix I: Growth curves and tables

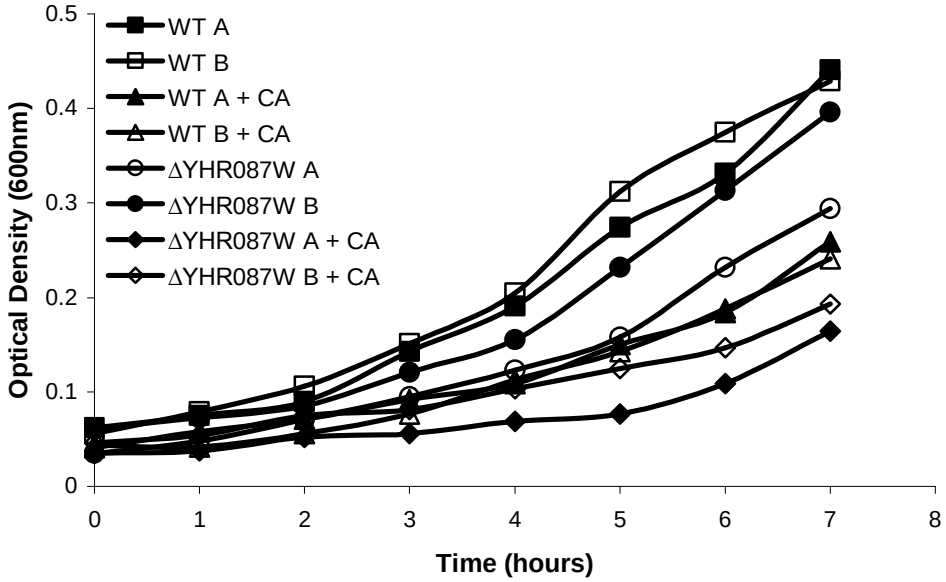


Figure & Table i: Growth rates of WT and Δ*YHR087W* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
WT	0.325	0.272	16.5
Δ*YHR087W*	0.307	0.179	41.7
		% Sensitivity Difference (Δ – WT)	**25.2**

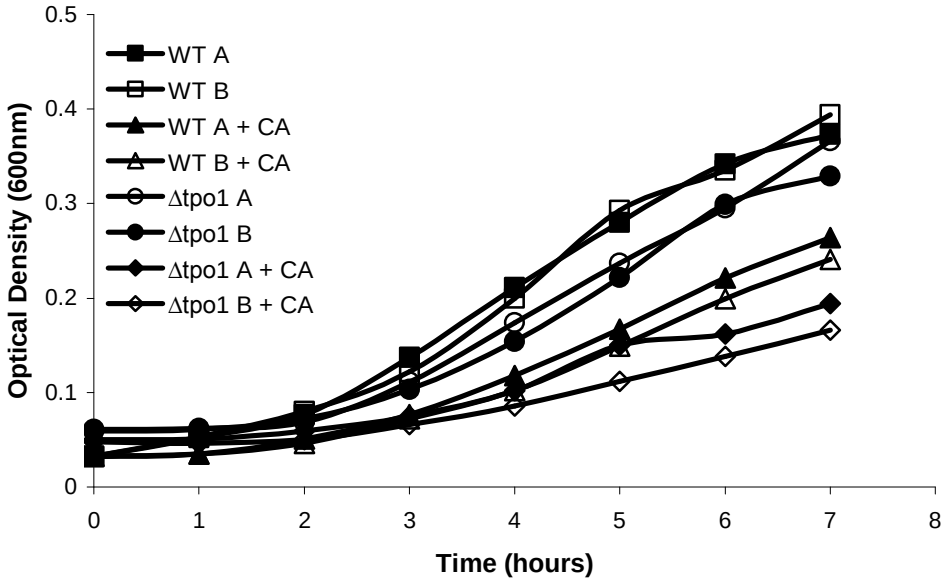


Figure & Table ii: Growth rates of Wt and Δ*tpo1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.334	0.199	40.5
Δ*tpo1*	0.323	0.192	40.6
		% Sensitivity Difference (Δ – WT)	**0.1**

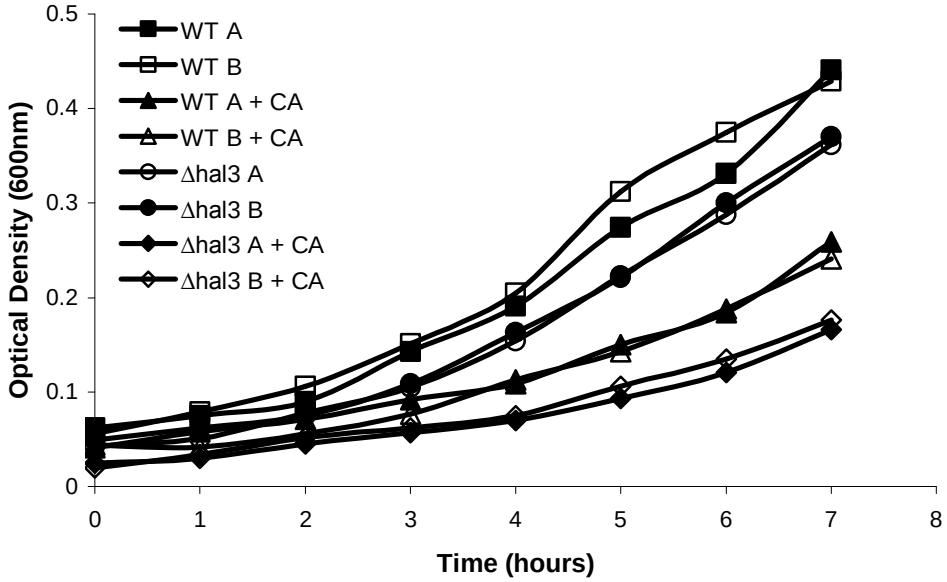


Figure & Table iii: Growth rates of Wt and Δ*hal3* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.325	0.272	16.5
Δ*hal3*	0.342	0.246	28.3
		% Sensitivity Difference (Δ – WT)	**11.8**

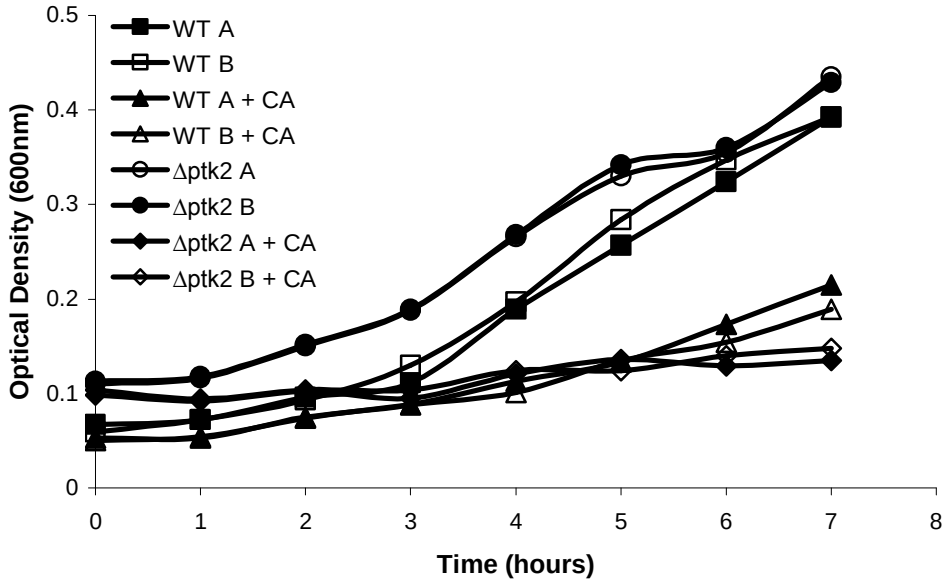


Figure & Table iv: Growth rates of Wt and Δ*ptk2* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.334	0.199	40.5
Δ*ptk2*	0.230	0.080	65.3
		% Sensitivity Difference (Δ – WT)	**24.8**

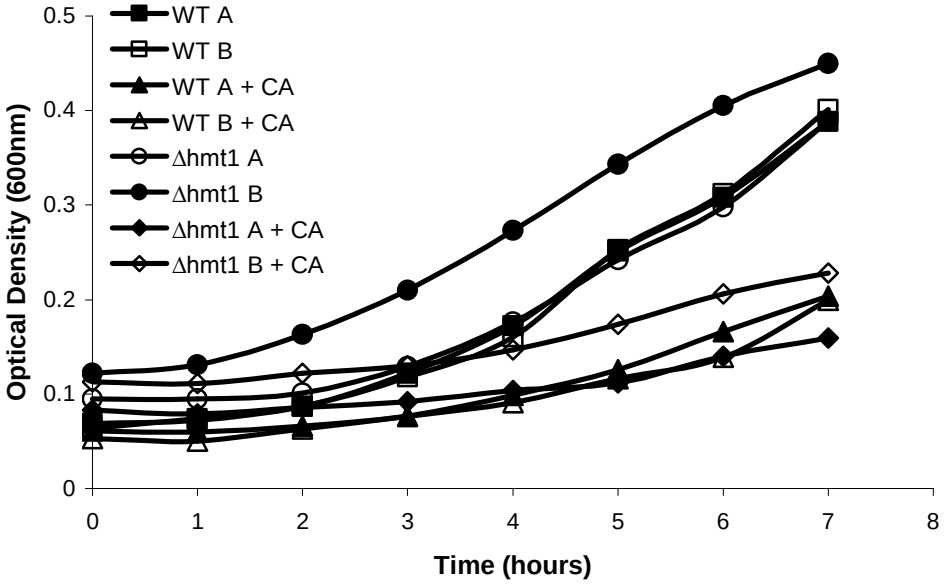


Figure & Table v: Growth rates of Wt and Δ*hmt1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.328	0.217	33.9
Δ*hmt1*	0.255	0.126	50.8
		% Sensitivity Difference (Δ – WT)	**16.9**

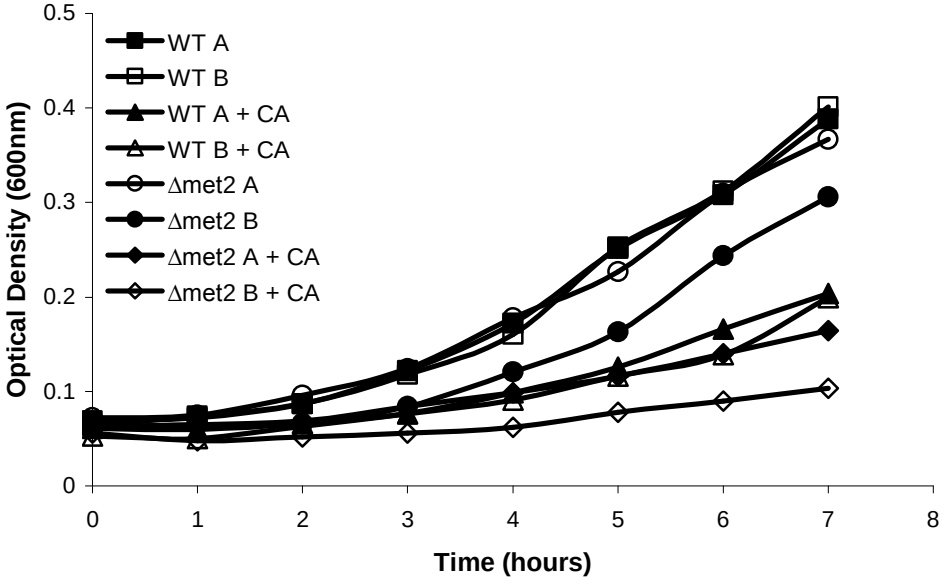


Figure & Table vi: Growth rates of Wt and Δ*met2* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.328	0.217	33.9
Δ*met2*	0.311	0.158	49.2
		% Sensitivity Difference (Δ – WT)	**15.3**

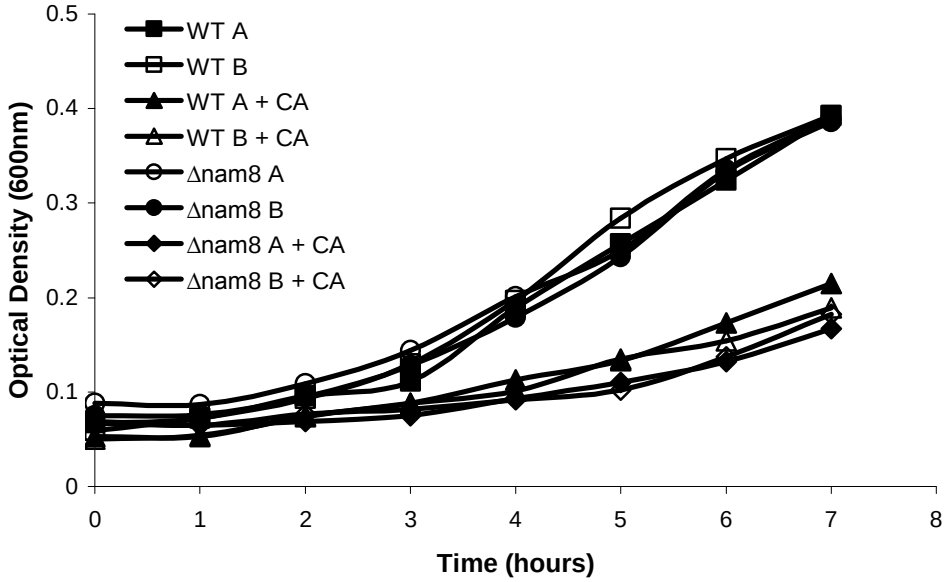


Figure & Table vii: Growth rates of Wt and Δ*nam8* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.328	0.217	33.9
Δ*nam8*	0.297	0.153	48.7
		% Sensitivity Difference (Δ – WT)	**14.8**

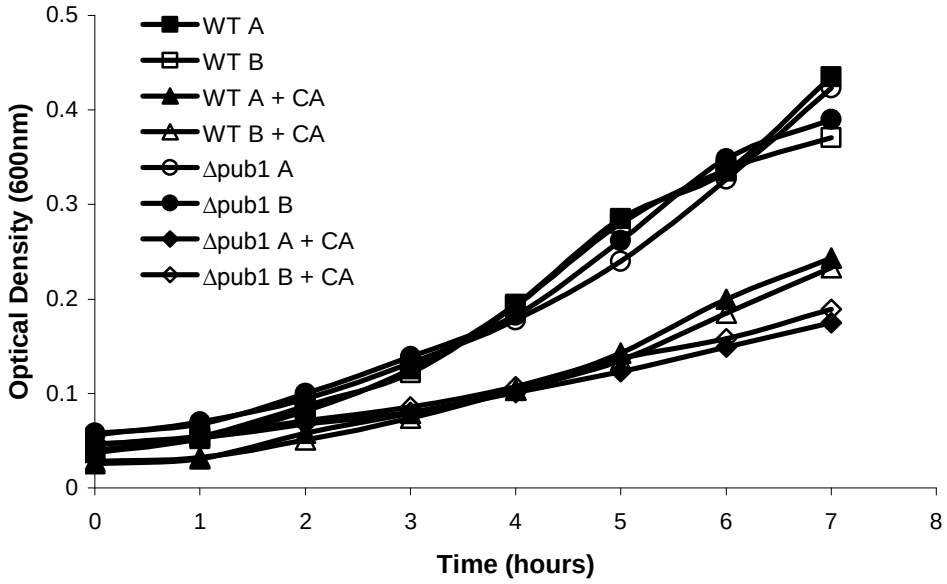


Figure & Table viii: Growth rates of Wt and Δ*pub1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.360	0.312	13.2
Δ*pub1*	0.311	0.203	34.7
		% Sensitivity Difference (Δ – WT)	**21.5**

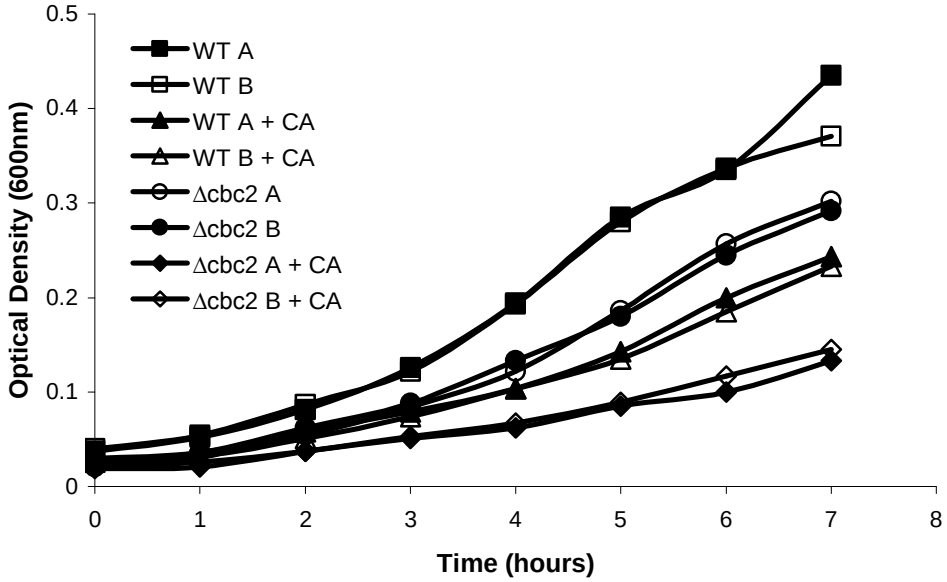


Figure & Table ix: Growth rates of Wt and Δ*cbc2* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.360	0.312	13.2
Δ*cbc2*	0.367	0.266	27.5
		% Sensitivity Difference (Δ – WT)	**14.3**

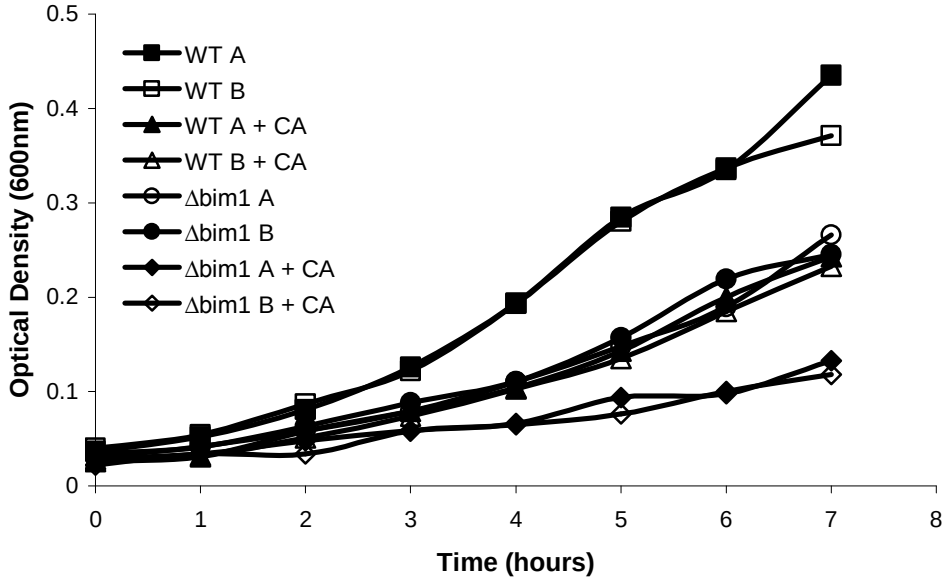


Figure & Table x: Growth rates of Wt and Δ*bim1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.360	0.312	13.2
Δ*bim1*	0.302	0.216	28.4
		% Sensitivity Difference (Δ – WT)	**15.2**

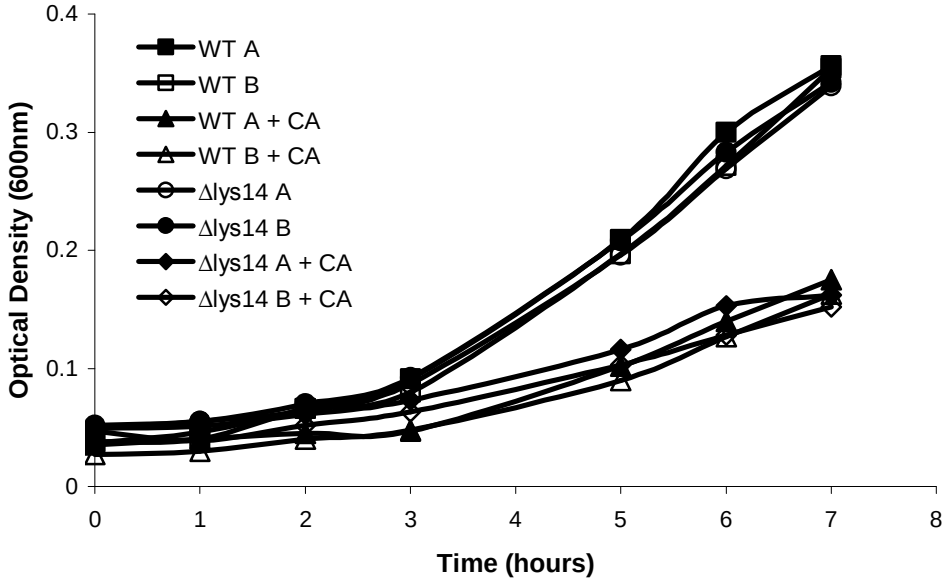


Figure & Table xi: Growth rates of Wt and Δ*lys14* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.380	0.299	21.2
Δ*lys14*	0.365	0.230	37.0
		% Sensitivity Difference (Δ – WT)	**15.8**

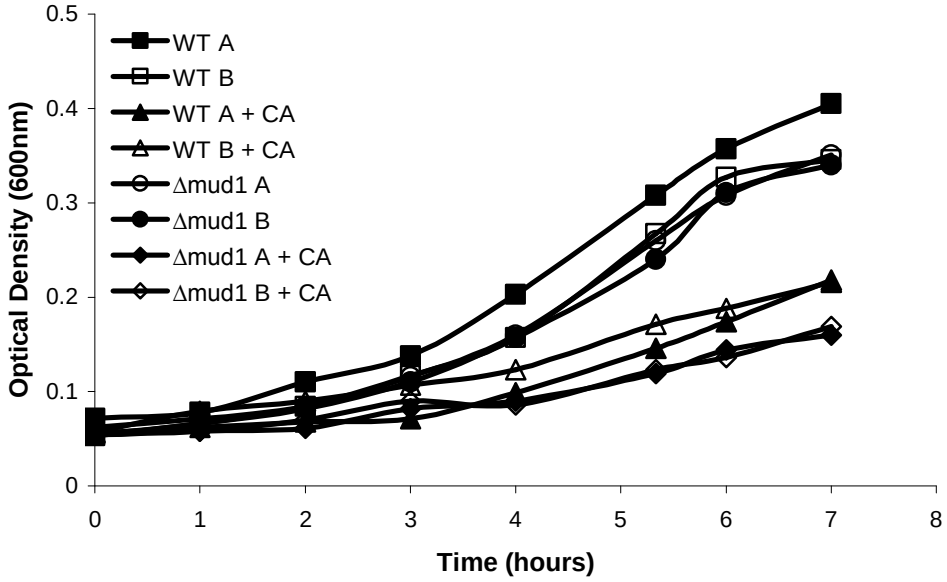


Figure & Table xii: Growth rates of Wt and Δ*mud1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.335	0.227	32.2
Δ*mud1*	0.343	0.187	45.6
		% Sensitivity Difference (Δ – WT)	**13.4**

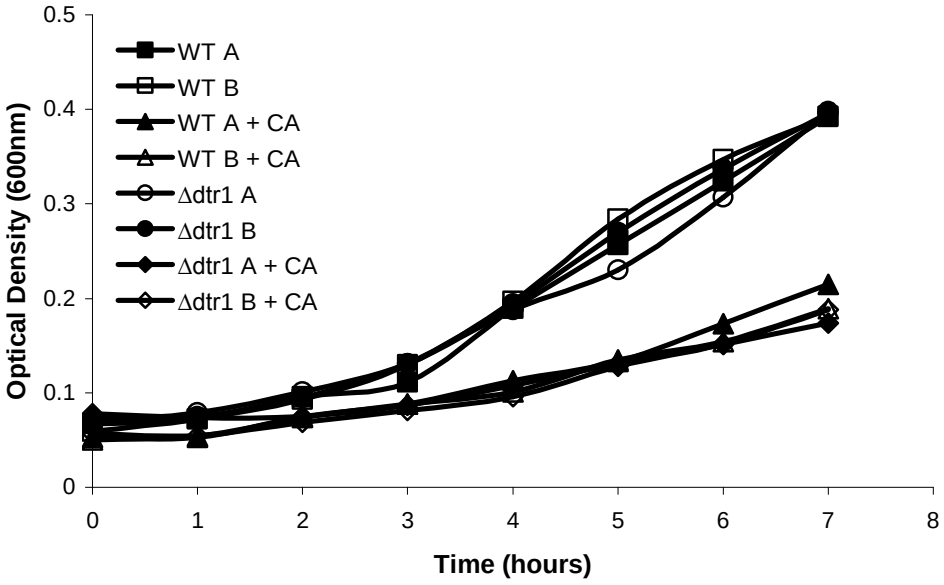


Figure & Table xiii: Growth rates of Wt and Δ*dtr1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.334	0.199	40.5
Δ*dtr1*	0.302	0.192	36.4
		% Sensitivity Difference (Δ – WT)	**-4.1**

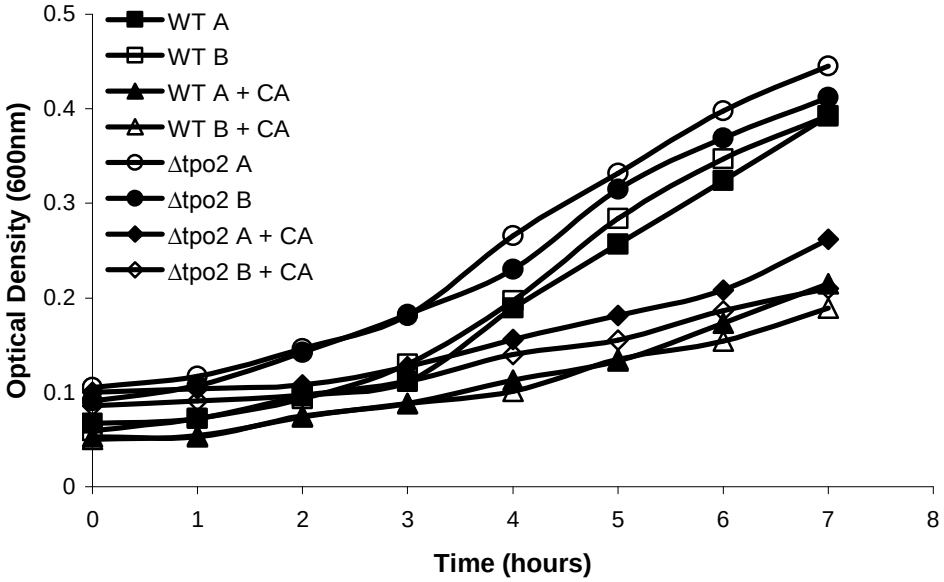


Figure & Table xiv: Growth rates of Wt and Δ*tpo2* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.334	0.199	40.5
Δ*tpo2*	0.254	0.165	35.4
		% Sensitivity Difference (Δ – WT)	**-5.1**

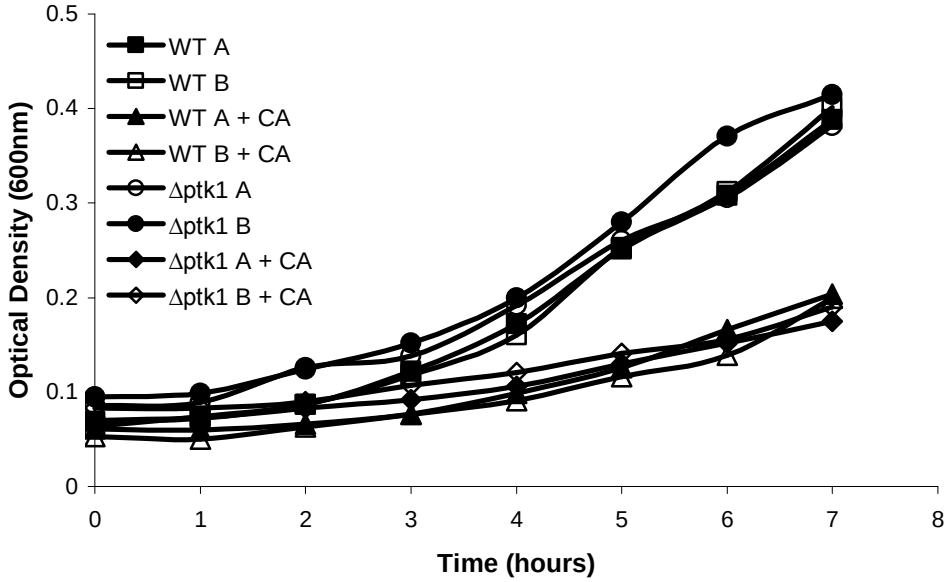


Figure & Table xv: Growth rates of Wt and Δ*ptk1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.328	0.217	33.9
Δ*ptk1*	0.233	0.147	37.1
		% Sensitivity Difference (Δ – WT)	**3.2**

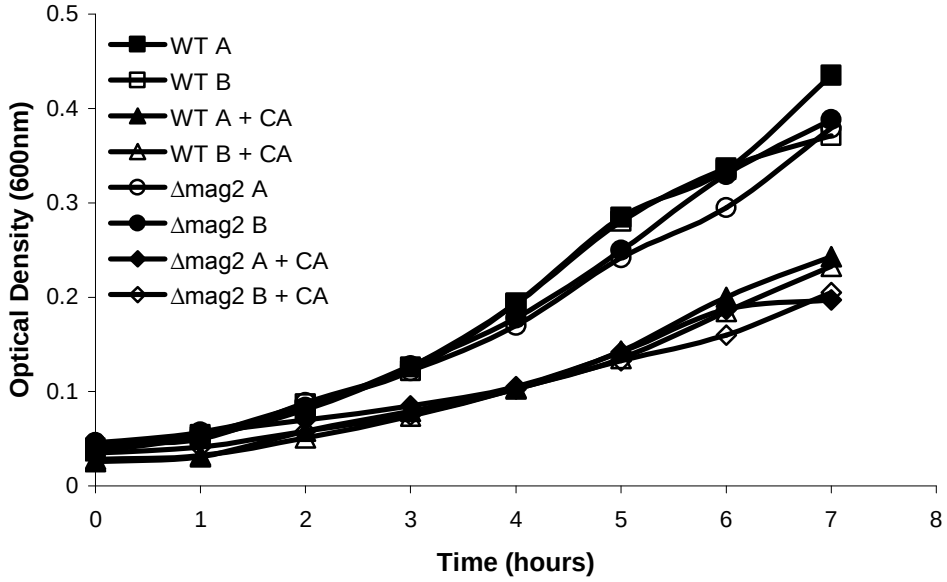


Figure & Table xvi: Growth rates of Wt and Δ*mag2* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.360	0.312	13.2
Δ*mag2*	0.327	0.254	22.3
		% Sensitivity Difference (Δ – WT)	**9.1**

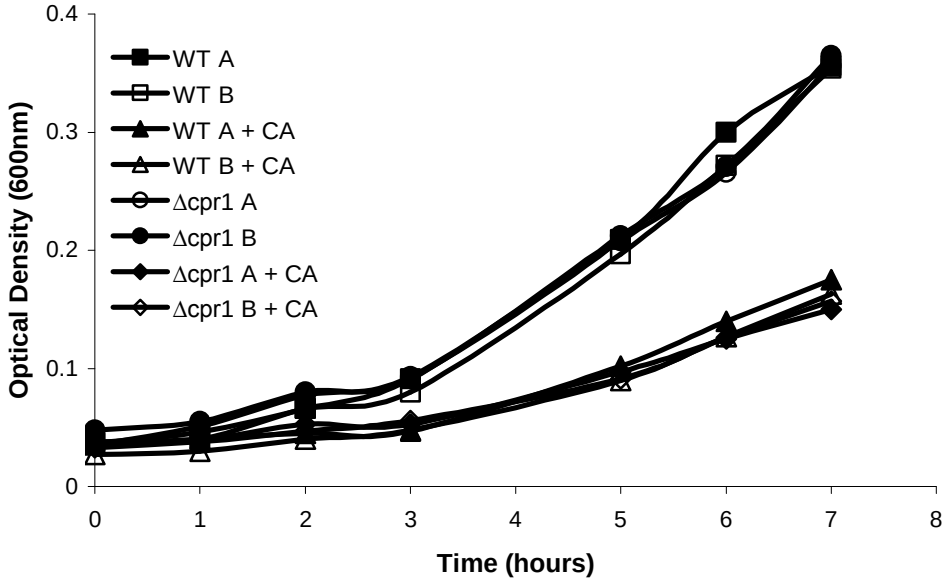


Figure & Table xvii: Growth rates of Wt and Δ*cpr1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.380	0.299	21.2
Δ*cpr1*	0.329	0.239	27.3
		% Sensitivity Difference (Δ – WT)	**6.1**

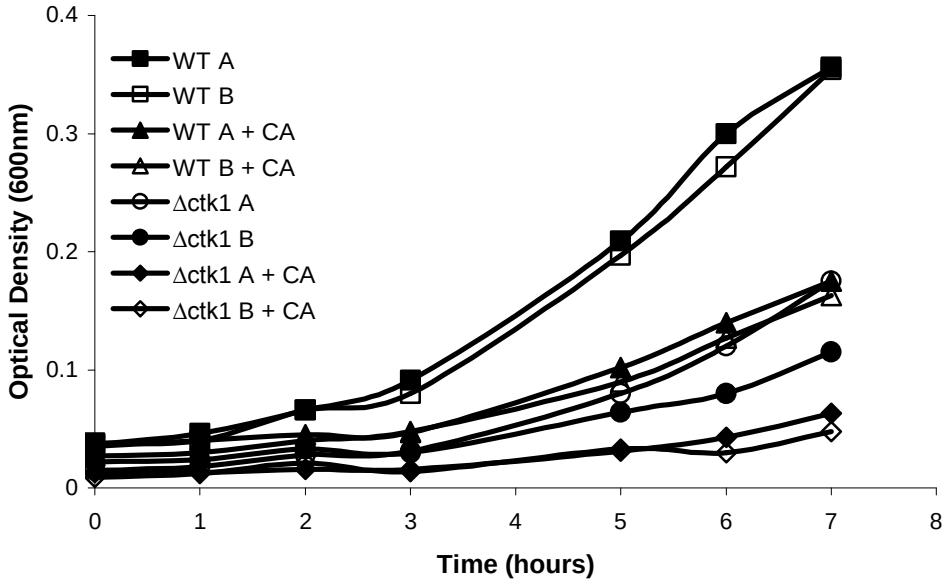


Figure & Table xviii: Growth rates of Wt and Δ*ctk1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.335	0.227	32.2
Δ*ctk1*	0.197	0.137	30.5
		% Sensitivity Difference (Δ – WT)	**-1.7**

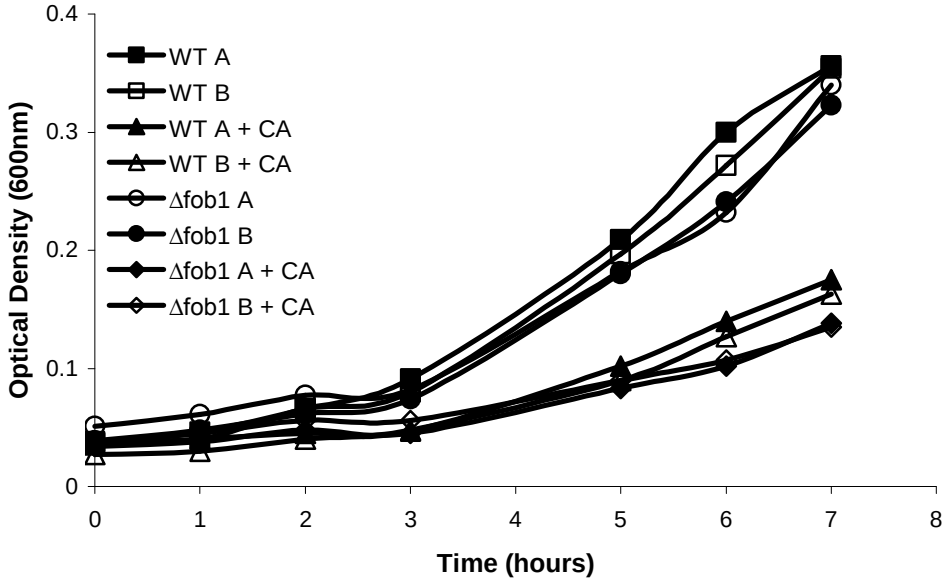


Figure & Table xix: Growth rates of Wt and Δ*fob1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.335	0.227	32.2
Δ*fob1*	0.327	0.208	36.4
		% Sensitivity Difference (Δ – WT)	**4.2**

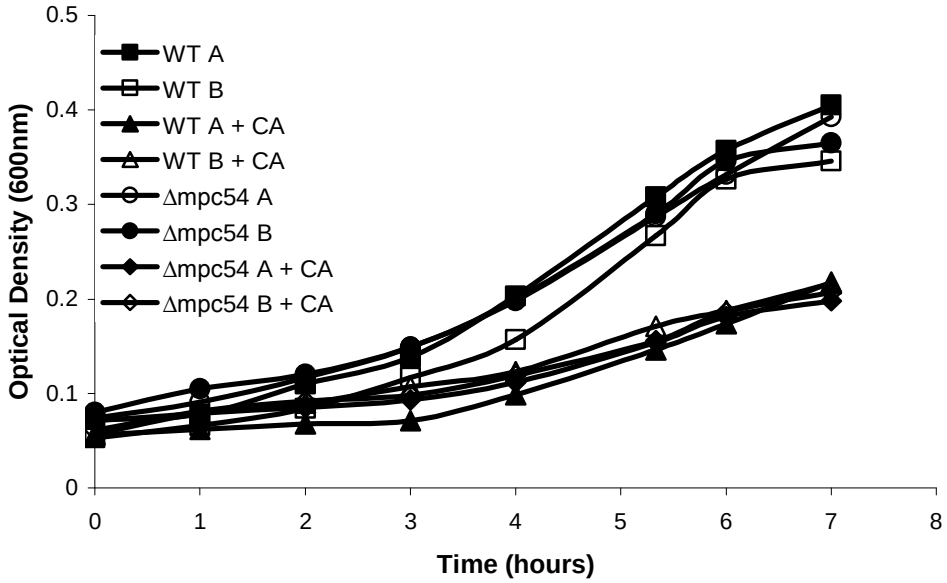


Figure & Table xx: Growth rates of Wt and Δ*mpc54* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.335	0.227	32.2
Δ*mpc54*	0.276	0.195	29.6
		% Sensitivity Difference (Δ – WT)	**-2.6**

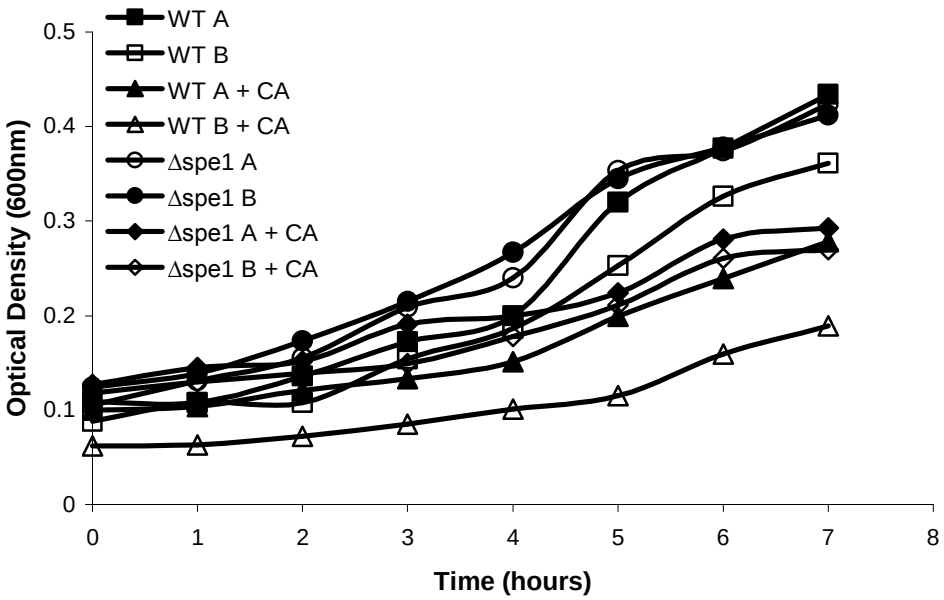


Figure & Table xxi: Growth rates of Wt and Δ*spe1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.268	0.183	32.0
Δ*spe1*	0.216	0.150	30.7
		% Sensitivity Difference (Δ – WT)	**-1.3**

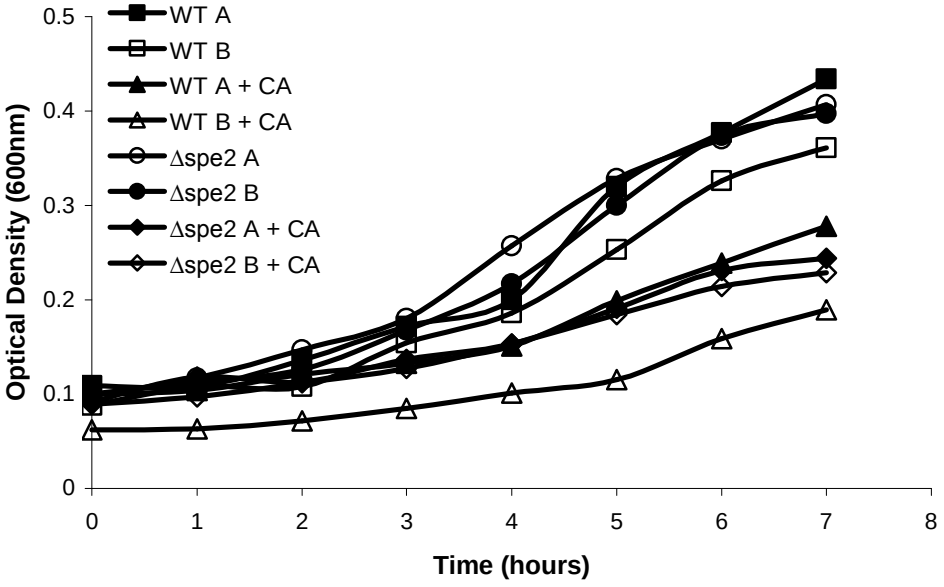


Figure & Table xxii: Growth rates of Wt and Δ*spe2* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.268	0.183	32.0
Δ*spe2*	0.260	0.171	34.1
		% Sensitivity Difference (Δ – WT)	**2.1**

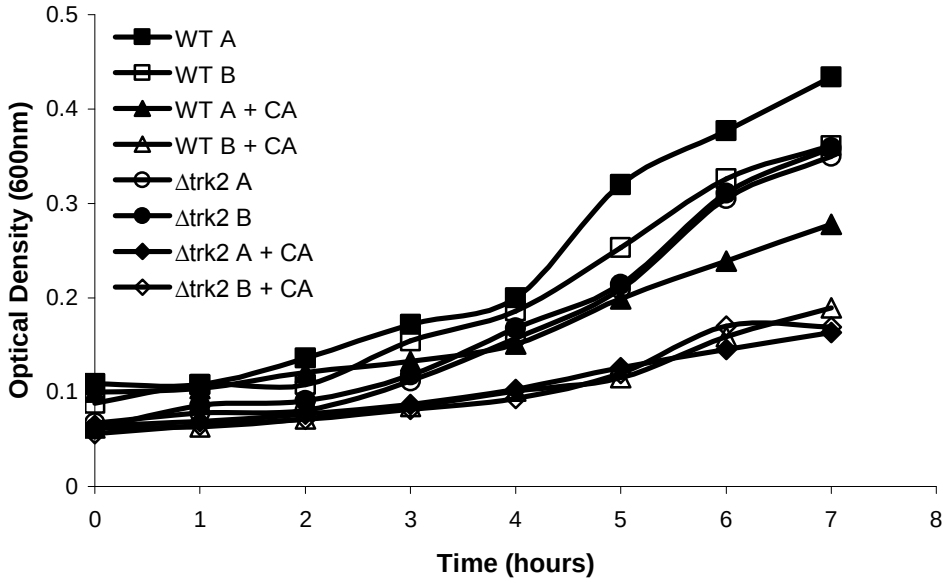


Figure & Table xxiii: Growth rates of Wt and Δ*trk2* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.268	0.183	32.0
Δ*trk2*	0.316	0.188	40.5
		% Sensitivity Difference (Δ – WT)	**8.5**

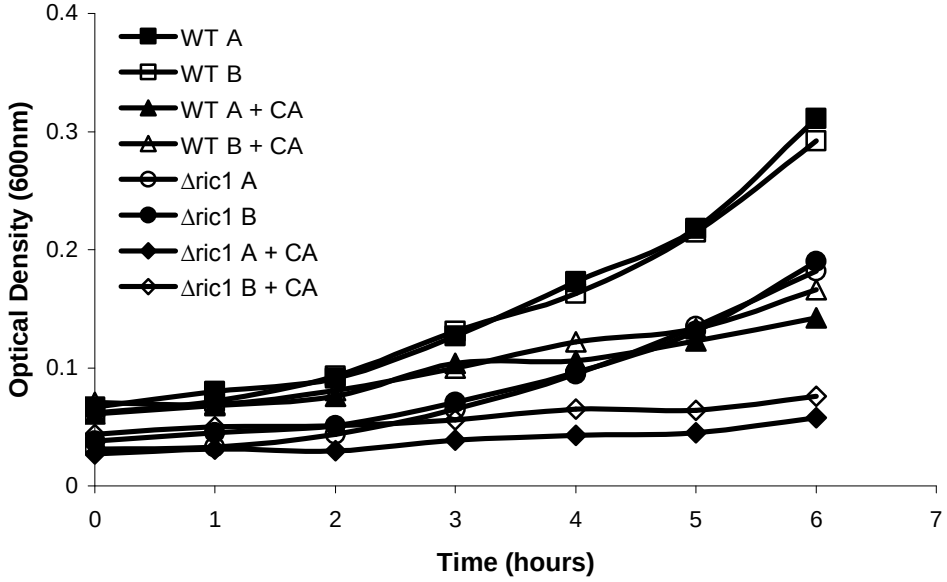


Figure & Table xxiv: Growth rates of Wt and Δ*ric1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.262	0.149	43.2
Δ*ric1*	0.295	0.103	65.0
		% Sensitivity Difference (Δ – WT)	**21.8**

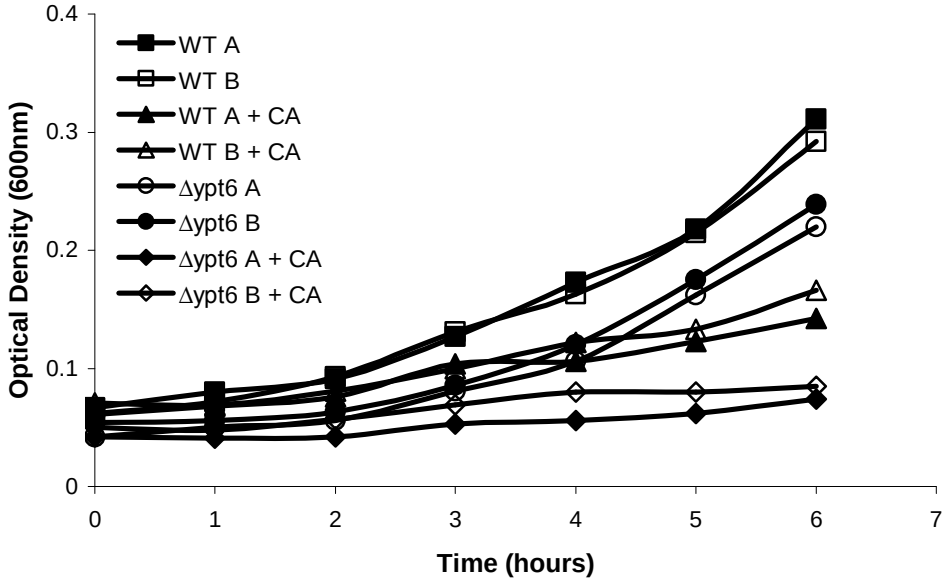


Figure & Table xxv: Growth rates of Wt and Δ*ypt6* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.262	0.149	43.2
Δ*ypt6*	0.274	0.103	62.5
		% Sensitivity Difference (Δ – WT)	**19.3**

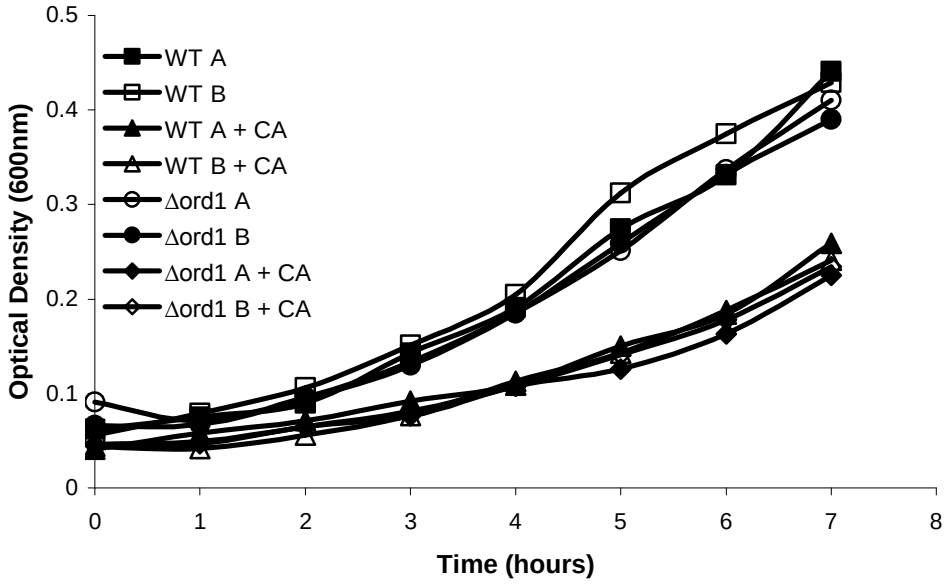


Figure & Table xxvi: Growth rates of Wt and Δ*ord1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.325	0.272	16.5
Δ*ord1*	0.318	0.246	22.7
		% Sensitivity Difference (Δ – WT)	**6.2**

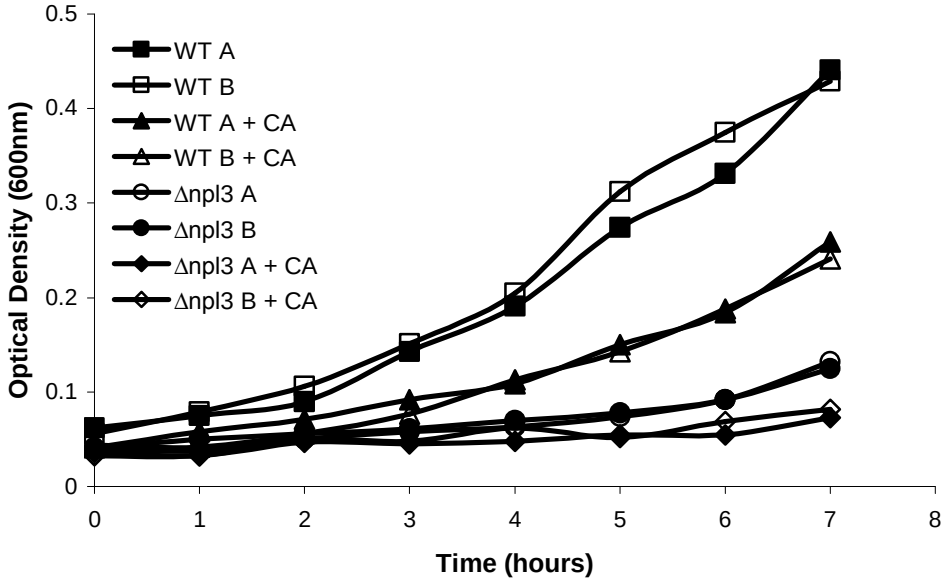


Figure & Table xxvii: Growth rates of Wt and Δ*npl3* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.325	0.272	16.5
Δ*npl3*	0.135	0.062	54.1
		% Sensitivity Difference (Δ – WT)	**37.6**

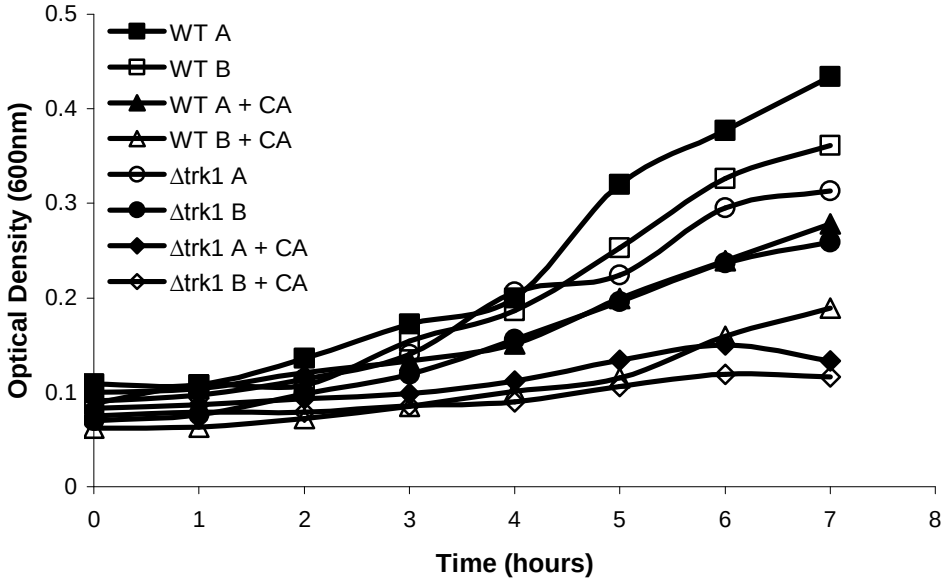
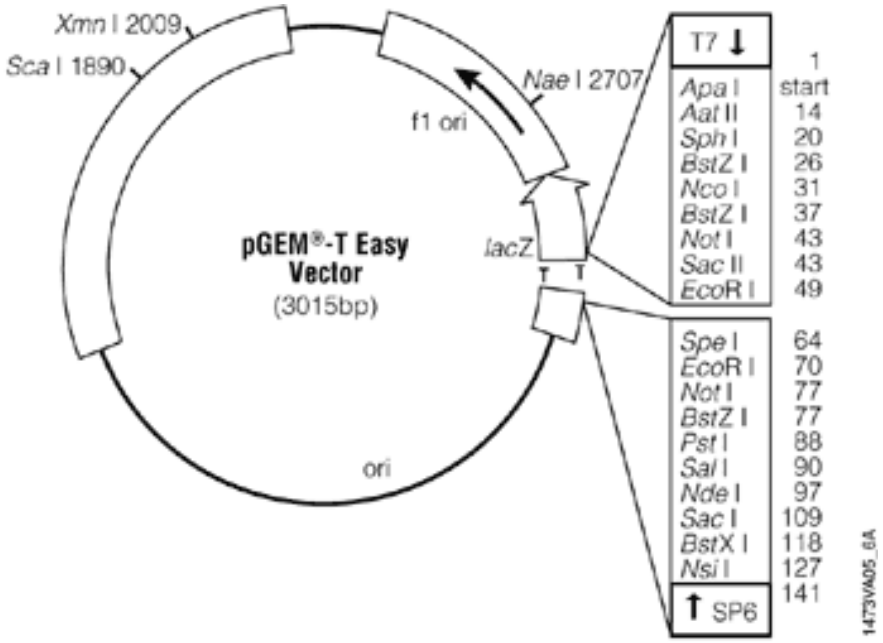


Figure & Table xxviii: Growth rates of Wt and Δ*trk1* and % sensitivity to citric acid

Strain	Growth Rate - No Citric acid	Growth Rate -300mM Citric Acid	% Sensitivity to Citric acid
Wt	0.268	0.183	32.0
Δ*trk1*	0.232	0.114	50.7
		% Sensitivity Difference (Δ – WT)	**18.7**

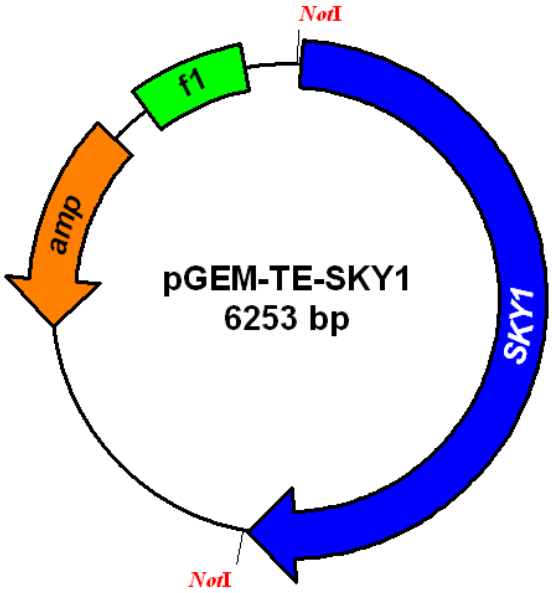
Appendix II: Plasmid maps

P-GEM-T-Easy Vector plasmid map (Promega Ltd., Hampshire, UK)



(URL: http://www.promega.com/figures/frame.asp?fn=1473va)

pGEM-TE-SKY1 plasmid map



pRS313 & pRS423 Plasmid maps, (Sikorski, 1989).