Molecular Genetic Analysis of Presevative Resistance In Zygosaccharomyces bailii.

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ABSTRACT

The ascomycetous yeast Zygosaccharomyces bailii (Z. bailii) is one of the microorganisms most commonly associated with the spoilage of foods and beverages. This
yeast can adapt to become resistant to the highest levels of those weak acid food
preservatives (primarily bisulphite, sorbic, benzoic, acetic and propionic acids) allowed
in food preservation. Also, under aerobic conditions, it can catabolise some of these
preservatives. Z. bailii is also tolerant of in excess of 20% ethanol and a major cause of
serious spoilage of bottled wine. This project has been the first to isolate genes of Z.
bailii and prove that it is possible to investigate genetically the mechanism of weak acid
adaptation in the food spoilage yeast Z. bailii.

Z. bailii sequences that complement the ura3 and trp1 mutations of Saccharomyces cerevisiae were initially isolated and sequenced. Also isolated was a Z. bailii gene (ZbYME2) that confers upon S. cerevisiae cells the ability to utilise the preservative benzoic acid as sole carbon source.

Using DNA cassettes containing dominant selectable markers (KanMX4, hphMX4 and new gene disruption cassette (SFA1MX4)), together with methods that were originally devised for gene deletion in S. cerevisiae, the two copies of ZbYME2 in the genome of Z. bailii strain 1427 were sequentially deleted. Unlike the original wild type isolate, the homozygous Zbyme2/Zbyme2 deletant strain lacks the ability to catabolise benzoate. This is a proof of the principle that it is possible to delete genes in Z. bailii, especially those genes that are suspected to be important for the growth of this yeast in preserved foods and beverages. Physiological analysis of this and other Z. bailii deletant strains should reveal the molecular mechanisms that provide this yeast with an exceptional capacity to adapt to conditions of food preservation. This in turn will allow the rational design of new food preservation strategies. Genes from both S. cerevisiae and Z. bailii that act as the multicopy suppressors of the S. cerevisiae weak acid sensitive pdr12 mutant were also isolated. The isolated S. cerevisiae genes (YOR114w, YPL246C) are ORFs of unknown function. The Z. bailii DNA fragments contained a YOR114w homologue and the gene for a small internal fragment of apocytochrome b. The later appears to be a fragment of mitochondrial DNA that has "escaped" to the nucleus. These may represent new genes of weak acid resistance in Z. bailii.

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This thesis is dedicated to those who encouraged me throughout my research.

ABBREVIATIONS

A absorbance

ATP Adenosine-5'-triphosphate

a_w water activity bp base pair

bis-acrylamide N,N'-methylene bisacrylamide

BSA bovine serum albumin C-terminal carboxy terminal $Ci(\mu Ci)$ curies (miro-curies)

d day

dATP adenosine deoxyribonucleoside triphosphate dCTP cytidine deoxyribonucleoside triphosphate

DEPC diethylpyrocarbonate

dGTP guanosine deoxyribonucleoside triphosphate

dH₂O de-ionised plus 1 x distilled water

DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid

dNTP equimolar mixture of dATP, dCTP, dGTP, dTTP

DTT dithiothreitol

dTTP thymine deoxyribonucleoside triphosphate

EDTA ethylenediaminetetraacetic acid

GFP green florescent protein

h hour

HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])

HSP heat shock protein

Kb kilobase KDa kilodalton

LFH long flanking homology

min minutes

mRNA messenger ribonucleic acid

mtDNA mitochondria deoxyribonucleic acid

N-terminal amino terminal
OD optical density
ORF open reading frame

PDR pleiotropic drug resistance PMSF phenylmethanesulphonyl floride

RNA ribonucleic acid

RNase ribonuclease rpm revolution per m

rpm revolution per minute
SDS sodium dodecyl DNA
SFH short flanking homology

TE Tris-EDTA buffer

TPCK toluene-4-sulphonamido-2-phenyl ethyl chloromethyl ketone

UV ultraviolet

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Chapter 1. Introduction

For centuries, man has applied natural preservatives and preservation methods, generally without knowing how these have helped to protect food from spoilage. Natural preservatives exist in herbs and spices, also plants such as onion, garlic and chives. Not only they can be responsible for the characteristics of taste and smell, but they can also influence microbiological stability. The active essential oils and related substances in herbs often act to make the cell membrane of fungi permeable, causing the contents to leak out. Other naturally occurring preservatives are weak organic acids. They include benzoic, acetic and sorbic acids. These three acids, together with propionate and sulphite, constitute the acids most widely used in large-scale food and beverage preservations. The use of some of these preservatives can be traced back many centuries, for example, burning of sulphur to sterilise the wooden vessels used in cider making and the use of acetic acid in vinegars and pickles.

1.1. Zygosaccharomyces yeasts and the problems posed by their unique stress resistances.

The conditions imposed by many preserved food materials (either low water activity (a_w); low pH; the presence of high preservative levels, carbon dioxide or ethanol; or the absence of oxygen) do not represent the ideal environments for microbial growth. The major spoilage threat to those materials of low a_w and/or low pH is frequently from yeasts and fungi. Most food-relevant bacteria, in contrast, thrive only at pH values closer to neutrality (some, though, such as the *Lactobacilli* will grow down to about pH4.5) (Deak, 1991; Fleet, 1992).

Species of the yeast genus Zygosaccharomyces are responsible for spoilage of beverages and preserved foods. This is partly a problem associated with achieving sterility in large-scale manufacture, since it is frequently contamination of the processing equipment that leads to contamination of foods with these yeasts (Deak, 1991; Fleet, 1992). The unique stress resistance properties of Zygosaccharomyces yeasts also contribute to the problem of food contamination. Z. rouxii, Z. bisporus, Z. bailii, and

Z. lentus pose the most serious problems to the food industry. Z. rouxii appears to constitute the most important Zygosaccharomyces from the standpoint of "xerotolerance" (the ability to grow at low aw). It can grow in substrates with high sugar concentrations that impose aw values as low as 0.76. Z. bailii and Z. lentus display an unusually high resistance to weak acids, including the small number currently approved for use as food preservatives (primarily bisulphite, sorbic, benzoic, acetic and propionic acids) (Thomas and Davenport, 1985). Z. bailii can often adapt to become resistant to the highest levels of these weak acids allowed in food preservation, at pH values below the pKa's of these acids (Thomas and Davenport, 1985). Z. bailii can also degrade preservatives such as sorbate and benzoate, provided oxygen is present (Mollapour and Piper, 2000; Müncnerova and Augustin, 1994; Praphailong and Fleet, 1997) and it can also tolerate ethanol levels in excess of 20%. This yeast is therefore a major cause of spoilage of bottled wine (Kalathenos, et al., 1995; Thomas and Davenport, 1985). Z. bailii tends to form spontaneous resistant colonies when grown on high concentrations of sorbate at low pH (Steels, et al., 2000). When Z. bailii cells were spread on 15mM and 20mM sorbate spontaneous resistance colonies were formed (Figure 1.1a,b) (P.W. Piper and M. Mollapour, unpublished data). This "inoculum effect" was used to explain the high Z. bailii resistance to sorbate (Steels, et al., 2000). However the reasons for this inoculum effect have not been explained at the molecular level.

Z. bailii colonies are usually white to cream cone and smooth, often with brownish top. The cells are cylindrical and/or ellipsoidal and slightly larger than S. cerevisiae cells (Figure 1.2a). In the literature there is reference to Z. bailii ascospores and the conjugation of cells prior to ascospore development (Thomas and Davenport, 1985). However these processes have never been described in any detail. Preliminarily observation of Z bailii sporulation over 14 days at 25°C on sporulation medium (0.3% malt extract, 0.5% peptone) revealed the cells forming the protrusions and spores (denoted "conjugation tubes" and "ascospores" in (Thomas and Davenport, 1985) seen earlier (Figure 1.2b). However many of the cells eventually sporulated without forming protrusions and the large zymolase-resistant structures formed on sporulation medium appear to be vegetative, not meiotic spores. So far I have not obtained any evidence of a sexual cycle in Z. bailii.

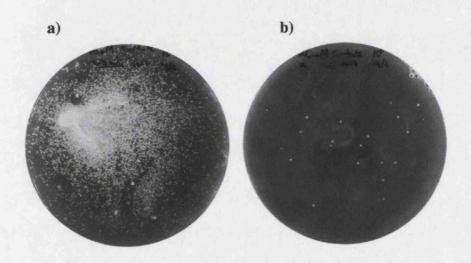


Figure 1.1. Z. bailii spontaneous resistant colonies were formed when 10^8 mid-log grown cells were spread on pH4.5 YPD with a) 15mM or b) 20mM sorbate. Plates were photographed after 5d incubation at 30°C .

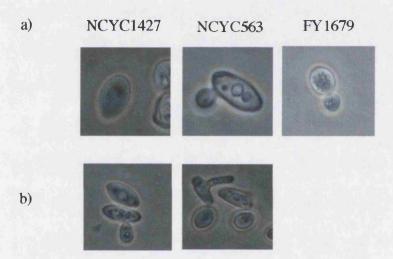


Figure 1.2. a) Z. bailii cells NCYC1427, NCYC563 and S. cerevisiae cells FY1679. b) Conjugation of Z. bailii cells prior to ascospore development.

1.2 What is weak acid stress?

At neutral pH, residues of acetic acid (pKa 4.75), sorbic acid (pKa 4.76) or benzoic acid (pKa 4.19) pose little threat and may even provide microbes with a potential carbon source. In contrast at low pH, these acids are potent growth inhibitors. Figure 1a shows the general perception of how they inhibit microbial growth. At low pH a substantial fraction of the acid will exist in the undissociated state (XCOOH; Figure 1.3a), a form that is readily able to diffuse across the cell membrane and dissociate in the higher pH environment of the cytosol. Such dissociation generates protons and the acid anion (XCOO-; Figure 1.3a). The proton release might acidify the cytosol, which in turn will inhibit many metabolic functions (Krebs, et al., 1983). The acid anion can also cause a problem since, being charged it cannot very readily diffuse from the cell. It will therefore accumulate in the cell to very high levels, potentially causing an abnormally high intracellular turgor pressure. This high anion accumulation can also influence free radical production, severe oxidative stress being one major factor associated with weak acid stress in *S. cerevisiae* (see section 1.8).

While the antimicrobial effects of weak organic acids at low pH have often been attributed to this intracellular acidification and anion accumulation (Russell, 1991; Salmond, et al., 1984), it is most unlikely that this represents the complete explanation of their actions or that all weak acids are operating in an identical manner. There have been a number of side-by-side comparisons of the effects of acetate and sorbate on yeast (Bracey, et al., 1998; Stratford and Anslow, 1996; Stratford and Anslow, 1998). These show that while acetate could be exerting its main inhibitory effects as in figure 1.3a, more hydrophobic acids such as sorbate are exerting their effects mainly through a disordering of membrane structure. A strong action of many weak organic acid preservatives on membranes is indicated by the tendency of these acids to become much more potent growth inhibitors as they become more lipophilic (Holyoak, et al., 1999; Piper, et al., 1998). For example, quite high concentrations (100-200mM) of acetic acid (pKa 4.75) are needed to effectively inhibit the growth of S. cerevisiae at pH4.5. In contrast only 1-5mM of the more liposoluble benzoic (pKa 4.19) or sorbic (pKa 4.76) acid to achieves the same degree of inhibition (Piper, et al., 1998; Stratford and Anslow, 1996). Growth inhibition is therefore determined not just by the level of undissociated acid (XCOOH; Figure 1.3a), but also by the lipophilicity of this acid. The effects on membranes will in turn have major effects on

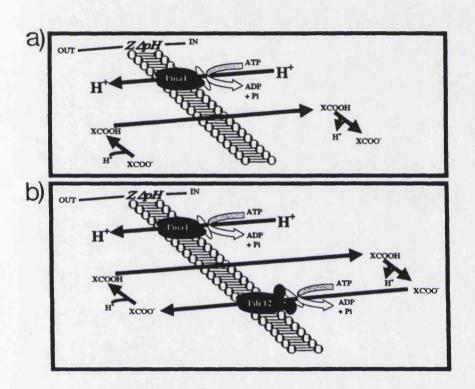


Figure 1.3. Schematic model of how the Pdr12 ABC transporter may help S. cerevisiae counteract the inhibitory effects of water-soluble weak organic acids. In both unadapted cells (a) and cells which have adapted to grow in the presence of the weak acid (b) the protonated, uncharged form of the acid (XCOOH) is shown as freely permeable to the cell membrane and readily entering by diffusion. In unadapted cells (a) the concentration of XCOOH inside and outside should be the same, governed by the pH either side of the membrane and the dissociation constant of the acid. A higher pH on the cytosolic side of the membrane will cause a substantial fraction of this acid to dissociate to the anion (XCOO), a form which is relatively membrane-impermeant and which therefore accumulates inside the cell. This dissociation also releases protons, potentially causing a cytoplasmic acidification that may inhibit many metabolic processes. The electrochemical potential difference across the plasma membrane ($Z\Delta pH$), a potential maintained largely through plasma membrane H⁺-ATPase (Pma1)-catalysed proton extrusion, is essential for many aspects of homeostasis. The weak acid influx in (a) will act to dissipate the ΔpH , though not the charge (Z), component of this gradient.

membrane transport processes, energy coupling and free radical formation. It is known that sorbate- and benzoate-stressed cells of *S. cerevisiae* and *Z. bailii* are experiencing very severe energy (ATP) depletion (Piper, et al., 1997; Warth and Nickerson, 1991). In addition they are, with oxygen present, suffering from an excessively high production of superoxide free radicals by the mitochondrial electron transport chain (Piper, 1999)(see section 1.8). It is noteworthy that no decreases in intracellular pH were observed in a recent study of the effects of inhibitory sorbate levels on *S. cerevisiae* cells (Bracey, et al., 1998). Such sorbate-stressed yeast may be suffering mainly from the effects of appreciable disruption to membrane organisation and/or the oxidative stress resulting from the sorbate anion accumulation (Piper, 1999).

Even at neutral pH, when sorbate and benzoate should be fully dissociated, high levels of these acids still exert some inhibitory effects (Stratford and Anslow, 1996). Sorbate also induces a transcriptional response in *S. cerevisiae* at pH6.8 (Piper, *et al.*, 1998).

1.3 The main advantage of weak acid adaptation for yeasts is probably to assist survival in slightly acid environments.

Weak organic acids will often be present in the plant materials where fungi grow as saprophytes, environments where growth does not need a high degree of evolutionary specialisation and where the competition among different microbes will often be extreme. Acetate or lactate can be present at quite high concentrations in these situations, since they are products of bacterial fermentation. Acetate is also secreted in high levels by certain yeast genera, such the *Brettanomyces* and *Dekkera* that have attracted attention as spoilage agents in wine fermentations (Pretorius, 2000). In wine fermentations the *S. cerevisiae* is often inhibited, especially at early stages of fermentation, by the acetate production by such competitor microbes. Therefore, while most of the interest in weak acid adaptation stems from the problems that it poses for food preservation, such adaptation has probably evolved to facilitate growth in environments of slightly acid pH, where the presence of high organic acid levels can pose a threat. As described below, it appears that yeasts have developed a stress response that minimises the possibility of water-soluble mono-carboxylic acids accumulation to toxic levels in their cells, a response that enhances acid resistance.

Coincidentally the same response increases resistance to the major weak acid food preservatives, so that it is often essential to employ weak organic acid preservatives at millimolar rather than micromolar level in order to prevent yeast spoilage of foods and beverages.

As mentioned above, it is *Zygosaccharomyces* yeasts, notably *Z. bailii* that constitutes the most weak acid-tolerant organisms known. However, relatively little is known about how *Z. bailii* acquires this remarkable stress resistance. Baker's yeast (*S. cerevisiae*) can only adapt to slightly lower amounts of sorbate and benzoate at pH4.5 as compared to *Z. bailii* and some *S. cerevisiae* strains are food spoilage yeasts (Steels, *et al.*, 2000). Because of inoculum effect, it is actually difficult to place quantitative values to the extent at which their resistance exceeds that of *S. cerevisiae*, since the upper limit of *Z. bailii* resistance increases with the size of the inoculum (Steels, *et al.*, 2000). Also the *S. cerevisiae* weak acid adaptation process is readily amenable to molecular genetic analysis. Therefore, this section focuses mainly on *S. cerevisiae*, where adaptation appears to involve a stress response that is quite distinct from other more widely-studied stress responses, such as those induced by osmostress or heat shock. A picture is emerging of an inducible response that acts to reduce the risk that water-soluble weak organic acids will accumulate to high, potentially toxic levels in the cells of the adapted yeast.

1.4 Weak acid adaptation in S. cerevisiae

In S. cerevisiae weak acid adaptation is readily induced by addition of subinhibitory levels (0.5-2.5mM) of sorbic or benzoic acid to vegetative pH4.5 cultures. Unlike Z. bailii, this yeast can not oxidatively-degrade sorbate or benzoate, so that the presence of these acids effectively provides continuous weak acid stress to S. cerevisiae cultures. Different S. cerevisiae mutants can be readily assessed for differences in resistance to sorbate or benzoate simply by plating strains onto pH4.5 media containing increasing levels of these weak acids (Figure 1.4). There is actually quite a large effect of both genetic background and physiological state on the weak acid resistance of laboratory S. cerevisiae strains. For reasons that are not yet understood, inactivation of the tryptophan biosynthetic pathway

Sorbate

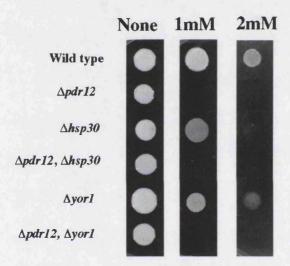


Figure 1.4. Differences in the sensitivities of different yeast strains to any weak organic acid are readily determined by plating dilutions onto low pH media containing increasing amounts of the acid. OD600=0.25 suspensions of stationary glucose (YPD)-grown cultures;

were replicated onto pH4.5 YPD medium containing the indicated amounts of sorbate. The plates were photographed after 2d incubation at 30°C. All the mutants were generated in strain FY1679-28c (Piper, 1998).

leads to a large increase in sensitivity to both benzoate and sorbate (P.W. Piper and M. Mollapour, unpublished data).

Immediately following the application of such subinhibitory weak acid stress, S. cerevisiae cells usually exit the cell cycle and enter a long period of stasis. Eventually, after several hours, they resume growth (Holyoak, et al., 1999; Piper, et al., 1998; Piper, et al., 1997). They are now weak acid-adapted, in that they will not display any transient growth arrest if re-inoculated into fresh pH4.5 medium containing the same levels of sorbate or benzoate. Remarkably, non acid pre-treated cells of the S. cerevisiae cmk1 mutant do not display this requirement for a long period of adaptation and can resume growth almost immediately following application of weak acid stress (Holyoak, et al., 2000). Evidently therefore the weak acid adaptation system is repressed by the Cmk1 isoform of Ca²⁺/calmodulin-dependent protein kinase, a repression that must be relieved in wild-type cells as they undergo weak acid adaptation (Holyoak, et al., 2000).

1.5 The weak acid response of S. cerevisiae leads to the induction of a

discrete ATP-binding cassette (ABC) transporter

In our laboratory weak acid stress was initially shown to inhibit yet another stress response, the heat shock response (Cheng and Piper, 1994). Subsequently it was found that sorbate was inducing an alternative stress response, which lead to dramatic changes to the protein composition of the *S. cerevisiae* plasma membrane. Two integral plasma membrane proteins were found to be induced strongly, Pdr12 (an ATP-binding cassette (ABC) transporter) and Hsp30 (the latter so called because it is also a heat shock protein)(Panaretou and Piper, 1992; Piper, et al., 1998; Piper, et al., 1997). So strong is the induction of Pdr12, that this transporter becomes one of the most abundant yeast plasma membrane proteins (Figure 1.5).

Pdr12 is the product of one of the 31 genes for ABC transporter proteins in the S. cerevisiae genome (Bauer, et al., 1999). Its gene (PDR12) is relatively unresponsive to a wide range of different stresses (heat shock, ethanol, osmostress, oxidative stress), yet moderately activated by growth at low pH (pH4.5) and activated very strongly by weak organic acid stress (K. Hatzianthis, unpublished data). This weak acid-induction of PDR12

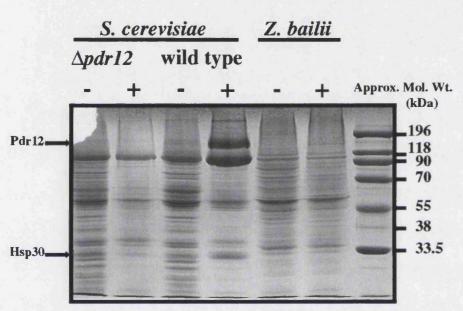


Figure 1.5. Plasma membrane proteins of *pdr12* and wild-type *S. cerevisiae*; also *Z. bailii* NCYC1427, grown 16h on liquid pH4.5 YPD, either in the absence (-) or the presence (+) of 1mM sorbate. These conditions cause strong induction of Pdr12 (arrowed) in wild type *S. cerevisiae*, whereas only a very weak induction of a protein of similar size is apparent in the *Z. bailii* plasma membranes. 30mg total protein from sucrose-gradient purified plasma membranes were loaded in each lane of this 12.5% SDS gel; proteins being detected by staining with Coomassie blue as described earlier (Panaretou and Piper, 1992). (This gel was prepared by Dr. C. Ortiz-Calderon).

is independent of Pdr1p/Pdr3p, Yap1p, Msn2p/Msn4p (Piper, et al., 1998) or Yrr1p (K. Hatzianthis, unpublished data), transcription factors that are known to control the expression of a number of the other ABC transporters of yeast (Bauer, et al., 1999), as well as several stress genes. The transcription factor that directs the weak acid induction of PDR12 therefore still awaits a positive identification.

1.6 These alterations to plasma membrane activities are important for

S. cerevisiae to adapt to growth in the presence of weak organic acids.

Cells lacking the Pdr12 transporter (the Pdr12 S. cerevisiae mutant) are hypersensitive to water-soluble mono-carboxylic acids of relatively short aliphatic carbon chain length (Holyoak, et al., 1999; Piper, et al., 1998). They are not sensitive to highly lipophilic, long chain fatty acids, whose toxic effects are thought to be due almost exclusively to a detergent disruption of membranes (Holyoak, et al., 1999). The activity of the Pdr12 transporter in vivo is readily measured as the ability of the cells to catalyse an active extrusion of fluorescein. Such fluorescein extrusion is competitively inhibited by the presence of weak organic acid preservatives (Holyoak, et al., 1999). Probably therefore Pdr12 counteracts weak acid stress by catalysing the active extrusion of water-soluble acid anions from the cytosol (Figure 1.3b). It appears that Pdr12 is an ABC transporter dedicated to counteracting weak acid stress, since its loss does not seem to render cells hypersensitive to compounds that are substrates for other yeast ABC transporters (Bauer, et al., 1999).

Even though the Pdr12 transporter is induced by growth at low pH (pH4.5) in the absence of weak acid stress (Piper, et al., 1998), it may not be active as an acid anion transporter in such situations. Unstressed CMK1+ cells in pH4.5 growth express this transporter protein yet, in contrast to cells of the cmk1 mutant, do not display any appreciable capacity for active efflux of fluorescein (Holyoak, et al., 2000). The Pdr12 protein levels in these pH4.5 cultures of CMK1+ and cmk1 cells are however identical (Holyoak, et al., 2000). The Cmk1 protein kinase may therefore keep Pdr12 inactive until the Pdr12-catalysed efflux of acid anions is required.

Another important activity counteracting weak acid stress is the plasma membrane H⁺-ATPase, an ATP-driven proton pump (Pma1; Figure 1.3). H⁺-ATPase is present at the plasma membrane of all fungal and plant cells where, both in unstressed and stressed cells, it performs functions vital for the maintenance of homeostasis. It constitutes the main activity contributing to the generation of the electrochemical potential across the cell membrane (ZΔpH; Figure 1.3a, b), a potential that drives nutrient uptake and which regulates ion and pH balance (Serrano, 1991). Any acidification of the cytosol due to the intracellular dissociation of weak organic acids could, in principle, is counteracted by increased H⁺-ATPase-catalysed proton extrusion from the cell (Figure 1.3a, b). A strong activation of plasma membrane H⁺-ATPase does indeed occur in weak acid-stressed cells, as revealed both by measurements of H⁺-ATPase activity in purified plasma membranes (Piper, *et al.*, 1997; Viegas and Sa Correia, 1991) and measurements of proton extrusion by intact cells (Holyoak, *et al.*, 1996). Reduced *PMA1* gene expression is also associated with an increased sensitivity to weak acids (Holyoak, *et al.*, 1996).

Acid influx as in figure 1.3a will act to dissipate the pH, though not the charge (Z) component of the electrochemical potential at the plasma membrane. The same would also occurs with the addition of a classical uncoupler such as 2,4-dinitrophenol (the effects of sorbate and 2,4-dinitrophenol on S. cerevisiae being remarkable in their similarity (Stratford and Anslow, 1996)). The extents to which increased H⁺-ATPase activity alone can counteract the intracellular acidification that accompanies weak acid influx (Figure 1.3a) may be limited, since there is a finite limit to the extent that H⁺-ATPase can enhance the charge component (Z) of the electrochemical potential (ZΔpH). One way to avoid this problem is to ensure the movement of a charge that compensates for the charge on the H⁺-ATPase-extruded proton. Anion (XCOO-) exit from the cell, as through a membrane pore, could satisfy this requirement and could in principle be driven by the membrane potential (positive outside, negative inside). However, according to the model in figure 1.3b the movement of this compensating charge is satisfied in adapted S. cerevisiae cells with the action of the Pdr12 transporter. There might thus be two beneficial effects of a catalysed extrusion of the acid anion in stressed cells: (i) a lowering of intracellular anion levels, thereby helping to normalise turgor pressure and reduce oxidative stress and (ii) the movement of a charge balancing the charge on a H⁺-ATPase-extruded proton, thereby

allowing higher levels of *catalysed* proton extrusion. Combined action of H⁺-ATPase and Pdr12 may therefore help weak acid-stressed *S. cerevisiae* restore homeostasis to the point where growth can resume (Figure 1.3b). This is undoubtedly an energetically very expensive process; at least 2 ATP molecules are needed in counteracting each weak acid molecule that enters the cell (Figure 1.3b). This high energy requirement of counteracting weak acid stress is reflected in the dramatic reductions in biomass yield for those cultures grown in the presence of this stress (Warth, 1988; Verduyn, *et al.*, 1992; Piper, *et al.*, 1997).

What is the role played by the other weak acid-induced plasma membrane protein, Hsp30? The stress activation of H⁺-ATPase is considerably greater in the *hsp30* mutant, indicating that Hsp30 somehow limits the activation of H⁺-ATPase by stress (Piper, *et al.*, 1997). *hsp30* mutant cells also take longer to adapt to weak acid stress and, when stressed, have abnormally low ATP levels. Probably this reflects their excessive H⁺-ATPase activity (Piper, *et al.*, 1997). We have therefore suggested that Hsp30 might have an energy conservation function (Piper, *et al.*, 1997). However the *hsp30* mutant is considerably less sensitive to acid inhibition than the *pdr12* mutant (Figure 1.4), indicating that Hsp30 is less important for weak acid resistance than Pdr12.

Even though Pdr12 appears to be the major activity catalysing active efflux of weak acid anions in *S. cerevisiae* (Holyoak, *et al.*, 1999; Piper, *et al.*, 1998), it is not the only membrane transporter exerting an influence on weak acid resistance. Yor1, another plasma membrane ABC transporter, also contributes to sorbate and benzoate resistance (Bauer, *et al.*, 1999; Cui, *et al.*, 1996). The contributions of Yor1 to these resistances are though relatively minor compared to those of Pdr12 (Figure 1.4). The loss of Pdr1, a transcription factor that regulates a number of the ABC transporter genes in *S. cerevisiae*, also elevates sorbate resistance (Piper, *et al.*, 1998). A number of the Pdr1 targets (including probably genes for ABC transporters other than Pdr12) may therefore counteract weak acid resistance. Weak acid adaptation therefore, while it involves inducing Pdr12, may also require the down regulation of several other transporter proteins. The mRNA for the Pdr5 ABC transporter, a major determinant of drug resistance in yeast (Bauer, *et al.*, 1999), has been observed to disappear in response to weak acid stress (Piper, *et al.*, 1998).

Undoubtedly, several aspects of the weak acid adaptation process still remain to be uncovered. This thesis describes identification of two genes (YOR114w and YPL246w) that when over-expressed, act as multicopy suppressors of the sorbate sensitivity of the pdr12 mutant (Chapter 5). Both are open reading frames of unknown function which, when disrupted, do not give a weak acid-sensitive phenotype. There is also the issue of whether yeasts can compensate for the increased turgor pressure with a high acid anion pool by losing osmolytes. Acetate-stressed Escherichia coli partly counteracts the increase in osmolarity through the release of glutamate (Roe, et al., 1998). Whether S. cerevisiae can also counteract weak acid stress through osmolyte release is not known, although those osmostress conditions that cause accumulation of the major S. cerevisiae osmolyte (glycerol) tend to reduce, rather than elevate, sorbate resistance (unpublished observations).

1.7 How do yeasts avoid a futile cycle of diffusional entry and active extrusion of organic acids?

The induction of high levels of active proton and acid anion extrusion from cells would seem pointless without some limitation to the diffusional uptake of the undissociated acid (XCOOH; Figure 1.3b). Without such limitation, acid could diffuse into the cells as fast as it is pumped out in a futile, and energetically very expensive, cycle. Nevertheless, Pdr12 action clearly enhances acid resistance (Figure 1.4). It has long been known though that acid-adapted S. cerevisiae and Z. bailii can maintain an intracellular versus extracellular distribution of benzoate that is not in equilibrium (Warth, 1977; Warth, 1988; Warth, 1989; Henriques, et al., 1997; Piper, et al., 1998). This is indicative of the induction of a process whereby the adapted cells can reduce their intracellular weak acid pool. It is unlikely that active anion extrusion alone would be sufficient to achieve this (Figure 1.3b). Instead, adapted cells must also restrict the diffusional entry of weak acid across the cell envelope. How this occurs is essentially unknown. The adaptation of Z. bailii to growth in the presence of 2mM benzoic acid is associated with a 40% reduction in the permeability of the cells to benzoate but, remarkably, no decrease in permeability to propionate (Warth, 1989). A recent screen for sorbate-sensitive S. cerevisiae mutants uncovered defects in

ERG6 and MNN5 as leading to sensitivity (S. Thompson, unpublished data). Changes to cell wall architecture or membrane sterol content may therefore be important in the adaptation process. Of the various mnn mutants of S. cerevisiae (Rayner and Munro, 1998), it is mnnl and mnn6 cells that are the most sorbate-sensitive (S. Thompson, unpublished data). These mutants lack the terminal additions of β1-3 linked phosphomannoses within the cell wall structure (Rayner and Munro, 1998). Electrostatic interactions within the cell wall architecture may therefore be important in resistance.

1.8 The influences of oxygen on weak acid resistance

S. cerevisiae cells are more sensitive to weak organic acids when oxygen is present. This is because these acids cause a strong enhancement to endogenous production of superoxide free radicals by the mitochondrial electron transport chain (Piper, 1999). Thus, with oxygen present, weak acid stress is also associated with severe oxidative stress. Indeed the extreme sensitivity of the pdr12 mutant to weak acids on pH4.5 plates (Figure 1.4) is due largely to the production of superoxide free radicals, since it is substantially reversed with the loss of superoxide dismutase activities (Piper, 1999). This free radical production also causes weak acids to be highly mutagenic towards the mitochondrial genome, as shown by the high rates of petite segregation by acid-stressed aerobic (but not anaerobic) S. cerevisiae (Piper, 1999).

Z. bailii is a petite-negative yeast, so weak acid-stressed aerobic cultures of Z. bailii do not segregate respiratory-deficient cells. Instead, Z. bailii can catalyse an oxidative degradation of sorbate and benzoate and can use these components as sole carbon source for growth ((Mollapour and Piper, 2000 and Chapter 3). In contrast S. cerevisiae is unable to oxidatively degrade benzoate probably because it lacks a benzoate-4-hydroxylase (Chapter 3). In this thesis it is described how, by introducing a library of Z. bailii sequences into S. cerevisiae cells and then selecting for growth on benzoate plates, a gene was isolated that confers to S. cerevisiae the ability to catabolise benzoate, sorbate and phenylalanine ((Mollapour and Piper, 2000 and Chapter 3). This gene is denoted ZbYME2, since it encodes a product with 74% amino acid sequence homology to the N-terminal domain of the S. cerevisiae mitochondrial protein Yme2p and complements a number of the

phenotypes associated with yme2 S. cerevisiae cells. The N-terminal domain of S. cerevisiae Yme2p is located within the mitochondrial matrix (Leonhard, et al., 2000; Hanekamp and Thorsness, 1996). Previously the loss of YME2 in S. cerevisiae was characterised as causing: (i) a high rate of DNA escape from the mitochondria to the nucleus; (ii) an inability to grow on non-fermentable carbon sources at 30°C in cells of yme1 genetic background; and (iii) an ability to suppress the cold-sensitive growth phenotype of yme1 strains at 14°C (Hanekamp and Thorsness, 1996; Hanekamp and Thorsness, 1999; Thorsness and Fox, 1993).

As shown in chapter 4, the product of ZbYME2 expressed in S. cerevisiae is also mitochondrial. Probably therefore the presence of ZbYME2 confers a mitochondrial mono-oxygenase function with benzoate-4-hydroxylase activity in yeast. In addition both copies of the ZbYME2 gene were deleted (Chapter 3) in Z. bailii, the first time that yeast gene knockout technology has been successfully applied to this spoilage yeast. The resulting mutant totally lacks the ability to grow on benzoate as sole carbon source and is more sensitive to benzoate or sorbate inhibition under aerobic conditions on pH4.5 glucose plates ((Mollapour and Piper, 2000 and Chapter 3). Therefore, in the presence of oxygen, ZbYME2 facilitates preservative degradation and thereby contributes to the weak acid resistance of Z. bailii. This thesis thus describes the first genetic proof of a Zygosaccharomyces gene involved in the resistance to food preservatives.

1.9 Z. bailii and S. cerevisiae appear not to use identical strategies for the acquisition of weak acid resistance.

The induction of Pdr12 in acid-stressed S. cerevisiae is so strong, that the levels of this ABC transporter in the plasma membrane approach those of the most abundant plasma membrane protein, the H⁺-ATPase (Piper, et al., 1998)(Figure 1.5). However, there is no equally strong induction of a putative weak acid transporter in the plasma membranes of acid-stressed cells of the more weak acid-resistant Z. bailii (Figure 1.5). Changes to the protein composition of Z. bailii plasma membrane do occur with weak acid adaptation, but they are not as dramatic as seen in S. cerevisiae (C. Ortiz; unpublished data). Why, therefore, is Z. bailii more weak acid resistant than S. cerevisiae? One can only surmise that

the former yeast has developed much more efficient ways of changing its cell envelope so as to limit diffusional entry of the acid. This, in turn, will dramatically reduce the need for any active extrusion of protons and acid anions. In 1989 Warth reported that the sensitivities of several different yeast species to benzoate inhibition are inversely proportional to the rates of diffusional entry of propionate into their cells. Of the nine yeasts that he investigated, it was Z. bailii that displayed the slowest uptake of propionate (Warth, 1989). The strategy adopted by S. cerevisiae, whereby resistance is largely conferred through high levels of anion extrusion (see Figure 1.3a, b), potentially has one fundamental flaw. Active (mainly Pdr12-catalysed) anion extrusion at the plasma membrane can only export the weak acid as far as the periplasm (Figure 1.3a, b). From there this acid might just as readily diffuse back into the cell as out through the cell wall. It seems more sensible to restrict the initial diffusion of the weak acids through the cell wall so lower amounts initially reach the periplasm. It is conceivable that Z. bailii puts more reliance on this latter strategy and that therein lies the secret of its extreme weak acid resistance. This would explain why acidadapting Z. bailii has no apparent need for any dramatic induction of a weak acid transporter (Figure 1.5). Z. bailii is also endowed with the ability to break down of sorbate and benzoate, a property which significantly enhances its resistance under aerobic conditions ((Mollapour and Piper, 2000 and Chapter 3).

1.10 Outline of this project.

Z. bailii possesses an unusually high resistance to food preservatives and ethanol. Despite the economic importance of this organism, very little is known about the stress adaptations that allows it to be an important agent of large-scale food spoilage. In Chapter 3 it is described how Z. bailii sequences were isolated that complement the ura3 and trp1 mutations of S. cerevisiae. Also isolated was a Z. bailii gene (ZbYME2) that confers to S. cerevisiae cells the capacity to use the preservative benzoic acid as sole carbon source. Using DNA cassettes containing dominant selectable markers and methods that were mostly originally devised for gene deletion in S. cerevisiae, the two copies of ZbYME2 in the Z. bailii genome were sequentially deleted. Unlike the wild type, a homozygous Zbyme2/Zbyme2 Z. bailii deletant strain lacks the ability to utilise

benzoate as sole carbon source and is generally more weak acid-sensitive during growth on glucose. This demonstrates that ZbYME2 is important for the growth of Z. bailii under conditions of weak acid stress; also that it is possible to delete in Z. bailii genes suspected as being important for the growth of this organism in preserved foods and beverages. This is also the first time that yeast gene knockout technology has been applied to Z. bailii.

In Chapter 4 it is described the further analysis of the ZbYME2 gene. The S. cerevisiae yme1-1, yme2-1 mutant that is unable to grow on non-fermentable carbon sources at 30°C (Hanekamp and Thorsness, 1996) is shown to be also weak acid sensitive. Heterologous expression of ZbYME2 in this strain reversed several yme1-1, yme2-1 phenotypes, including the lack of respiratory growth, the weak acid sensitivity and the increased rate of escape of mtDNA to the nucleus.

The final result chapter (Chapter 5) describes how libraries of Z. bailii and S. cerevisiae DNA fragments in the yeast-E. coli shuttle vector pRS415 were used to reverse the weak acid sensitivity of the $\Delta pdr12$ S. cerevisiae mutant. This led to isolation of genes that act as multicopy suppressors of the weak acid sensitivity of $\Delta pdr12$ cells. Two such sequences were isolated from S. cerevisiae (YOR114w and YPL246C) and also another two from Z. bailii (ZbYOR114, a homologue of S. cerevisiae YOR114w and a nuclear gene encoding a fragment of the mitochondrial gene for apocytochrome b). Preliminary analysis of these genes is also described.

Chapter 2. Materials and Methods.

2.1 Materials

Standard reagents:	AR grade supplied by Sigma and BDH.
Microbiological media:	Supplied by Difco. Ampicillin and auxotrophic requirements supplied by Sigma.
Electrophoresis reagents:	40% acrylgel 2.6 solution (40% w/v solution of acrylamide 'electran' and NN'-methyene bis acrylamide 'electran' in dH ₂ O, final ratio 37:1) and ammonium persulphate supplied by BDH. TEMED and agarose supplies by Sigma.
Restriction endonucleases:	Supplied by New England Biolabs (NEB).
Radiolabelled nucleotides:	(α- ³² P)-dCTP (3000 Ci/mmol; 10mCi/ml).
Other reagents:	SDS (biochemical grade) was supplied by BDH. All buffer salts (Tris and MES), DEPC, 5-FOA and DNase-free RNase supplied by Sigma.

Sources of any other materials are stated where appropriates in the text.

2.2 Yeast strains

Table 2.1 The strains of S. cerevisiae used in this study.

Strain	Genotype	Reference/ Source
FY1679	MATa/α ura3-52	(Delaveau, 1994)
FY1679-	MATa ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63	(Delaveau, 1994)
28C		(Belaveau, 1991)
	MATERIAL 11 G 2 50 11 2 4000 1 2 4 1	(D) 1 1000)
YYM19	MATa $\Delta pdr12$::hisG ura3-52 his3- $\Delta 200$ leu2- $\Delta 1$	(Piper, et al., 1998)
	trp1-∆63	
PMY1	MATα his4- leu2- Δl	gift from P. Meacock
BJ2168	MATa ura3-52 leu2-Δ1 trp1-Δ63 gal2	gift from H.R.B.
		Pelham
PM1	Diploid formed by mating PMY1 and BJ2168	This study
PTY44	MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 [rho ⁺	Gift from Dr. P.
	TRP1J	Thorsness
PTY55	MATα ade2 ura3-52 lys2 leu2-3,112 trp1-Δ1 1	Gift from Dr. P.
	yme1-1 yme2-1 [rho+ TRP1]	Thorsness
CEN.PK2	MATa ura3-52 his3-1 leu2-3,112 trp1-289	Euroscarf
CEN.SR3	MATa ura3-52 his3-1 leu2-3,112 trp1-289	Euroscarf
4-3C	yor114w::HIS3	
CC1	MATa ura3-52 his3-1 leu2-3,112 trp1-289	This study
	pdr12::kanMX4	
CC2	MATa ura3-52 his3-1 leu2-3,112 trp1-289	This study
	yor114w::HIS3 pdr12::kanMX4	
SCC1	MATa ura3-52 his3-1 leu2-3,112 trp1-289	This study
	ypl246w::kanMX4 pdr12::kanMX4	
SCC2	MATa ura3-52 his3-1 leu2-3,112 trp1-289	This study
	ypl247w::kanMX4 pdr12::kanMX4	

Table 2.2 The strains of Z. bailii used in this study. Genotype is Unknown; strains isolated from instance of food and beverage spoilage.

Strain	Source
NCYC 1427	NCYC Norwich
NCYC 563	NCYC Norwich
NCYC 1766	NCYC Norwich
NCYC 128	NCYC Norwich
NCYC 1416	NCYC Norwich

2.3 E. coli strains

Table 2.3 The strains of E. coli used in this study.

Strain	Genotype	Source
DH5α	deoR, endA1, gyrA96, hsdR17, $(r_k^- m_k^+)$ recA1,	Clontech
	relA1, supE44, thi-1, Δ (lacZYA-argFV169),	Laboratories, Inc
	φ80δ lacZΔM15, F ⁻ , λ ⁻	
JM110	Rpsl (Strr), th,r leu, thi-1, lacY, galK, galT, ara	Clontech
	tonA, tsx, dam, dcm, supE44, Δ(lac-proAB), [F'	Laboratories, Inc
	traD36 proAB lacIqZ \(\Delta M15 \)]	
XL1-Blue	RecA1, end A1, gyrA96, thi-1, hsdR17, supE44,	Stratagene®
	relA1, lac[F' proAB lacFZ∆M15 Tn10 (Tet)]	

2.4 Plasmids

Table 2.4 The plasmids used in this study.

Plasmids	Reference/ Source
PAG32	(Goldstein and McCusker,
	1999)
YCplac111	(Gietz and Sugino, 1988)
YEplac181	(Gietz and Sugino, 1988)
pRS415	(Sikorski and Hieter, 1989)
pSL1183	Gift of Drs. T. Chappell and B.
	Panaretou
pGFP-C-FUS	(Niedenthal, et al., 1996)
pGFP-N-FUS	(Niedenthal, et al., 1996)
pUG6	(Güldener, et al., 1996)
pYES2	Invitrogen Co.

2.5 Yeast methods

2.5.1 Growth media and culture conditions

Yeast cultures were grown in rich media (YPD) or synthetic defined minimal media (SD). The recipes for all the yeast media are given below (all % values are w/v):

YPD: 2% D-glucose, 2% bactopeptone, 1% yeast extract (pH adjusted with HCl or NaOH prior to autoclaving).

YPG: 3% glycerol, 2% bactopeptone, 1% yeast extract.

YPEG: 2% glycerol, 2% ethanol, 2% bactopeptone, 1% yeast extract

YPgalactose: 2% galactose, 2% bactopeptone, 1% yeast extract.

SD: 2% D-glucose, 0.67% yeast nitrogen base (without amino acids) plus one or more of the following auxotrophic requirements where appropriate; L-leucine (30 mg/l), L-lysine (30 mg/l), L-tryptophan (20 mg/l), L-histidine (20 mg/l), uracil (20 mg/l) and adenine (20 mg/l)

SB: 0.67% yeast nitrogen base (without amino acids), 1 mM benzoate (pH adjusted with HCl or NaOH)

SHB: 0.67% yeast nitrogen base (without amino acids), 1 mM hydroxy-benzoate (pH adjusted with HCl or NaOH prior to autoclaving).

SP: 0.67% yeast nitrogen base (without amino acids), 1 mM phenylalanine (pH adjusted with HCl or NaOH prior to autoclaving).

ST: 0.67% yeast nitrogen base (without amino acids), 1 mM tyrosine (pH adjusted with HCl or NaOH prior to autoclaving).

Pre-sporulation: 10% D-glucose, 0.3% bactopeptone, 0.8% yeast extract.

Malt extract: 0.3% malt extract, 0.5% bactopeptone

2% bacto agar was used for the preparation of solid media. Plates were left on the bench for 1-2 days after pouring to dry. Liquid media cultures were grown at the appropriate temperature with rapid agitation in a media volume 1/5 of the flask volume. Yeast strains were maintained as frozen stocks in 2X YPD plus 15% glycerol at -70°C. All solutions and glassware were sterilised by autoclaving at 15psi for 20 min..

2.5.2 Monitoring of cell growth

Yeast cell growth in liquid media was monitored by taking OD_{600} readings at appropriate time intervals and, when necessary, OD_{600} readings were correlated to cell number by using an improved Neunauer haemocytometer (Hawksley).

2.5.3 Yeast transformation

Transformation of S. cerevisiae and Z. bailii strains with DNA was as described by (Gietz, et al., 1992) or by electroporation (Thompson, et al., 1998).

2.5.4 Extraction of total cell protein

Yeast cells were pelleted by centrifugation (6000 rpm, 5 min.) and the supernatant was discarded. Two volumes of acid washed glass beads (BDH, 40 mesh), were added to the cell pellet, together with a sufficient volume of protein extraction buffer to just cover both the cells and the beads. This extraction buffer was composed of the followings (50mM Tris-HCL[pH 8.0], 1mM MgCL₂, 2mM EDTA, 1mM DTT, 1mM PMSF, 0.5mM TPCK and $2\mu g/ml$ Pepstatin A). Cells were disrupted by vortexing for 30 seconds, followed by chilling on ice for 30 seconds. This procedure was repeated 10 times to ensure complete cell breakage. Beads and cellular debris were pelleted by centrifugation (4000rpm, 2min.) and the soluble protein-rich supernatant was removed. Subcellular fractions were isolated as described by Panaretou and Piper (1992).

2.5.5 Determination of protein concentration

Protein concentrations were determined by Bradford dye-binding assay and used to Bio-Rad Proteins Assay Kit and BSA as standards. Both standard and unknown protein concentrations were assayed as manufacturer instruction.

2.5.6 Separation of proteins by SDS polyacrylamide gel electrophoresis (PAGE)

Proteins and their subunits can be separated by size using a discontinuous electrophoresis system (Laemmli 1970). Slab gels were cast in the Bio-Rad Mini-protein system, all gel solutions are listed below.

The Laemmli system consists of a Stacking gel, containing 4% acrylamide and a Resolving gel containing the appropriate percentage of acrylamide to successfully separate sample proteins. Both gels were cast using a 30% acrylamide:bis-acrylamide stock and polymerised with the addition of 0.05% (v/v) TEMED and 0.7% (w/v) APS.

Prior to loading, protein samples were boiled for 10 min, in sample buffer. These samples were loaded onto wells formed within stacking gel, and electrophoresis proceeded at 100V for 45min., in electrode buffer (compositions given below). Molecular weight markers (Stratagene®) were ran alongside samples to indicate protein size.

2.5.7 SDS-PAGE solutions

30% Acrylamide stock (BDH) 29.2% acrylamide and 0.8% Bis-acrylamide

APS 10% (w/v) solution stored at 4°C

Stacking gel 0.125M Tris-HCL [pH6.8]; 0.1%SDS; and 4%

polyacrylamide.

Electrode Buffer (pH 8.3) 0.025 trizma base; 0.192M glycine and 0.1% SDS

Protein sample buffer 0.125M Tris-HCL [pH 6.8]; 10% (v/v)glycerol,

5% (v/v) b-mercaptoethanol; 2% SDS and 0.013% (w/v)

bromophenol blue.

2.5.8 Analysis of protein after SDS-PAGE

After electrophoresis was completed the gel plates were seperated and the stacking gel discarded. Proteins within the resolving gel were visualised by one of the following methods.

2.5.8.1 Direct staining

Staining with Coomassie blue R-250 allows the detection of abundant proteins (1 μ g or more). Gels were incubated, for 1 hour at room temperature, in 0.05% (w/v) Coomassie blue, 50% methanol and 10% acetic acid. Gels were destained by their gentle agitation in a solution of 10% methanol and 7.5% acetic acid at room temperature. Destain solution was changed periodically.

2.5.8.2 Western blotting

SDS-PAGE separated proteins can be blotted onto a nitrocellulose or PVDF membranes by means of electrophoresic transfer. The electrophoresed gel, transfer membrane, 6 pieces of Whatmann filter paper were pre-equilibrated in transfer buffer (25mM Tris and 150mM glycine [pH 8.3] with 29% methanol to minimise swelling of the gel during transfer). A gel-blot sandwich was constructed of 3 pieces of filter paper, the gel, the membrane and the remaining sheets of paper. The sandwich was immersed within a buffer-filled Western blotting tank, with gel towards the cathode and membrane towards the anode. The electrophoretic transfer proceeded at 50V, 10°C, overnight. Efficiency of protein transfer was verified by staining the membrane with Ponceau-S. The transient stain was water soluble and did not affect further analysis of blotted proteins. By running pre-stained protein marker during SDS-PAGE, both the efficiency of transfer and molecular weight of sample proteins were identified.

2.5.8.3 Immunodetection of proteins on blotted membranes

All immunodetection steps were carried out at room temperature with constant agitation. Phosphate Buffer Saline + 0.1% Tween (PBS-T) was used as both a base for blocking agent (5% non-fat dried milk powder dissolved in PBS-T), and as between-step washes. Antibodies were diluted in blocking agent and applied to the blot in a final volume of 0.1mlcm⁻¹ of membrane.

After Ponceau –S staining, the remaining protein-binding sites on the blot were blocked by incubation in blocking agent for 1 hour. Excess blocking agent was removed with 3X ten min. washes in PBS-T. Primary antibody was diluted in blocking agent and used to probe the blot for 1 hour. Superfluous block and antibody were removed with PBS-T.

The Secondary antibody was diluted in blocking agent and applied to the membrane for a further hour. Again surplus antibody was removed by washing the blot 3 times with PBS-T. Antibody binding was visualised by means of enhance chemiluminesence (ECL), (Amersham). Equal volume of reagents A and B were mixed and applied to the blot for 1 min. The localised fluorescence can be detected by exposure to Fuji X-ray film.

2.6 Recombinant DNA techniques

2.6.1 Restriction enzyme digest

0.1 to 4 μ g DNA (in dH₂O or TE buffer) was digested with restriction endonuclease (1 to $5U/\mu$ g DNA) and with 1/10 volume 10x restriction buffer for 1 hour at specific temperature recommended by NEB.

2.6.2 Polymerase chain reaction (PCR)

The PCR was used for DNA amplification and site-directed mutagenesis (Chapter 3). A 50 μ l preparetive PCR reaction contained 5 μ l of 10X ExpandTM high fidelity (HF) buffer with 15 mM MgCl₂ {20 mM Tris-HCL, pH 7.5, 100 mM KCl, 1 mM dithiothreito (DTT), 0.1 mM EDTA, 0.5% Tween® 20(v/v), 0.5% Nonident® p40 (v/v), 50% glycerol (v/v)}, 100 μ M dNTP mix (100 μ M of each of dATP, dCTP, dTTP, dGTP (PE Applied Biosystems)), 100 pmol of each primer, and 1.14 unit ExpandTM HF polymerase (Boehringer Mannheim, Germany). The PCR conditions and elongation time are given in tables 2.5 and 2.6 respectively.

Table 2.5 The PCR conditions (Boehringer Mannheim, Germany).

Number of cycles	Conditions		
1x	Denature template 2 min. at 94°C		
35-40x	Denaturation at 94°C for 30 sec		
	Annealing usually at 45-65°C* for 30 sec		
	Elongation at 72 or 68°C** for 45sec-8min Table 2.6.		
1x	Final elongation at 72°C for 4 min		

^{*} Annealing temperature depends on the melting temperature of the primers used.

Table 2.6 The PCR elongation time.

Elongation time	45 sec	1 min	2 min	4 min	8 min
PCR fragment length (kb)	<0.75	1.5	3.0	6.0	10.0

2.6.3 Purification and precipitation of DNA

Plasmid DNA was purified by vortexing with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1). The top layer was recovered after centrifugation. Routine precipitation of DNA was achieved by adding 2 volumes of 100% ethanol and 1/10 volume 6M ammonium acetate. The mixture was stored at -20°C for 20 min. and centrifuged at 12 000g for 20 min.. The pellet was washed twice with 70% ethanol to remove precipitated salt and resuspended in dH2O or TE buffer (10mM Tris HCl pH 7.0; 1 mM EDTA).

2.6.4 Agarose gel electrophoresis of DNA

DNA fragments between 0.5 and 25 kb were routinely separated using the following protocol. 0.8% w/v agarose gel was prepared using 0.5x TBE electrophoresis buffer (90mM Tris base, 90mM boric acid, 2mM EDTA) and appropriate weight of agarose. The mixture was melted in a microwave oven, mixed, cooled to 55°C and after

^{**} Elongation temperature depends on the length of amplification product: 72°C are used for amplification up to 3.0 kb; 68°C are used for amplification >3.0 kb.

adding ethidium bromide (EtBr) $0.5\mu g/ml$, it was poured to a sealed platform with the gel combs. The DNA samples were electrophoresed (at 1 to 10 V/cm of agarose gel) with an appropriate amount of 10x DNA loading buffer (0.5xTBE, 10% glycerol (v/v), 0.1% bromophenol blue (w/v)) and appropriate DNA molecular weight markers. Gels were viewed using short wave UV transilluminator and photographed.

2.6.5 Isolation and purification of DNA fragments from agarose gels

DNA fragments of 70 bp to 10 kbp were extracted and purified from agarose gel using the QIAquickTM gel extraction kit (QIAGEN Ltd.).

2.6.6 Dephosphorylation of 5' end of DNA and oligonucleotides

An appropriate amount of DNA or oligonucleotide was dephosphorylated using the Calf Intestine Alkaline Phosphotase (CIP) kit (Promega Cor.).

2.6.7 Phosphorylation of 5' end of DNA and oligonucleotides

An appropriate amount of DNA or oligonucleotide was incubated with 5 mM ATP, 1 mM DTT, 10 U T4 polynucleotide kinase (PNK) and 1/10 volume 10x linker kinase buffer for 30 min. at 37°C. The PNK was inactivated at 70°C for 10 min..

2.6.8 Ligation of DNA fragments to plasmid vectors

Ligations were routinely carried out as described in (Maniatis, et al., 1989).

2.6.9 Site-directed mutagenesis

In vitro site-directed mutagenesis of SFA1MX (Chapter 3) was carried out by QuikchangeTM site-directed mutagenesis kit (Stratagene®). The method utilises a double-stranded vector and two synthetic oligonucleotide primers (containing the desired mutation), each complementary to opposite strands of the vector. Mutant DNA is extended by PCR and the mutated plasmid containing the staggered nicks is generated. Following temperature cycling the product is treated with DpnI (tagged sequence: 5'- $G^{m6}ATC$ -3') specific for methylated and hemimethylated DNA found in almost all E.

coli strains. The partial DNA template is dam methylated and susceptible to DpnI digestion while PCR generated mutant plasmid DNA is unmethylated and resistant to digestion leading to a high mutation efficiency (Figure 2.1). After PCR extension and DpnI digestion, mutated plasmid DNA was transformed in Epicurian coli® XL1-Blue Supercompetent Cells as described in the Stratagene® instruction manual (catalogue number 200815). Plasmid DNA was isolated from single Epicurian coli® colonies, and checked by restriction digest and sequenced.

2.6.10 E. coli growth media and culture conditions

E.coli strains were grown in LB (Luria-Bertani), (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl and 1mM NaOH final concentration) at 37°C. Cultures for plasmid transformation and selection were grown in LB plus ampicillin to a final concentration of 100µg/ml.

2.6.11 Preparation of competent E. coli

Competent *E. coli* cells were prepared according to a calcium chloride technique as described in (Maniatis, *et al.*, 1989). The competent cells were resuspended in ice cold 0.1M CaCl₂ plus 15% glycerol and stored in 0.1 ml aliquots at -70°C.

2.6.12 E. coli transformation

Competent *E. coli* cells were thawed on ice and 100 μ l pipetted into a chilled ependorf. An appropriate amount of plasmid DNA was added and the cells were incubated on ice for 15 min. The cells were heat-shocked at 42°C for 90 seconds and placed on ice for 5 min. The cell were then resuspended in 900 μ l of SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5mM KCl, 10 mM MgCl₂, 20 mM MgSO₄, 20 mM Dglucose) and incubated at 37°C for 45 min. The cells were centrifuged at 6,000g for 30 seconds. The pellet was resuspended in 100 μ l of the

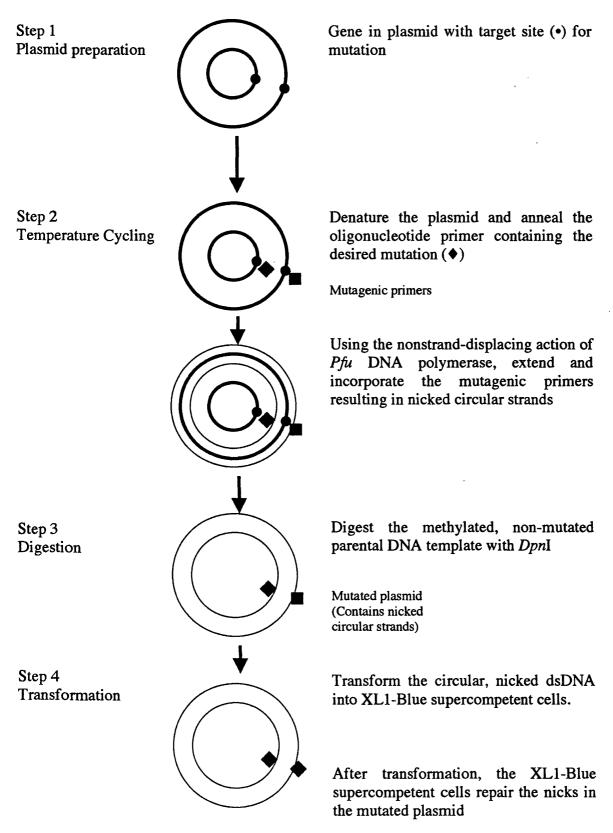


Figure 2.1 Strategy for generating specific base changes using Quikchange™ Site directed Mutagenesis kit (from Stratagene®, catalogue number 200518).

supernatant and then plated on LB plus ampicillin to a final concentration of 100µg/ml and incubated at 37°C overnight.

2.6.13 High efficiency transformation by electroporation

Electro-competent $E.\ coli$ cells were prepared as described in (Zabarovsky and Winberg, 1990). 50 μ l aliquots of electro-competent $E.\ coli$ cells and an appropriate amount of DNA (10 μ l) were added to a Flowgen cuvette and electroporated (at 2,500V, 0.25mF, 201 Ω , 5msec) in an EasyjectT+ electroporator (Flowgen, Inc.). Immediately after electroporation 1 ml of LB or SOC was added to the cuvette and quickly but gently resuspended the cells. The cells suspension was then incubated at 37°C for 45 min. The cells were centrifuged at 6 000g for 30 seconds. The pellet was resuspended in 100 μ l of the supernatant and then plated on LB plus ampicillin to a final concentration of $100\mu g/ml$ and incubated at 37°C overnight.

2.6.14 Preparation of plasmid DNA from E.coli.

Approximately 20-100 μ g of high-copy plasmid DNA was obtained from 5ml or 3ml overnight cultures of *E.coli* using QIAprep® Miniprep or Midiprep kits respectively as described in the appropriate QIAprep® handbooks (QIAGEN Ltd.).

2.7 Procedures for nucleic acid analysis

2.7.1 Isolation of yeast total genomic DNA

Yeast genomic DNA was prepared as previously described by (Adams, et al., 1997), except that proteinase K (200 μ g/ml final concentration) was added at the point of spheroblast lysis. The lysate was extracted by phenol:chloroform:isoamylalcohol (25:24:1) and DNA was precipitated by addition of an equal volume of isopropanol. High molecular weight DNA was recovered by spooling and washed twice in 70% ethanol. DNA was resuspended in (TE or dH₂O) and treated with ribonuclease A and respooled before final resuspension in (TE or dH₂O).

2.7.2 Construction of yeast total genomic DNA libraries.

Yeast genomic DNA was digested with Sau3A1, the restriction fragments were separated by sucrose density ultracentrifugation and fractions containing fragments in the 1.0-8.0 kb range pooled. After recovery, this DNA was ligated to the BamHI site of pRS415, following treatment of the shuttle vector with calf intestinal alkaline phosphatase to prevent recircularisation (Sikorski and Hieter, 1989). After transformation of E. coli DH5\alpha by electroporation approximately 25000 colonies were screened. This was calculated to be 99% representative of S. cerevisiae or Z. bailii genome, assuming the genome of Z. bailii is identical in size to that of S. cerevisiae (12,068 kbp).

2.7.3 Isolation of yeast mitochondrial DNA (mtDNA)

Yeast mitochondrial DNA was isolated using a method previously described by (Querol and Barrio, 1990). The method is based on: preparation of spheroplast , lysis of the cellular membrane, isopycnic purification of mitochondria, lysis of the mitochondial membranes, and isolation of mtDNA without using CsCl gradient centrifugation.

2.7.4 Isolation of yeast RNA

Yeast RNA was isolated as previously described by (Piper, 1994), all solutions, glassware and plasticware being treated with diethyl pyrocarbonate (DEPC) to deactivate RNase.

2.7.5 Quantification of DNA and RNA

DNA and RNA samples were quantified as described in (Maniatis, et al., 1989). An A_{260} of 1.0 was taken to indicate $50\mu g/ml$ double stranded DNA, 37 $\mu g/ml$ single stranded DNA or 40 $\mu g/ml$ RNA. The ratio of A at 260 and 280nm was used to indicate nucleic acid purity.

2.7.6 Southern analysis

Following electrophoresis the DNA was denatured and agarose gels were blotted by capillary transfer of DNA as described in (Maniatis, et al., 1989). Blotting was carried out overnight with Hybond-N nylon transfer membrane (Amersham, Ltd.) in 20x SSC (3M NaCl, 0.3M sodium citrate; pH 7.0). Membranes were wrapped in 3MM Whatman filter paper and vacuum dried for 2 hours at 80°C. Colony hybridisation were carried out by the following method. Yeast and E. coli cells were transferred onto Hybond NA-45 nitro-cellulose filters (Amersham, Ltd.) using forceps. The original plates were stored at 4°C. The filters were soaked for 5 min. (cell side up) in three metal trays lined with Whatman 3MM filter paper saturated with denaturation solution (0.5 M NaOH, 1.5 M NaCl), neutralisation (0.5 M Tris-HCL pH 7.5, 1.5M NaCl) and 2x SSC (0.3M NaCl, 30mM sodium citrate; pH 7.0). The filters were wrapped in 3MM Whatman filter paper, vacuum dried at 80°C for 2 h.

2.7.7 Northern analysis

Yeast RNA was denatured by treating samples with a combination of glyoxal and DMSO, subjected to electrophoresis and blotted according to the method of (Maniatis, et al., 1989). Membranes were wrapped in 3MM Whatman filter paper and vacuum dried for 2 hours at 80°C prior to hybridisation. The blots were washed for 30 min. at 60°C in 20mM Tris.Cl, pH 8.0 prior to hybridisation.

2.7.8 In vitro labelling of single stranded DNA probes.

DNA probes were prepared as described by (Feinberg and Vogelstein, 1983). Double stranded DNA was first denatured by boiling 100 ng of DNA in 10 μ l of sterile dH2O for 3 min.. The DNA was cooled rapidly on ice and 12.5 μ l of oligonucleotide labelling mix (25 μ l nucleotide stock: 100 μ M dGTP, dATP, dTTP, 250 mM Tris.Cl, pH 8.0, 25 mM MgCl2; 25 μ l 1M HEPES pH 6.6; 7 μ l pd(N)6 sodium salt (100 ODU/ml (Pharmacia Biotech Ltd., UK), 20 μ Ci of (α -³²P)-dCTP and 5 units of Klenow were added. The probe was centrifuged at 12 000g for 10 seconds and incubated

at 37°C for 1-2 hours. After annealing, the reaction was quenched by adding 50 μ l of 20mM EDTA. The probe was denatured with 100 μ l of 0.2M NaOH for 5 min. at 37°C.

2.7.9 Hybridisation analysis of DNA probes to membrane bound nucleic acid

Hybridisation analysis was carried out by a modified method described by (Meinkoth and Wahl, 1984). Dried membranes were pre-hybridised in siliconised glass bottles (Hybaid) in hybridisation solution (0.125 ml/cm2; 5x Denhardt's solution, 0.5% (w/v) SDS, 10% dextran sulphate and 100 mg/ml single stranded salmon sperm DNA) for at least 30 min. at the appropriate temperature with constant agitation. The denatured probe was added to the pre-hybridisation buffer and hybridisation was carried out overnight at the appropriate temperature. After hybridisation, the blot was washed at room temperature for 15 min. in 2x SSC plus 0.1% (w/v) SDS. This was followed by a 15 min. moderate stringency wash at the appropriate temperature in 2x SSC plus 0.1% (w/v) or a min. high stringency wash in 65°C 0.1x SSC plus 0.1% SDS (w/v). All washes were carried out in duplicate with constant agitation.

2.7.10 Autoradiography

Membranes were placed in direct contact with Fuji RX film in light proof autoradiographic cassettes for the appropriate length of time at -70°C with an intensifying screen. X-ray film was developed according to the manufacturers instruction.

2.7.11 Removal of probe from hybridised membrane

The hybridised probe was removed from membranes by boiling in 0.1% SDS for 10 min. followed by cooling to room temperature. The membranes were wrapped in Saranwrap and stored at room temperature.

2.7.12 DNA sequencing analysis

Nucleotide sequences were determined using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Corp.) For each reaction the following reagents were added on ice to a PCR tube:

Terminator ready reaction mix

 $8 \mu l$

Template:

Single-stranded DNA

50-100 ng

Double-stranded DNA

200-500 ng

PCR product DNA

30-90 ng

Primer

3.2 pmol

The volume was made up to 20 μ l by adding dH2O. Reagents were mixed and spun briefly. The tube was placed in GENIUS TECHNE thermal cycler and the following cycle was repeated 25 times.

96°C for 30 seconds.

50°C for 30 seconds.

60°C for 4 min.

After completion of temperature cycle; 2 μ l of 3 M sodium acetate pH 4.6 and 50 μ l of 100% ethanol were added to each extension reaction and placed on ice for 10 min. The tubes were centrifuged at 12 000g for 15 min. The pellet was washed twice with 250 μ l 70% ethanol, dried in a vacuum centrifuge for 1-3 min. and then electrophoresed on the ABI PrismTM 377 DNA sequencer Kit (PE Applied Biosystems, Corp.). Signals were analysed using ABI Automated DNA Sequence Viewer version 1.0 software (PE Applied Biosystems, Corp.).

Chapter 3. Targeted gene deletion in Z. bailii.

3.1 Introduction

Despite the economic importance of the Zygosaccharomyces (see chapter 1) very little is known about their genetics or the adaptations to conditions of low a_w or high weak acid preservative levels, which permit them to become agents of large-scale food and beverage spoilage (Chapter 1). Z. bailii, Z. bisporus and Z. rouxii are petite-negative and contain circular DNAs (Toh-E, et al., 1984). The structures of these plasmids indicate that they probably replicate like the 2μ extra-chromosomal DNA of S. cerevisiae (Toh-E and Utatsu, 1985). Small-subunit rRNA gene sequencing has also revealed these Zygosaccharomyces to be very closely related. Over a 1832 nucleotide region, the rDNA of Z. bailii and Z. bisporus are 99.8% identical, 99.2% identical to the rDNA of Z. rouxii and 97.5% identical to the rDNA of S. cerevisiae (James, et al., 1994). In this, study Z. bailii genes that are functional homologues of the URA3, YME2, and TRP1 genes of S. cerevisiae were isolated by complementation cloning in the latter yeast. Also described is the first deletion of a Z. bailii gene important for growth of this organism in the presence of food preservatives. A Z. bailii deletant strain was generated and used to show that this ZbYME2 sequence contributes to weak organic acid preservative resistance.

3.2 Results

3.2.1 Isolation of the URA3 and TRP1 genes of Z. bailii by functional complementation in S. cerevisiae

At the start of this project the only Z. bailii sequence in the database was ribosomal RNA-encoding (James, et al., 1994). It was therefore necessary to first isolate a few sequences that could be used in gene deletion studies. Fragments of genomic DNA (1-5kb, generated by partial Sau3A1 digestion) were prepared (as in section 2.7.2), from two Z. bailii isolates, NCYC563 and NCYC1427 (Table 2.2). These were ligated to BamH1-cleaved pRS415 (a S. cerevisiae-E. coli shuttle vector (Sikorski and Hieter, 1989)), and then transformed into E. coli to produce libraries of Z. bailii genomic DNA fragments (see Methods). These libraries were initially transformed into S. cerevisiae YYM19 (Table 2.1) by selection for leucine prototrophy. Approximately 25,000 LEU+ transformants from each Z. bailii library were then replica plated onto SD tryptophan plus histidine, SD uracil plus histidine and SD uracil plus tryptophan plates. One TRP+ colony was obtained from the Z. bailii 563 library; also one TRP+ and one URA+ colony from the Z. bailii 1427 library. However no HIS+ colonies were obtained from either library. Plasmids rescued from these URA+ and TRP+ S. cerevisiae transformants (as in section 2.5.3) were shown to have the capacity to reconfer either uracil prototrophy or tryptophan prototrophy, respectively, when re-transformed back into S. cerevisiae YYM19. The Z. bailii 1427 and 563 DNA fragment inserts of these plasmids were then sequenced using the BigDye™ terminator cycle sequencing ready reaction kit (PE Applied Biosystem) and primers shown in Figures 3.1a, 3.2a and listed in Table 3.2. The sequence of the Z. bailii 1427 DNA fragment that conferred uracil prototrophy to S. cerevisiae YMM19 (Figure 3.1a) revealed two complete ORFs. One of these (ZbURA3, Genbank Accession: AF279259) encoded a product with 82-83% amino acid sequence identity with the URA3 products of S. cerevisiae (Figure 3.1b) and Candida glabrata. The other ORF (ZbTIM9, Genbank Accession: AF279260) had strong (85%) sequence conservation with the essential S. cerevisiae gene TIM9 (Figure 3.1a). An identical juxtapositioning of URA3 and TIM9 sequences is also to be found in the S. cerevisiae genome, showing this to be a gene order conserved between S. cerevisiae and Z. bailii.

```
a) 1 GATCTGGTAT TACTTTCCAG CCATATTTAC TCAATGCCTT CGCCAAGATT CCCAAACTTG AGTTTTCTAG GTAATAAAAG
      TATTTTGGGT TCAAAATCTT AAACCTTCTG TGTCTTGATT CCTGCATTGG TCTTTTCTCC TCACCCGTCA AAACGACCCT CAAGTAGTGC AAATAGTCAA AGCGAGCAGG CCTGAAAAGT TTTAAGGGCG TTCGAAGATA GAAACTGGTC ATTTGATACA
       CTGCAGTCCT ACCGCCTGCC GTGAGGGCAC CGATAATAGA ACTTCGCTGA GCCGTTGACG GCGATGCATT TTGTCCAGTT
 321 GGATCAGTGT GGTCAACGTT AGTAGTCATA TITAATGCAT CTGATATGTT TTCGTACGTT CAATAATCAA GCCCCAGCTT
 401 TCATAGCGTT ACGATGTGGA TTTTGCTGTA GCATTTGGTG TGGCCAAATC GGACTTTTTT TTTCTGCAAT ATGCTGGAGC
 481 GCAAGGCGGA TCGAACGAAA AATTGCAAGT TATAGTCCAA TATCAATTGT ATCTATAAAT CAACCAAGAT ACCTCAGAAC
            URA3 >>>
 561 GCTTCATGAG TACCGCTACT TATCACGATA GGGCCGCTAA ACATGCAAGC CCGCTTGCAA GCAAGCTCCT TGAGTTGATG

1 M S T A T Y H D R A A K H A S P L A S K L L E L M
      CATGAGAAAC AAACTAATTT GTGTGCCTCA CTAGATGTAG ACACGACAAC AGAACTTCTC CGTCTCGTGG ATATACTTGG
  26 HEK QTNL CAS LDV DTTT ELL RLV DILG
 721 CCCTTACATT TOCCTAGTGA AGACCCATGT TGATATTTTG TCCGACTTTT CAATGGACGG CACTGTGAAG CCTTTGAAAG
  52 PYICLV KTHV DIL SDF SMDG TVK PLK
 3UT7
801 AGTTGGCTGC CAAACACAAA TTCCTGATTT TTGAGGATAG AAAATTTGCA GACATTGGAA ATACTGTTAA ATTGCAATAC
  79 E L A A K H K F L I F E D R K F A D I G N T V K L Q Y
 881 TCTTCTGGTG TCTACCAAAT CTCCCAATGG GCTGATATAA CTAATGCACA CGGCGTGACT GGCCCAGGAA TTGTACAGGG 106 \, S S G V Y Q I S Q W A D I T N A H G V T G P G I V Q G
 961 TCTTAAGCAA GCAGCCGAGG AGACTACACA GGAGCCTCGC GGACTGCTAA TGCTAGCGGA ATTATCTTCT AAGGGGTCCC 132 L K Q A A E E T T Q E P R G L L M L A E L S S K G S
1041 TTGCCCACGG TGAATACACT AAGGGGACAG TGGAAATCGC CAAGACTGAC AAGGACTTCG TGATCGGCTT TATAGCTCAA
 159 LAHGEYT KGT VEIA KTD KDF VIGFIAQ
 .121 AGAGACATGG GTGGCAGAGA TGAAGGCTAC GATTGGCTTA TCATGACTCC AGGTGTAGGA CTTGAAGACA AGAGTGATGC
1121
 .201 ATTGGGACAA CAGTATCGTA CGGTTGAAGA CGTGATCTCT ACAGGCTCAG ATATCATTAT TGTAGGAAGA GGCCTATTTG
212 L G Q Q Y R T V E D V I S T G S D I I I V G R G L F
1201
1281 GAAAAGGCCG TGACCCCAAA ATTGAAGGAG ATCGCTATAG AAAAGCAGGT TGGGAGGCAT ATCTGAAGAG ATGTAATCCA
 239 FG K G R D P K I E G D R Y R K A G W E A Y L K R C N P
1361 TAGATGTGAT AGCTGCGAAT TITCTACTGT ATAGACTGTA TICTGTAACT CGCAAATATC TITAATTTTT TTAGTTTACC
1441 CGCACACTCA AAATTTTCAA TGCTGTCTGC ACATTTATAA AGGCCAACAT CGGCAACTGC GAACCAATTT CGAGTCAATA
                                                            TIM 9 >>>
1521 TIGCTCTTCC TITGGGGGAA GTAGTCAGAG CACATATATT ATCGAAGATG GACTCTCTAA ACCCCAGAGA ACAGCAGGAG
                                                         1 M D S L N P R E Q Q E
1601 TITTCTAAGC TCATTGAGCA CAAGCAAATG AAGGATTITA TGCGCCTTTA CTCCAACTTG GTTGAAAGAT GCTTTACGGA 12 F S K L I E H K Q M K D F M R L Y S N L V E R C F T D
  81 TIGTGTGAAT GATTTCACTT CTTCTAAACT AACTAGTAAG GAGCAAACCT GCATCAGCAG GTGCTCCGAA AAGTTTTTGA
38 CVNDFTSSKLTSKEQTCISRCSE
1761 AGCATAGCGA ACGTGTGGGG TTGCGCTTCC AGGAGCAGAA TGCCGCTATG GGGCAAGGTC TCGGGCGCTA GCGCATACAG
  65 N H S E R V G L R F Q E Q N A A M G Q G L G R
```

1841 CTTATATTAT AGTACATGTA AATACTTACG AAGTTTATTT TTTTTTTAGA TC

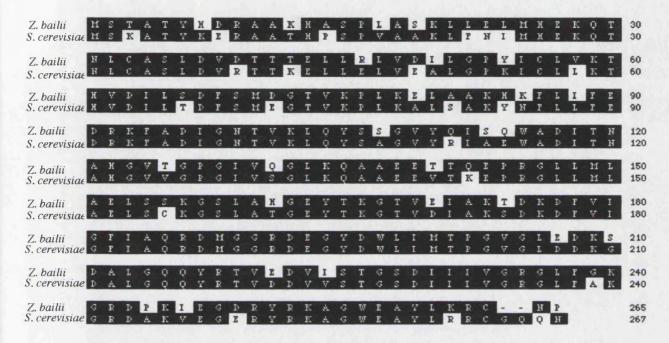


Figure 3.1 a. The Z. bailii 1427 DNA sequence which complemented the S. cerevisiae ura3 mutation, showing the ZbURA3 and ZbTIM9 ORFs (GenBank Accession AF279259 and AF279260). Arrows represent the primers used to sequence this DNA fragment.; b. Amino acid sequence alignment of Ura3p from Z. bailii and S. cerevisiae.

The Z. bailii 563 DNA fragment that conferred tryptophan prototrophy to S. cerevisiae contained three potential ORFs, of which two were complete and one incomplete (Figure 3.2a). One of these ORFs was denoted ZbTRP1, (Genbank Accession: AF279262), since its product shares 59% sequence identity with the TRP1 product of S. cerevisiae. Trp1p of Z. bailii is however significantly smaller than the corresponding S. cerevisiae protein (205 amino acids, as compared to 224 amino acids for the latter (Figure 3.2b)). Upstream of this ZbTRP1 ORF is what is evidently a histone H3 gene (ZbHHT1, Genbank Accession: AF279270), with 86% sequence identity with the histone H3 gene of S. cerevisiae, HHT1. Downstream of ZbTRP1, on the opposite DNA strand, is an incomplete ORF with high homology to S. cerevisiae gene for inorganic pyrophosphatase, IPP1 (Figure 3.2a). It is noteworthy that an identical juxtapositioning of HHT1, TRP1 and IPP1 sequences is also to be found in Kluyveromyces lactis (Stark and Milner, 1989), but not in S. cerevisiae. Z. bailii Trp1p is however slightly closer in sequence to the Trp1p of S. cerevisiae (Figure 3.2b) than to the Trp1p of K. lactis (not shown).

3.2.2 Isolation of the ZbYME2 gene from Z. bailii

One of the primary objectives of this study was to isolate, and then delete, genes in Z. bailii that might be important in weak organic acid resistance. In simple growth tests we observed that Z. bailii could use the food preservative benzoate as its sole carbon source, provided oxygen is present. S. cerevisiae cannot catabolise benzoate, apparently because it lacks a benzoate-4-hydroxylase activity (Chapter 4). Therefore, the Z. bailii DNA libraries were screened for DNA fragments that could confer to S. cerevisiae the ability to grow on benzoate. These libraries were transformed into the diploid S. cerevisiae strain PM1, a leucine auxotroph (Table 2.1). Approximately 20,000 transformants that were leucine prototrophs were initially selected on SD plates. These were then replica plated onto SB plates (pH6.0 minimal plates containing 1mM benzoate as sole carbon source; see Methods). A single colony from the Z. bailii 1427 library grew on the latter plates. The pRS415-derived plasmid (pZB1) of this transformant was rescued, then used to retransform S. cerevisiae PM1. This confirmed that pZB1 could

104 S A S P V E S A A A A S Y T P T D T L T T N T L S I G

2081 CGTGGCTCTC AATAGACCTG GGAAGTACTT CTCCACGTCC TCAATGTCAT GCAACTTGGG TGCCAATGGA TC 24 TARLLGPFYKEVDEIDHLKPALP

CCTTGCTGTC AGAGACTIGA CCATGGATCA ATTGCTTCCA GGATTCGTGA GTCTCTCTAA TGATGTCTAG AGCGTACTTC 77 KSDSVQGHILQKWSEHTERIIDLAYK 2001 TIGTICTIGG CCTCACCAGA GAAGGCAAAT TGGTTCTCGG GCTTGCCATC GGGAATTITG TAAATTCTGA ACCATTCGTT 50 K N K A E G S F A F Q N E P K G D P I K Y I R F W E N

<<ZbIPP1 (part)

1921



Figure 3.2 a. The Z. bailii 563 DNA sequence which complemented the S. cerevisiae trp1 mutation, showing the ZbTRP1 and ZbHHT1 ORFs (GenBank Accession AF279262 and AF296170) and the C-terminal part of an ORF with homology to S. cerevisiae IPP1. Arrows represent the primers used to sequence this DNA fragment; b. Amino acid sequence alignment of Trp1p from Z. bailii and S. cerevisiae.

confer both leucine prototrophy to *S. cerevisiae* cells (from the *LEU2* gene within the pRS415 sequences of pZB1) and the ability to use benzoate as a carbon source (Figure 3.3). The 1.625kbp *Z. bailii* DNA insert of pZB1 was sequenced (Figure 3.4a) and found to contain a number of small ORFs, only one of which encoded a product with any significant similarity to a *S. cerevisiae* gene. This ORF is denoted *ZbYME2* (Genbank Accession: AF279261), since its product has 74% sequence identity with the N-terminal domain of a much larger *S. cerevisiae* protein, the nuclear gene-encoded mitochondrial protein Yme2p/Rna12p (Figure 3.4b). Within *S. cerevisiae* this N-terminal domain of Yme2p is located within the mitochondrial matrix (Hanekamp and Thorsness, 1996). Just the *ZbYME2* ORF of the pZB1, when over-expressed as a GFP fusion, can confer benzoate utilisation in *S. cerevisiae* (see section 4.2.4), proof that this is the ORF in the pZB1 insert that can confer benzoate metabolism in *S. cerevisiae* (Figure 3.3). Since *ZbYME2* appeared to be a gene that might be involved in weak acid resistance it was selected as a testbed for the *Z. bailii* gene deletion studies.

3.2.3 The design of a gene disruption strategy for Z. bailii

Preliminary experiments revealed that Z. bailii plated on SD medium containing uracil and 1mg/ml 5-fluoroorotic acid (FOA), conditions routinely used to select ura3 mutant cells of S. cerevisiae (Adams, et al., 1997), rapidly yielded large numbers of FOA-resistant colonies. None of these colonies were uracil auxotrophs, indicating that Z. bailii can readily acquire a high intrinsic resistance to FOA. It was suspected therefore that it would not be straightforward to obtain gene deletions in Z. bailii by first constructing a ura3 mutant strain; then employing disruption vectors that contain a URA3 gene and subsequently selecting for the loss of URA3 (a technique sometimes termed "URA-blasting" (Fonzi and Irwin, 1993)). Since Z. bailii isolates are generally prototrophic, the yeast gene deletion strategies based on dominant selectable markers seemed to be the most attractive. In growth inhibition tests we found that the Z. bailii isolates in the NCYC collection were totally inhibited by the inclusion of 30-40µg/ml G418 in YPD (Table 3.1). Therefore, Z. bailii is even more sensitive to G418 than S. cerevisiae. For this reason the kanMX4 cassette system (Güldener, et al., 1996;

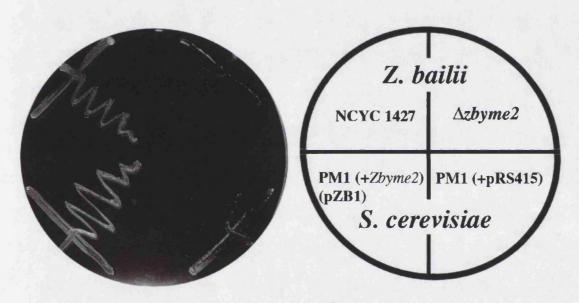


Figure 3.3. A plate containing benzoate as sole carbon source, streaked with *S. cerevisiae* PM1 containing either an empty vector (pRS415) or pRS415 with a *ZbYME2* fragment insert (pZB1); or wild type *Z. bailii* 1427 and a *zbyme2::kanMX4:zbyme2::hphMX4* homozygous deletant strain derived from *Z. bailii* 1427 (Δ*zbyme2*). The *ZbYME2* gene of pZB1 allows *S. cerevisiae* to grow on benzoate, whereas inactivation of the same gene in *Z. bailii* abolishes the intrinsic capacity of the latter yeast to utilise benzoate. The plate was photographed after 3d growth at 30°C.

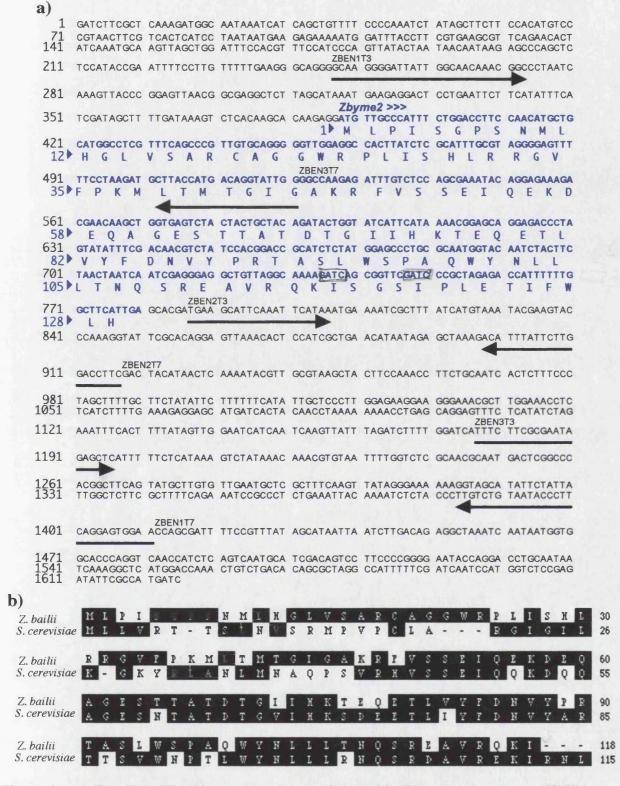


Figure 3.4. a) The Z. bailii 1427 sequence insert of plasmid pZB1, showing the ZbYME2 ORF (GenBank Accession AF279261). The only other ORF of >100 amino acids within this sequence, on the opposite strand of the DNA at 993 to 1487, bears no discernable similarity to any gene in the sequence databases. Arrows represent the primers used to sequence this DNA fragment. b) Amino acid sequence alignment of full length ZbYme2p and the N-terminal region (amino acids 1-115) of S. cerevisiae Yme2p.

Wach, 1996; Wach, et al., 1994) was initially used for deletions of ZbYME2 in Z. bailii.

Table 3.1. The concentrations of different compounds needed for complete inhibition of YPD growth of Z. bailii strains 563 and 1427; also concentrations used in selecting Z. bailii transformants.

Compound	Growth inhibitory	Concentrations used in cassette			
	concentration ^a	selection.			
Sorbate ^a	8mM	-			
Benzoate ^a	8mM (563); 10mM (1427)	-			
Fluoroacetate	11mM	-			
Formaldehyde	6mM (563); 7mM (1427)	9-10mM (<i>SFA1MX4</i>)			
G418	30-40 μg/ml	60-100 μg/ml (kanMX4)			
CuSO ₄	0.5mM	-			
Hygromycin A	40-50 μg/ml	100-150μg/ml (hphMX4)			

^a All concentrations are for 30°C pH6.8 growth on YPD, except the values for sorbate and benzoate, which relate to pH4.5 YPD 30°C growth.

3.2.4 Selecting for resistance to G418.

kanMX4 cassettes, designed to replace a ZbYME2 allele in Z. bailii by homologous recombination, were prepared with both short (40bp; SFH) and long (400bp; LFH) flanking homologies to ZbYME2 (see Methods). Primers ZbYME2-SFH-F and ZbYME2-SFH-R (Table 3.2) were used to amplify the SFH-kanMX4 cassette using pUG6 (Güldener, et al., 1996) as PCR template. Each end of this SFH cassette had 40bp of sequence from the regions immediately flanking the coding region of Z. bailii ZbYME2 (Figure 3.5). A LFH-ZbYME2 cassette was also constructed, by first amplifying the 5' noncoding region of ZbYME2 using primers B15'F(KpnI) and B15'R(AscI) (Table 3.2). This PCR product was digested with Kpn1 and Asc1 and then ligated into KpnI/AscI-cleaved pSL1183. The resulting plasmid was BamHI/SmaI-cleaved, whereupon the 3' noncoding region of ZbYME2 was inserted as a BamHI/SmaI-cleaved PCR product originally amplified using primers B13'F(BamHI) and B13'R(SmaI) (Table 3.2). The loxP-kanMX-loxP cassette was then amplified by PCR

from pUG6, using primers LOXPF(AscI) and LOXPR(AscI) (Table 3.2). This cassette was then digested with AscI and ligated to the AscI-cleaved sites of the above vector to produce plasmids pSL1183-B1kanMX4 (Figure 3.6). This vector was then used as a template in PCR amplifications of the LFH-ZbYME2 disruption cassette, with primers ZbYME2-LFH-F/R (Table 3.2).

Z. bailii 1427 was successfully transformed to G418 resistance with these LFH and SFH kanMX4 cassettes, using transformation procedures originally developed for S. cerevisiae (Gietz, et al., 1995). G418-resistant colonies (approximately 20 per µg DNA) were isolated on YPD plates containing 60µg/ml G418 (Table 3.1). Using colony PCR techniques (Ling, et al., 1995), individual transformants were checked for the correct replacement of a ZbYME2 gene using primers B1, B2, B3, B4, K2 and K3 (Table 3.2, also Figures 3.5a,b, 3.7b). Of the 30 transformants obtained using the SFH cassette, just 2 had correctly integrated a cassette at a ZbYME2 allele (thus generating the Zbyme2::kanMX4/YME2 genotype; see below). Only a slightly higher fraction of the transformants generated using the LFH cassette (6 out of 40 tested) had integrated correctly. Proper targeting of gene disruption cassettes is therefore considerably less efficient in Z. bailii than in S. cerevisiae and appears to be improved only slightly by the use of longer flanking homologies to the target gene. Nevertheless ZbYME2-targetting kanMX4 cassettes can be used successfully in Z. bailii, an indication that the Ashbya gossypii transcriptional control sequences of kanMX4 are functional in this yeast.

3.2.5 Sporulation of Zbyme2::kanMX4/YME2 Z. bailii yielded vegetative, not meiotic spores.

The ploidy of the Z. bailii isolates from cases of food spoilage in the culture collections is unclear. None of the four Z. bailii strains investigated (Table 2.2) showed any spontaneous mutation to canavanine resistance when vegetative cells were plated on SD plates containing 60μ g/ml canavanine. A high rate of generation of can' mutants by haploid, but not diploid cells, is frequently used as a means to identify haploid S. cerevisiae (Adams, et al., 1997). The literature contains references to Z. bailii ascospores and the conjugation of cells prior to ascospore development (Thomas and

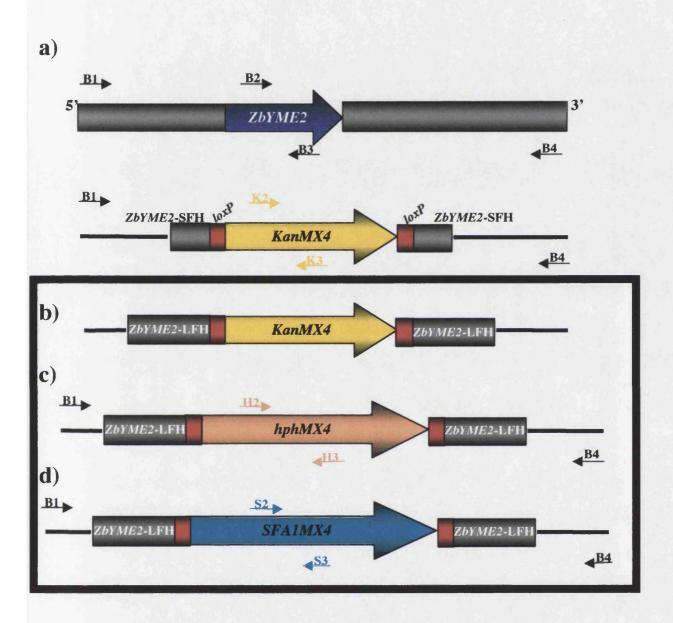


Figure 3.5. Schematic view of the general strategy used for PCR diagnosis of transformants. The primers B1, B2, B3, B4, K2, K3, H2, H3, S2, and S4 are used to check for correct integration of (a), (b) *KanMX4*, (c) *hphMX4* and (d) *SFA1MX4* cassettes at the alleles *ZbYME2*.

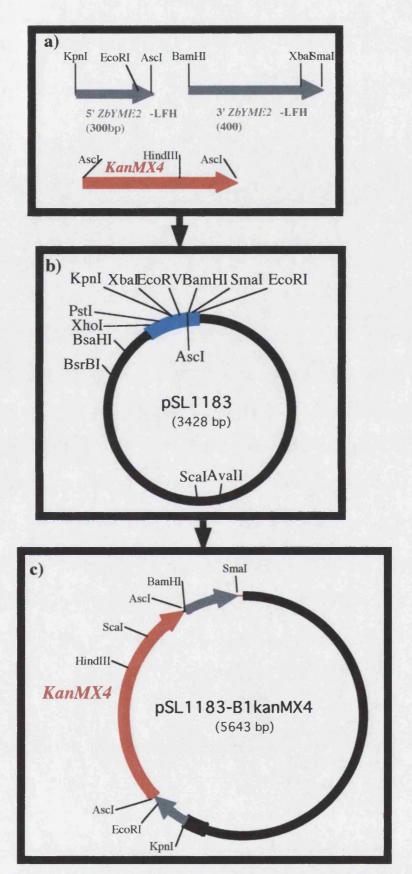


Figure 3.6. Schematic representation of the construction of pSL1183-B1kanMX4

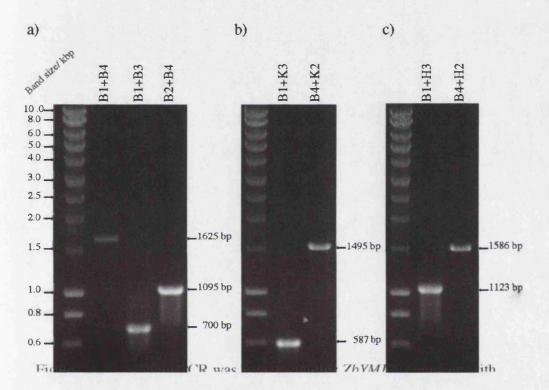


Figure 3.7. (a) Colony PCR was used to amplify *ZbYME2* fragments with primers B1+B4 and also the 5' and 3' regions of this gene were amplified by PCR using primers B1+B3 and B2+B4 respectively (See figure 3.5). The correct replacement of a *ZbYME2* with (b) *kanMX4* and (c) *hphMX4* cassettes were confirmed with primers B1+K3, B4+K2 and B1+H3, B4+H2 respectively.

Davenport, 1985). However these processes have never been described in much detail. Z. bailii sporulation was investigated here in the hope that cells would undergo meiosis and that the selection of a haploid spore colony from a heterozygous disruptant might eliminate the need for disruption of the second allele of our target gene. Over 14d at 25°C on sporulation medium (0.3% malt extract, 0.5% peptone) some Z. bailii cells formed the protrusions and spores (denoted "conjugation tubes" and "ascospores" in (Thomas and Davenport, 1985)) seen in earlier work (Figure 1.2 b). Most of the cells eventually formed 2-4 spores without forming protrusions. With very brief zymolase treatment the individual phase-bright spores in sporulating Z. bailii cultures were readily separated and the unsporulated cells lysed. Micromanipulation was used to separate 60 individual zymolase-treated spores from the Zbyme2::kanMX4/YME2 Z. bailii strain. All the spores germinated on YPD, yet retained their Zbyme2::kanMX4/YME2 genotype (not shown). It was concluded, therefore, that these were vegetative and not meiotic spores. So far no firm evidence of a sexual cycle in Z. bailii has been obtained, even though the Z. bailii 1427 strain may be diploid, as suggested by the presence of two ZbYME2 gene copies (see 3.2.7).

3.2.6 Construction of a new disruption cassette (SFA1MX4) that confers formaldehyde resistance

Over-expression of the S. cerevisiae SFA1 gene is known to confer formaldehyde resistance to S. cerevisiae (Van den Berg and Steensma, 1997). Therefore, the Tn903-derived coding sequence of kanMX4 was replaced with the coding sequence of S. cerevisiae SFA1 to generate a new disruption cassette (SFA1MX4). The sequence of SFA1MX4, like kanMX4, should be non-homologous to sequences within the Z. bailii genome.

pUG6 was digested completely with NcoI, then partially digested with ScaI in order to remove the E. coli transposon Tn903 sequences. SFAI coding sequences were amplified by PCR from S. cerevisiae FY1679-28C genomic DNA, using primers SFA1F and SFA1R (Table 3.2). This 1158 bp PCR product was then digested with NcoI and ScaI and ligated into NcoI/(partially)ScaI-digested, dephosphorylated pUG6. Finally the

ATG codon within the NcoI site of the resulting plasmid was mutated to TTG, using primers MUTSFA1F, MUTSFA1R (Table 3.2) and the QuickChangeTM Site-directed mutagenesis kit (Section 2.6.9). This point mutation was confirmed by sequencing across the mutagenised area. The mutagenised plasmid, pUG6-SFA1MX4 (Figure 3.8), conserves the loxP sites of pUG6 (Güldener, et~al., 1996) and contains a cassette, designated SFA1MX4, selectable from its ability to confer increased formaldehyde resistance.

3.2.7 Selection for formaldehyde or hygromycin B resistance in Z bailii and the construction of a Zbyme2::kanMX4/Zbyme2::hphMX4 homozygous mutant.

PCR analysis of those Zbyme2::kanMX4/YME2 Z. bailii colonies that had correctly integrated a kanMX4 cassette at a ZbYME2 sequence also revealed bands originating from an undisrupted copy of ZbYME2, showing that Z. bailii 1427 has at least two ZbYME2 alleles (Figure 3.7). As described in section 3.2.5, sporulation of this strain did not yield spore colonies totally lacking a functional ZbYME2 gene. Therefore, a strategy was required in order to delete the second ZbYME2 copy. Testing the sensitivity of Z. bailii to various compounds that could be used with dominant selectable markers, we observed that cells were sensitive to 6-7mM formaldehyde and 40-50µg/ml hygromycin B on YPD plates (Table 3.1).

Primers ZbYME2-SFH-F and ZbYME2-SFH-R (Table 3.2) were used, with either pUG6-SFA1MX4, or pAG32 (Goldstein and McCusker, 1999) as the DNA templates, in order to generate ZbYME2SFH-SFA1MX4 and ZbYME2SFH-hphMX4 cassettes. These were selectable on the basis of either formaldehyde or hygromycin resistance respectively.

For preparing ZbYME2-disrupting LFH cassettes the pSL1183-B1SFA1MX4 and pSL1183-B1hphMX4 plasmids were constructed. The loxP-SFA1MX4-loxP and loxP-hphMX4-loxP cassettes were amplified by PCR from pUG6-SFA1MX4 and pAG32 plasmids using primers LOXPF(AscI) /LOXPR(AscI) and LOXP-F26/LOXP-R26 (AscI) respectively (Table 3.2). These two cassettes were then digested with AscI and ligated to the AscI-cleaved sites of pSL1183-B1kanMX4 (Figure 3.6) to produce plasmids,

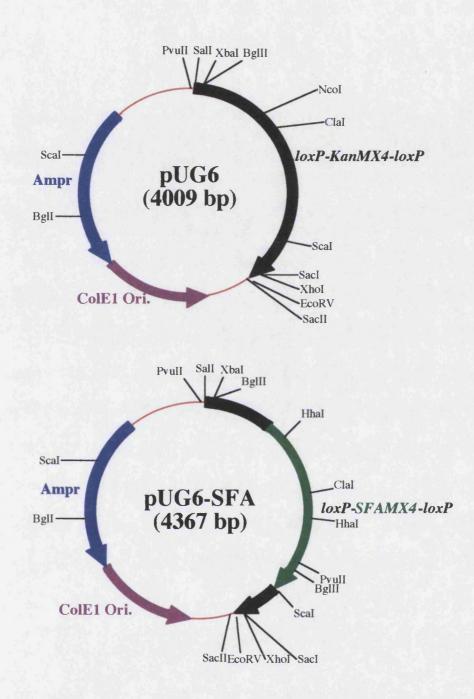


Figure 3.8. Schematic representation of the pUG6 and the pUG6-SFA1 vectors.

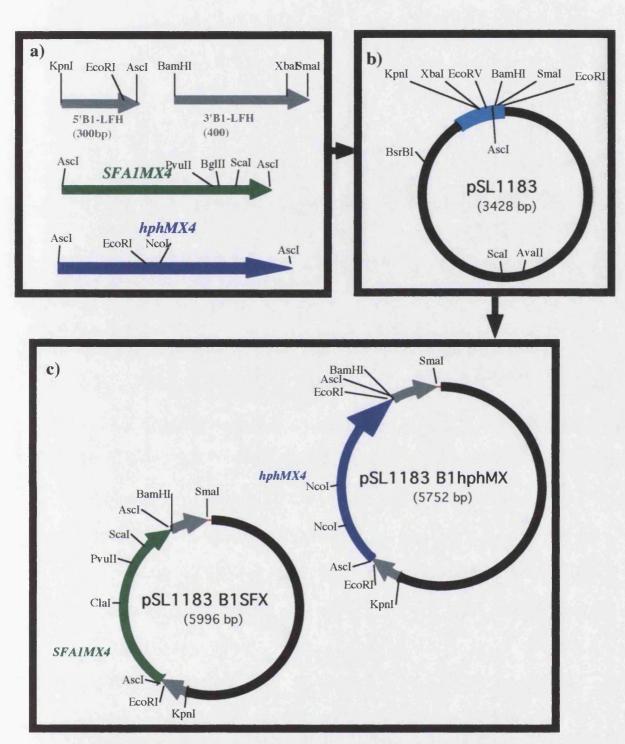


Figure 3.9a) Schematic representation of 5'and 3' regions of *ZbYME2*-LFH and the *SFA1MX4* and *hphMX4* cassettes. b) These were ligated into pSL1183 in order to construct c) pSL1183-B1SFA1MX4 and pSL1183-B1hphMX4

pSL1183-B1SFA1MX4 and pSL1183-B1hphMX4 (Figure 3.9). These two vectors were used as templates in PCR amplifications of the LFH *ZbYME2* disruption cassettes, with primers ZbYME2-LFH-F/R (Table 3.2).

The previously constructed Zbyme2::kanMX4/YME2 strain (section 3.2.4) and its Z. bailii 1427 parent were transformed using these ZbYME2-targeting LFH SFA1MX4 and hphMX4 cassettes, selecting for either formaldehyde resistance or hygromycin B resistance respectively, under conditions given in Table 3.1. Analysis of the resistant colonies by colony PCR (Ling, et al., 1995) using primers B1, B2, B3, B4, S2, S3, H2 and H3, (Table 3.2) revealed that both SFA1MX4 and hphMX4 cassettes had yielded deletant of ZbYME2 (Figure 3.7). However only 10-15% of the transformants tested had resulted from integration of the cassette at a ZbYME2 locus. Therefore it is possible to use these resistance cassettes in Z. bailii, even though there is only a 10-15% efficiency of correct gene targeting.

The phenotypes associated with the loss of ZbYME2 function in Z. bailii are described in Chapter 4. The most important phenotype is the inability of the cells to grow on benzoate as sole carbon source (Figure 3.3)

Table 3.2. PCR primers.

Oligonucleotide	Sequence and usage (restriction sites underlined)				
	Z. bailii NCYC 1427 ZbTIM9, ZbURA3 sequencing				
-UT3	TAATGCAGCTGGCACGACAG				
UT3	AATTAACCCTCACTAAAGGG				
1UT3	CATAGCGTTACGATGTGGAT				
2UT3	CCAATATCAATTGTATCTAT				
3UT3	GCAATACTCTTCTGGTGTCT				
4UT3	GACTGTATTCTGTAACTCGC				
-UT7	GATCGGTGCGGGCCTCTTCG				
UT7	GTAATACGACTCACTATAGGGC				
1UT7	TAAATGTGCAGACAGCATTG				
2UT7	GGATTACATCTCTTCAGATA				
3UT7	CCTGATTTTTGAGGATAGAA				
4UT7	CGTTCAATAATCAAGCCCCAGC				
	Z. bailii NCYC 563 ZbHHT1, ZbTRP1 sequencing:				
TT3	AATTAACCCTCACTAAAGGG				
3TT3	AAGTTGTCTTCTACATAACA				
4TT3	GCAGACAGCTAGAAAGTCT				
1TT7	GTTAGCTGTGAATCTTGAAA				
TT7	GTAATACGACTCACTATAGGGC				
3TT7	ATCTAATTCATACTCTTGCG				
4TT7	CCTGCTGAGCAGCCTCGACA				
<i>5</i> TT7	TGTTACTACCTCGTGGAGGTC				

	Z. bailii NCYC 1427 ZbYME2 sequencing:
T3	AATTAACCCTCACTAAAGGG
ZBEN1T3	GCAAGGGGATTATTGGCAACAAACGGC
ZBEN2T3	TGAAGCATTCAAATTCATAA
ZBEN3T3	TTTCTTCGCGAATAGAGCTC
T7	GTAATACGACTCACTATAGGGC
ZBEN1T7	TTCCACTCCTGAAGGGTATTACAGACAA
ZBEN2T7	GAAGGTCCAAGAATAAATGT
ZBEN3T7	CCAATACCTGTCATGGTAAG
	Construction of SFA1MX4 cassette:
SFA1F	CATGCCATGGATGTCCGCCGCTACTGTTGGT
SFA1R	AAAAGTCTATTTTATTTCATCAGACTTCAAG
MUTSFA1F	GAACATAAACAACCTTGGATGTCCGCCGCTA
MUTSFA1R	TAGCGGCGGACATCCAAGGTTGTTTATGTTC
MOTOTTING	Construction of vectors pSL1183-B1kanMX4,
	pSL1183-B1SFA1MX4 and pSL1183-B1hphMX4:
B13'F(BamHI)	AAAAAAGGATCCTGAGCACGATGAAGCATTCAAATTCA
B13'R(SmaI)	TTTTAACCCGGGAATGATCCAAAAGATCTAAATAACTT
B15'F(KpnI)	GGAAAAGGTACCTAATAATGAAGAGAAAAATGGATTT
B15'R(AscI)	GGGGGCGCCCATCCTCTTGTGCTTGTGAGACTTTAT
LOXPF(AscI)	TTTTGGCGCGCCTTCGTACGCTGCAGGTCGACAACC
LOXPR(AscI)	TTTTGGCGCGCCACTAGTGGATCTGATATCACCTAT
LOXP-F26	TTCGTACGCTGCAGGTCGAC
LOXP-R26 (AscI)	TTTT <u>GGCGCGCC</u> ACTAGTGGATCTGATATC
0032 100 (2202)	ZbYME2-SFH cassette amplification:
ZbYME2-SFH-F	GCGATTTTCATTTATGAATTTGAATGCTTCATCGTGCTCACCACTAGT
	GGATCTGATATC
ZbYME2-SFH-R	TCGATAGCTTTTGATAAAGTCTCACAAGCACAAGAGGATGTTCGTAC
	GCTGCAGGTCGAC
	ZbYME2-LFH cassette construction:
ZbYME2-LFH-F	CTAATAATGAAGAGAAAATGGATTT
ZbYME2-LFH-R	AATGATCCAAAAGATCTAAATAACTT
	Confirming ZbYME2 deletion in Z. bailii:
B1	GATCTTCGCTCAAAGATGGCAATA
B2	GATTTGTCTCCAGCGAAATACAGGAG
В3	GAAGTAGATTGTACCATTGCGCAGG
B4	GATCATGGCGAATATCTCGGAGAC
K2	GATTTTGATGACGAGCGTAAT
К3	TGTACGGGCGACAGTCACATC
S2	ATTTTAATCAAATGTTAGCG
S3	TGTACGGGCGACAGTCACATC
H2	CCTCGTGCACGCGGATTTCGGC
H3	GAACAGCGGCAGTTCGGTTTC
	Amplification of inserts in pRS415:
T3	AATTAACCCTCACTAAAGGG
T7	GTAATACGACTCACTATAGGGC
L <u></u>	

3.3 Discussion

This study is the first to show that Z. bailii genes are readily obtainable by complementation cloning; also that inactivation of these genes is feasible using the transformation and gene deletion procedures originally developed for S. cerevisiae. Even with the use of LFH gene disruption cassettes, correct cassette targeting by homologous recombination in Z. bailii appears to be no more than 10-15%. Several transformants must therefore be screened in order to obtain any desired deletion. Also, all the copies of the target sequence must be knocked out in turn, using different dominant selectable markers, in order to inactivate any gene function.

One disadvantage of using different dominant selectable markers to sequentially perform multiple gene disruptions is that eventually one runs out of markers to perform further disruptions. Further investigations can reveal if it is possible to insert a Cre recombinase gene into Z. bailii and, then to excise integrated cassettes that are flanked by loxP sites using Cre-catalysed recombination (a proven strategy for the reuse of cassettes in S. cerevisiae (Güldener, et al., 1996)). The cassettes that were used here for ZbYME2 deletion contained flanking loxP sites (Güldener, et al., 1996), although Crecatalysed recombination at these loxP sites was not part of the eventual strategy for deleting both ZbYME2 alleles.

Z. bailii does possess plasmids similar in overall structure to the 2μ DNA of S. cerevisiae (Toh-E, et al., 1984; Toh-E and Utatsu, 1985). The S. cerevisiae-E. coli shuttle vectors YCplac111 and YEplac181 (Gietz and Sugino, 1988), containing a kanMX4 gene insert (Wach, et al., 1994) for selection and maintenance on G418 media, are able to replicate in Z. bailii 1427 (data not shown). It appears that S. cerevisiae plasmid replication sequences function in Z. bailii.

The ability of the YEplac181-derived plasmid to replicate suggests that the endogenous 2μ-like plasmids of Z. bailii may be able to provide replication functions in trans to plasmids containing the S. cerevisiae 2μORI-STB region. Inserting into Z. bailii

cells a plasmid that bears a dominant gene for selection and maintenance in Z. bailii, together with a Cre recombinase gene under inducible promoter control, may be all that is needed to excise loxP-flanked cassettes. This would overcome any limitation to the number of gene knockout cassettes available, therefore the number of deletions that can be performed in any Z. bailii strain.

Chapter 4. Further characterisation of ZbYME2: Oxidative degradation of benzoic acid by Z. bailii

4.1 Introduction

Z. bailii can degrade sorbate and benzoate, although prior to this work, the mechanisms by which it degrades these weak acids had not been explained. Biodegradation of phenols and other aromatic compounds by fungi has been the subject of a number of studies. Rhodotorula yeasts and other fungi metabolise L-phenylalanine and other aromatic compounds by the \(\beta\)-ketoadipate pathway via benzoate (McNamee and Durham, 1985). p-hydroxybenzoate has been demonstrated in these yeasts as an in vivo oxidation product of benzoate, L-phenylalanine, and D,L-mandelate (Figure 4.1). Thus, these yeasts have a mono-oxygenase with benzoate 4-hydroxylase activity. This enzyme was first identified in Aspergillus niger and then later in yeasts such as Rhodotorula minuta. The CYP53 gene encodes a benzoate 4-hydroxylase and has the properties of cytochrome P450 (Fujii, et al., 1997).

The CYP51 cytochrome P450s provides the house keeping lanosterol 14-demetylase function and are well conserved in fungi and mammalian cells (Aoyama, et al., 1996). CYP51s of the ascomycete yeasts, S. cerevisiae and Candida tropicalis share 47% amino acid homology with those of basidiomycete fungus Ustilago maydis. It can therefore be suggested that CYP53s, encoding benzoate 4-hydroxylases, may be yet another conserved class of cytochrome P450 (Fujii, et al., 1997).

Chapter 3 shows that Z. bailii can utilise benzoate as sole carbon source (Figure 3.3) and also describes the isolation of a gene that facilitates this degradation. This gene (ZbYME2 (AF279261)) also confers to S. cerevisiae cells the capacity to catabolise L-phenylalanine and benzoate (Figure 4.1), compounds that S. cerevisiae is normally unable to use as sole carbon source. This suggests that ZbYME2 provides a benzoate 4-hydroxylase in S. cerevisiae. However it may not encode a cytochrome P450. The protein encoded by ZbYME2 has 74% amino acid sequence identity with the N-terminal mitochondrial matrix domain of the Yme2p/Rna12p of S. cerevisiae (Figure 4.4a).

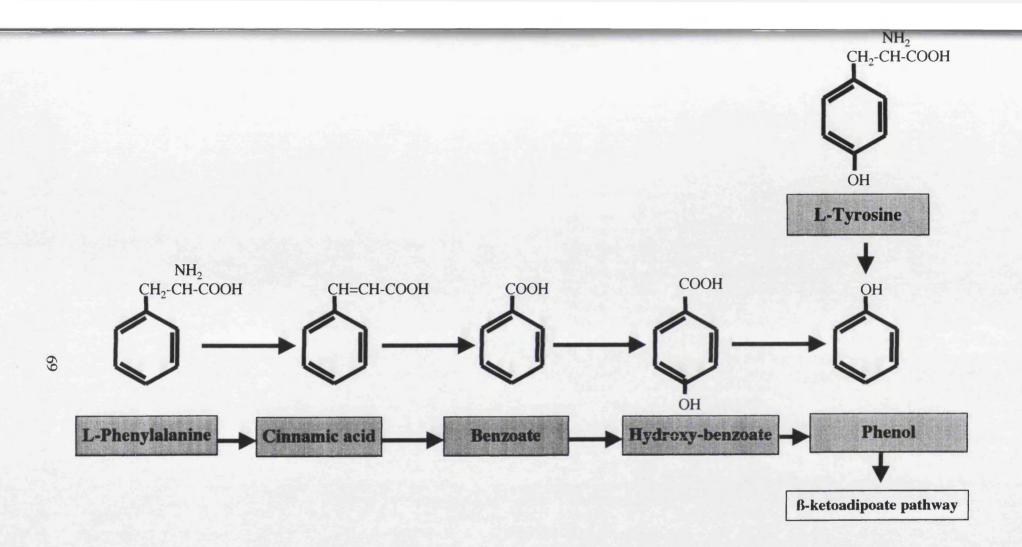


Figure 4.1. Degradation of phenylalanine and tyrosine in fungi.

It also has 34% partial amino acid sequence similarity to an insect (*Manduca sexta*) CYP450 (Figure 4.4b), but the haem-binding residues are not part of the conservation.

Yme2p of S. cerevisiae is a large integral inner mitochondrial membrane protein, comprising an N-terminal matrix domain, a single transmembrane domain and a C-terminal domain located in the intermembrane space (Hanekamp and Thorsness, 1996; Leonhard, et al., 2000). Its loss is characterised by three phenotypes: a high rate of DNA escape from mitochondria to the nucleus, an inability to grow on non-fermentable carbon sources at 30°C in cells of yme1 genetic background, and the ability to suppress the cold-sensitive growth phenotype of yme1 strains (Hanekamp and Thorsness, 1996; Hanekamp and Thorsness, 1999). As shown in this chapter, many of these phenotypes are suppressed with the heterologous expression of ZbYME2.

4.2 Results

4.2.1 Benzoate is utilised as carbon source by Z. bailii but not S. cerevisiae.

Z. bailii grows on SD plates over the pH ranges 3.0-6.0. At pH 5.0, it can also grow on 1200 mgl⁻¹ (~ 10mM) of benzoate or 750 mgl⁻¹ (~ 5mM) of sorbate (Praphailong and Fleet, 1997). Z. bailii strains 1427 and 563 grow on media containing benzoate or phenylalanine as sole carbon source (Figure 4.2a, b). Relative growth on different concentrations of benzoate or sorbate as sole carbon sources at pH 6.0 is given in table 4.1.

Table 4.1 Growth of Z. bailii on different concentrations of benzoate and sorbate at

pH6.0. Growth was scored after 7 day incubation at 30°C.

Strain	Benzoate concentration (mM)						
·	0.5	1.0	2.0	3.0	4.0	5.0	6.0
Z. bailii 1427	+++	+++	+++	+++	++	++	+
Z. bailii 5 63	+++	+++	+++	+++	++	++	++
	Sorbate concentration (mM)						
Strain			Sorbate o	concentrat	ion (mM)		
Strain	0.5	1.0	Sorbate 6 2.0	concentrat	ion (mM) 4.0	5.0	6.0
Strain Z. bailii 1427	0.5	1.0				5.0	6.0

⁺⁺⁺ Strong growth, ++ Moderate growth, + Poor growth.

Prototrophic S. cerevisiae (strains PM1 plus pRS415 and Σ 1278) can not use benzoate (Figure 4.2a) or phenylalanine (Figure 4.2b) as sole carbon source. However they are able to use 4-hydroxy-benzoate (Figure 4.3a) or tyrosine (Figure 4.3b), indicating that their inability to use benzoate or phenylalanine is due to the lack of a benzoate 4-hydroxylase (Figure 4.1).

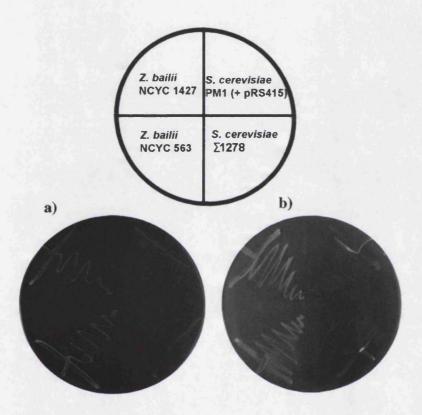


Figure 4.2. pH6.0 plates containing a) benzoate, or b) phenylalanine as sole carbon source, streaked with Z. bailii 1427 and 563, S. cerevisiae PM1 containing empty vector (pRS415) and a prototrophic S. cerevisiae Σ 1278. Plates were photographed after 5d at 30°C.

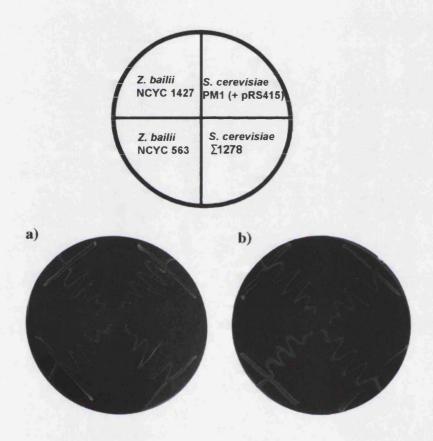


Figure 4.3. pH6.0 plates containing a) hydroxy-benzoate, or b) tyrosine as sole carbon source, streaked with Z. bailii 1427 and 563, S. cerevisiae PM1 containing empty vector (pRS415) and a prototrophic S. cerevisiae Σ 1278. Plates were photographed after 5d at 30°C.

4.2.2 Z. bailii ZbYME2 sequence analysis.

The ZbYME2 gene, isolated as a sequence that confers benzoate utilisation to S. cerevisiae cells, (Chapter 3), encodes a 14.6 kDa protein with 74% amino acid sequence similarity to the N-terminus of the Yme2p/Rna12p of S. cerevisiae (Figure 4.4a). Its closest similarity to a cytochrome P450 is to the CYP450 of the insect M. sexta (34%) amino acid sequence similarity), (Figure 4.4b)). In S. cerevisiae Yme2p is an integral inner mitochondrial membrane protein with one transmembrane domain (Figure 4.4a), an amino terminal domain located in the mitochondrial matrix and a carboxyl-terminal portion facing the inter-membrane space (Hanekamp and Thorsness, 1996). It is the major part of the matrix domain that has high homology to the much smaller ZbYme2p (Figure 4.4a). Since S. cerevisiae Yme2p undergoes processing to a smaller form during mitochondrial import (Leonhard, et al., 2000), it is possible that the non-homologous 43-47 amino acids at the N-termini of S. cerevisiae Yme2p and Z. bailii ZbYme2p contain mitochondrial targeting signals that are lost during mitochondrial import. Structure prediction suggests that ZbYme2p contain a hydrophobic amphipathic α-helix at its Nterminus, which may function as a mitochondrial targeting sequence (Kyte and Doolittle, 1982) (Figure 4.5).

4.2.3 Inability of the Z. bailii zbyme2::kanMX4/zbyme2::hphMX4 homozygous deletant to use benzoate as sole carbon source.

The zbyme2::kanMX4/zbyme2::hphMX4 homozygous deletant strain described in chapter 3, was investigated with regard to phenotype. Unlike the Z. bailii 1427 parent, this mutant lacks the ability to grow on benzoate as sole carbon source (Figures 3.3 and 4.6a). The ZbYME2 gene appears to be essential for Z. bailii to catabolise the preservative benzoic acid. The homozygous deletant strain was also more sensitive to benzoate and sorbate during growth on glucose at pH4.5 (Figure 4.6b, c), conditions that provide severe weak acid stress. It displayed no defect in growth at high temperatures (Z. bailii 1427 grows up to about 33-35°C) but, surprisingly, was impaired in respiratory growth at 15°C (YPEG medium; Figure 4.6d). Thus by generating a homozygous deletant strain

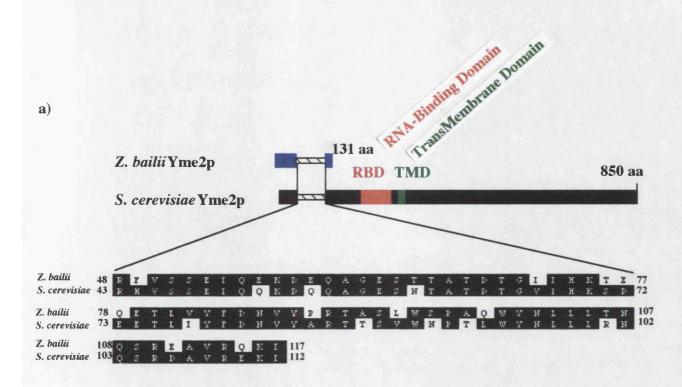






Figure 4.4. a) Sequence identity between ZbYme2p and the N-terminal region of S. cerevisiae Yme2p. b) Sequence identity between Zbyme2p and cytochrome P450p of M. sexta.

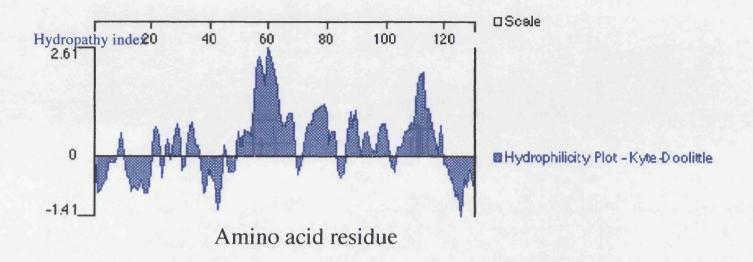


Figure 4.5. Hydropathy plot of the Z. bailii Zbyme2 protein. The plot was obtained after the algorithm of Kyte and Doolittle (Kyte and Doolittle, 1982)

it was possible to demonstrate that ZbYME2 is essential for the catabolism of benzoate by Z. bailii (Figure 4.6a). ZbYME2 also contributes to growth on glucose in the presence of weak acid stress and normal respiratory growth at low temperatures (Figure 4.6b, c, d). This is the first identification of a Z. bailii gene important in resistance to weak acid preservatives.

4.2.4 Confirmation of the mitochondrial localization of ZbYme2p when expressed in S. cerevisiae as a GFP fusion.

To investigate the intracellular localisation of ZbYme2p expressed in S. cerevisiae, two vectors for expression of GFP-tagged ZbYme2p were constructed. First the ZbYME2 ORF was amplified with PCR primersYM2gfpNF/ YM2gfpNR or YM2gfpCF/ YM2gfpCR (Table 4.2). The resulting PCR products were digested with HindIII/ XhoI or XbaI/ HindIII and cloned into HindIII/ XhoI cleaved pGFP-N-FUS and XbaI/ HindIII cleaved pGFP-C-FUS respectively (Niedenthal, et al., 1996; Figure 4.7a, b). The resulting pGFP-N-ZbYME2 and pGFP-C-ZbYME2 plasmids, for expression of ZbYme2p with GFP added at its N- or the C-terminus respectively, were then transformed into the diploid uracil auxotrophic S. cerevisiae strain FY1679 (Table 2.1), selecting for growth on SD plates with no supplements. Transformants were then streaked on SB plates without methionine in order to induce cells growth on benzoate and high level of fusion protein expression from the MET25 promoter.

Plasmid pGFP-C-ZbYME2 but not pGFP-N-ZbYME2 enabled the growth of these S. cerevisiae cells on SB plates (Figure 4.8). It appears that an N-terminal GFP extension abolishes the ability of ZbYme2p to confer benzoate utilisation to S. cerevisiae cells. This is possibly because it interferes with the functioning of mitochondrial targeting signals. In contrast, this protein is still functional with a C-terminal GFP extension (Figure 4.8).

Cells growing on benzoate, through expression of the functional ZbYme2p-GFP fusion, were next stained *in vivo* with MitoTracker (Molecular Probes Ltd., UK), and examined by microscopy, as described previously (Niedenthal, *et al.*, 1996). Figure 4.9 shows that much of the ZbYme2-GFP fusion co-localised with mitochondria at different

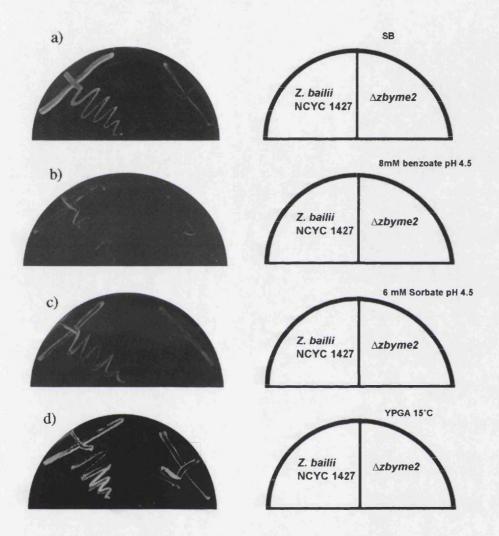


Figure 4.6. The growth phenotype of *Z. bailii* 1427 and its *zbyme2::kanMX4:zbyme2::hphMX4* homozygous deletant strain derivative. a) SB plate was photographed after 5d growth at 30°C. YPD pH 4.5 plates containing b) 8mM benzoate or c) 6mM sorbate were photographed after 3d growth at 30°C. d) The YPEG pH 6.0 plate was photographed after 7d growth at 15°C.

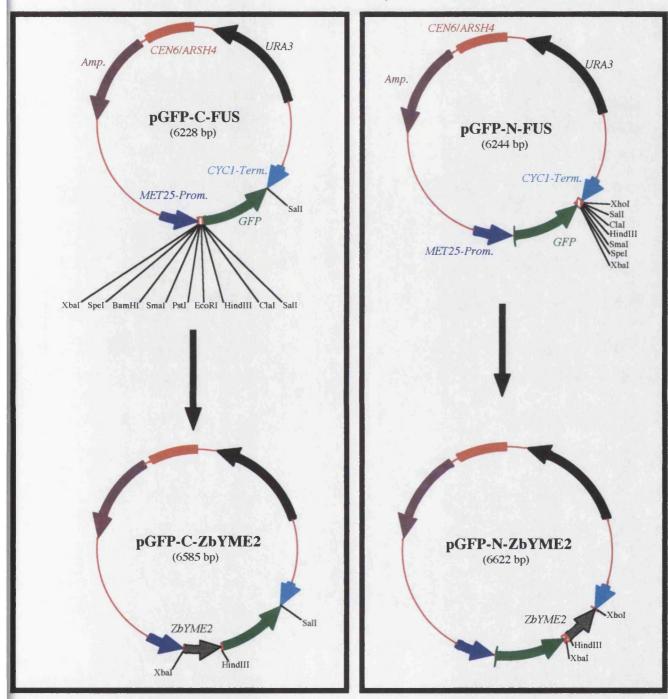


Figure 4.7. Vectors for over-expression of GFP linked to the a) C terminus of S. cerevisiae ZbYME2 (pGFP-C-ZbYME2) or b) to the N terminus of ZbYME2 (pGFP-N-ZbYME2).

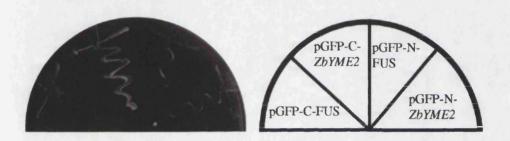


Figure 4.8. *S. cerevisiae* FY 1679 cells containing GFP fusions to the C- and N-termini of *ZbYME2* and also control plasmids lacking a *ZbYME2* insert were streaked on SB plates without methionine. Only cells expressing the pGFP-C-*ZbYME2* construct were able to grow on SB medium. Plates were photographed after 5d at 30°C.

stages of cell division. In some of the cells examined, almost all of the ZbYme2p-GFP was co-localised with mitochondria. In others, some of the ZbYme2-GFP was also present as what appeared to be cytosolic inclusion bodies. The presence of the latter may be a reflection of the high level of ZbYme2-GFPp expression from the MET25 promoter.

Cells expressing the ZbYME2-GFP fusion gene were sub-fractionated by differential centrifugation into plasma membrane, mitochondrial and soluble fractions as in section 2.5.4. These fractions were separated on a SDS-PAGE and western blotted. The over-expressed ZbYme2-GFP fusion protein was detected using anti-GFP monoclonal antibody (Clontech, Ltd., UK) in the whole-cell extract and mitochondrial fractions (Figure 4.10). The predicted molecular weight of this ZbYme2-GFP fusion (approximately ~41 kDa) was consistent with the molecular weight observed on SDS-PAGE and immunoblots (data not shown).

4.2.5 Heterologous expression of ZbYME2 reverses several phenotypes of the S. cerevisiae yme1-1,yme2-2 mutant.

Growth of *S. cerevisiae yme1* strains on non-fermentable carbon sources at 30°C is largely prohibited in a *yme2* background (Hanekamp and Thorsness, 1996; Thorsness and Fox, 1993). To see whether this phenotype is reversed with *ZbYME2* expression in a *yme1*, *yme2* background, the p*ZbYME2*(415) vector was constructed. The *ZbYME2* coding region plus 387 nucleotides of upstream sequence (the native promoter) was PCR amplified from pZB1, using primers ZBenzORF-F/R (Table 4.2). The PCR product was digested with *XhoI* and *HindIII*, then ligated into *XhoI/HindIII* cut pRS415 in order to produce p*ZbYME2*(415) (Figure 4.11). A *yme1-1 yme2-1 S. cerevisiae* (PTY55) and its isogenic wild type (PTY44) (Table2.1) were transformed with this p*ZbYME2*(415) vector, selecting for the *LEU2* gene of pRS415 (Sikorski and Hieter, 1989). Colonies were replica plated onto respiratory media (YPEG and YPG) and grown at 30°C. The presence of *ZbYME2* suppressed the poor respiratory growth of the *S. cerevisiae yme1,yme2* double mutant (PTY55), both on 3% glycerol (Figure 4.12a), and 2% glycerol plus 2% ethanol (Figure 4.12b).

ZbYme2-GFP MitoTracker Merged

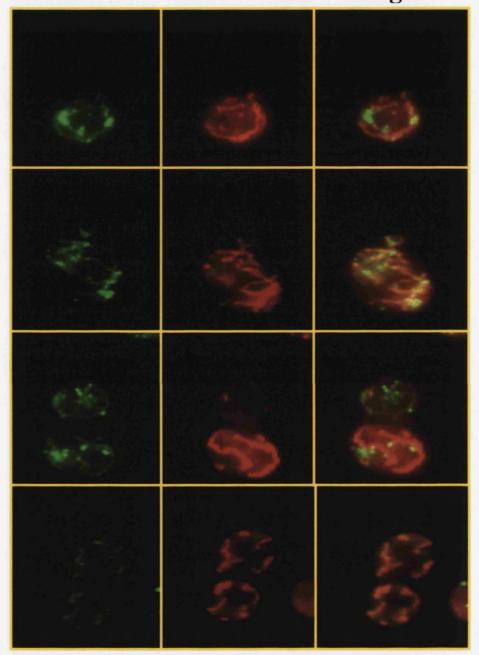


Figure 4.9. Subcellular localization of a ZbYme2-GFP fusion. *S. cerevisiae* FY1679 cells containing pGFP-C-ZbYME2 were grown on SB medium. GFP and MitoTracker were then visualised microscopically at different stages of division. In some cells (bottom figure) almost all of the ZbYme2p-GFP colocalised with mitochondria. In others (top 3 figures) some of the ZbYme2p-GFP appeared to be present as cytoplasmic inclusion bodies.

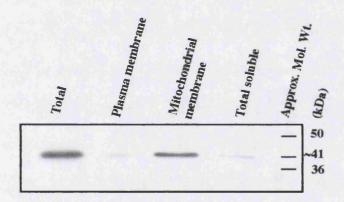
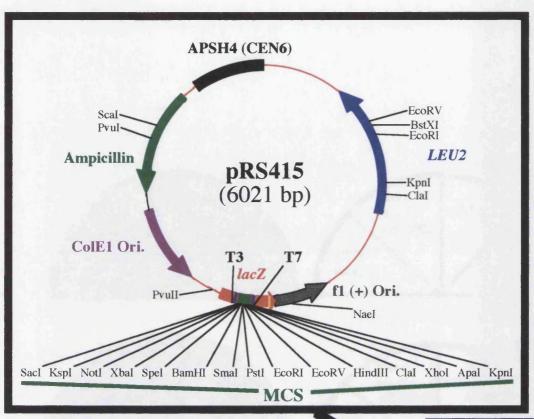


Figure 4.10. Western blot analysis of a ZbYme2-GFP fusion in fractions from disrupted FY 1679 cells. Proteins of total cell extracts, mitochondrial, total soluble, and plasma membrane fractions (approximately $10\mu g$ protein per lane) were immunoblotted and probed using an anti-GFP-tag monoclonal antibody.



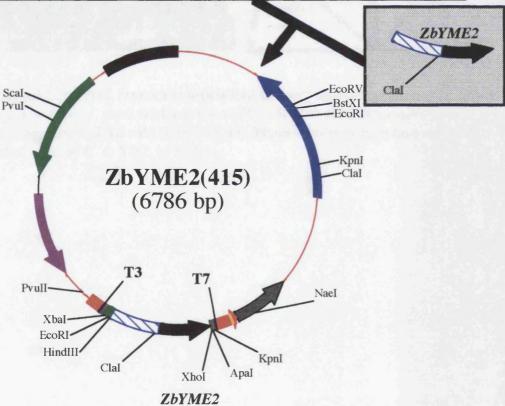


Figure 4.11. Schematic representation of the construction of vector *ZbYME2*(415).

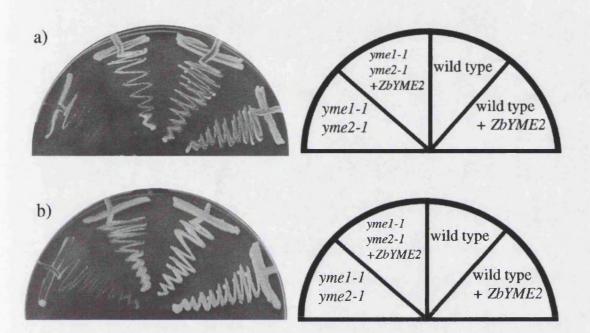


Figure 4.12. ZbYME2 expression suppressed the poor respiratory growth of the S. cerevisiae yme1, yme2 double mutant (PTY55). ZbYME2 expression did not effect the growth of the wild type (PTY44). Plates were photographed after 3d incubation at 30°C. a) YPG; b) YPEG.

The presence of ZbYME2 in the yme1-1 yme2-1 S. cerevisiae strain PTY55 also suppressed the escape of mtDNA to the nucleus (Figure 4.13). mtDNA escape in this strain is readily assayed as the appearance of TRP+ cells. This is due to the expression of a TRP1 sequence that is normally silent, since it is part of mtDNA in these S. cerevisiae strains (Thorsness and Fox, 1993). In Figure 4.13, cells were initially spread on minimal SD to provide a confluent lawn and then replica plated onto SD media lacking tryptophan, in order to identify TRP+ colonies. After 5 days at 30°C the number of TRP+ colonies in each plate was scored. (Hanekamp and Thorsness, 1999) reported the rate of mtDNA escape of the yme1 strain as 1.25x10⁻⁴ events/cell division; 25 times higher than in the wild type (Thorsness and Fox, 1993; Figure 4.13). This is still increased further in the yme1-1 yme2-2 double mutant (PTY55). The rate of mtDNA escape was substantially decreased in PTY55 transformed with pZbYME2(415) (Figure 4.13), but this was not observed in the control (PTY55+pRS415). ZbYME2 expression did not appear to suppress the cold-sensitive growth of yme1, yme2 cells described by (Hanekamp and Thorsness, 1996) (not shown).

In addition to the previously reported phenotypes, it was observed in this work that yme1-1, yme2-2 cells are sensitive to weak acid stress. They cannot adapt to grow on YPD at pH 4.5 in the presence of 3mM benzoate (Figure 4.14 a) or 1mM sorbate (Figure 4.14b), unlike the wild type. However expression of the heterologous Z. bailii ZbYME2 gene in yme1-1, yme2-2 S. cerevisiae (PTY55) restored capacity for growth on these media (Figure 4.14a, b).

4.2.6 Benzoate metabolism in a petite-negative S. cerevisiae expressing the ZYME2 gene.

Spontaneous respiratory-deficient (ρ) colonies of *S. cerevisiae* PM1 expressing the *ZbYME*2 gene were obtained by overnight growth on liquid YPD medium at 37°C, then confirmed as respiration-deficient from their total incapacity for growth on respiratory carbon sources. Respiratory deficiency led to an inability to grow on SB or SP plates (Figure 4.15a, b), although the cells still retained capacity for growth on glucose (SD) plates and therefore still maintained the plasmid. This indicates that the use

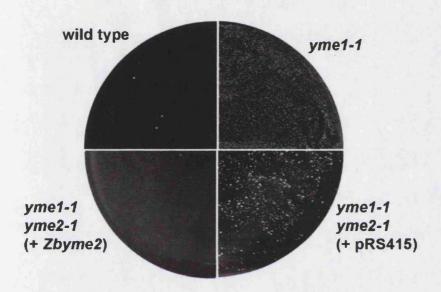


Figure 4.13. Detection of DNA escape from mitochondria to the nucleus. Confluent sectors were grown on SD medium supplemented with appropriate amino acids and then replica plated to SD medium with required amino acids except tryptophan. This led to the detection of Trp+ colonies resulting from the escape of *TRP1* from mitochondria. The plates were photographed after 5d incubation at 30°C.

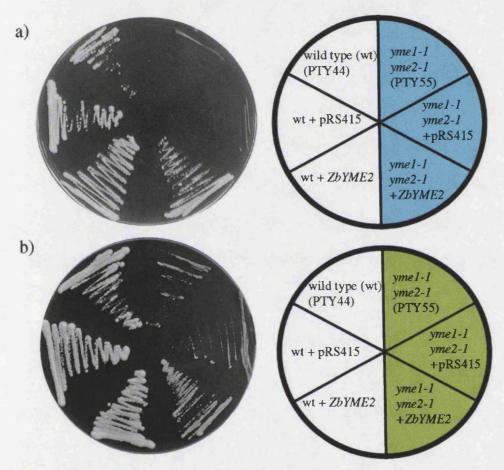


Figure 4.14. The growth of *S. cerevisiae* PTY44, and its *yme1-1 yme2-1* double mutant derivative (PTY55) on YPD at pH4.5 in the presence of a) 3mM benzoate and b) 1mM sorbate. Heterologous expression of *ZbYME2* in the *S. cerevisiae yme1-1 yme2-1* double mutant restored capacity for growth on these media.

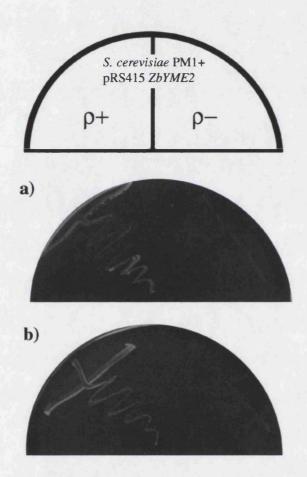


Figure 4.15. The growth deficiency of a respiratory-deficient (ρ -) derivative of *S. cerevisiae* PM1 expressing the *ZbYME2* gene on a) SB and b) SP media. The ρ + cells were able to grow on both media. Plates were photographed after 5d growth at 30°C.

of benzoate or phenylalanine as carbon sources requires a functional electron transport chain.

4.2.7 Deletion of the chromosomal copies of YME1 and YME2 in S. cerevisiae expressing ZbYME2 does not interfere with ability to metabolise benzoate.

To facilitate deletion of the native YME1/2 genes, diploid S. cerevisiae PM1 cells containing pRS415 or pZbYME2(415) were sporulated. Ten tetrads were dissected on SD medium and scored for leucine prototrophy. One-quarter of the spores did not yield viable haploids, although they germinated and formed microcolonies. The remaining spores produced viable haploids that were leucine prototrophs. Only those containing pZbYME2(415) were able to grow on SB medium.

The native YME1 and YME2 genes were deleted in haploid derivative of S. cerevisiae PM1 (MATa) containing either pRS415 or pZbYME2(415). Primers YME1-SF/SR and YME2-SF/SR (Table 4.2) were used to generate SFH gene disruption cassettes specific for YME1 (hphMX4) or YME2 (kanMX4) respectively. YME1 deletion was confirmed by colony PCR (Ling, et al., 1995), in reactions that used either primers YME1-F/H3 or YME1-R/H2 similarly YME2 deletion was confirmed using primers YME2-F/K3 or YME2-R/K2 (Table 4.2). The resultant yme1::hphMX4, yme2::kanMX4 double mutant containing pRS415 had totally lost any ability to grow on respiratory media at 30°C (Figure 4.16a). It also did not grow on YPD pH4.5 containing 4mM benzoate (Figure 4.16b) or 2mM sorbate (Figure 4.16c). However the S. cerevisiae YME1 and YME2 genes are non-essential for ZbYME2 to confer growth on SB medium (Figure 4.16d) and reverse the weak acid sensitivity of yme1, yme2 cells (Figure 4.16b, c). These results indicate that ZbYME2 can confer a number of the properties of S. cerevisiae YME2. It can also reverse weak acid sensitivity of glucose grown yme1, yme2 cells independently of the native YME1. Northen blot analysis also confirmed that the YME2 or YME1 are not induced when the cells are exposed to benzoate at low or high pH (data not shown).

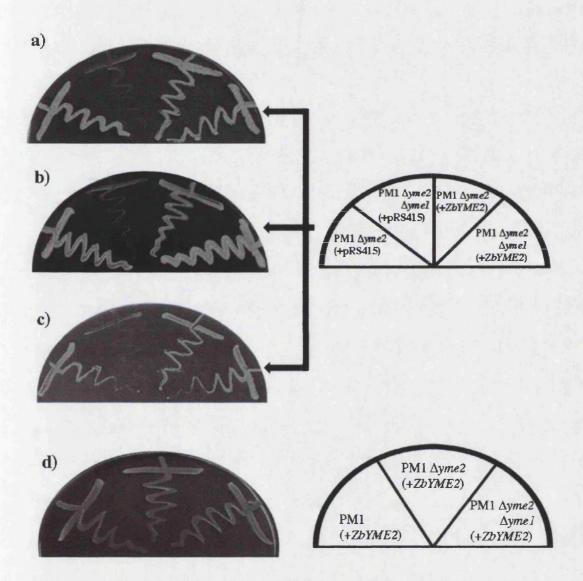


Figure 4.16. The growth phenotype of haploid PM1, and its yme2 or yme1 yme2 deletant strains with and without ZbYME2 expression. a) YPEG pH6.0 plate b) pH4.5 YPD plates containing 4mM benzoate and c) 2mM sorbate, all photographed after 3d at 30°C. d) The native YME1 and YME2 are not required for strains expressing ZbYME2 to grow on SB. (Plate photographed after 5d incubation at 30°C).

Table 4.2. PCR primers.

Oligonucleotide	Sequence and usage (restriction sites underlined)
	Construction of pGFP-N-ZYME2
YM2gfpNF(HindIII)	GGGGGGAAGCTTATGTTGCCCATTTCTGGACCTTCC
01	AAC
YM2gfpNR(XhoI)	GGGGGCTCGAGTCAATGAAGCCAAAAAATGGTCT
	CTA
	Construction of pGFP-C-ZYME2
YM2gfpCF(XbaI)	GGGGG <u>TCTAGA</u> ATGTTGCCCATTTCTGGACCTTCC
	AAC
YM2gfpCR(HindIII)	GGGGGAAGCTTATGAAGCCAAAAAATGGTCTCTA
	GCG
	Construction of ZbYME2(415)
ZBenzORF-F(XhoI)	GGGG <u>AAGCTT</u> GATCTTCGCTCAAAGATGGCAATA
ZBenzORF-R(HindIII)	GGGG <u>CTCGAG</u> TCAATGAAGCCAAAAAATGGTCTC
	Amplification of SFH-YME1 cassette and checking for
	deletion
YME1-SF	AATACATTGTGGATAGAACGAAAACAGAGACGTGA
	TAGATGTTCGTACGCTGCAGGTCGAC
YME-SR	GGTAGGTTCCTTCATACGTTTAACTTCTTAGAATAA
	AATCACCACTAGTGGATCTGATATC
YME1-F	AATGCAAAATGCTATATGCTTTTAACG
YME2-R	TGACCAGGAGAAGATGTAGATTCTGAAAG
H2	CCTCGTGCACGCGGATTTCGGC
H3	GAACAGCGGCAGTTCGGTTTC
	Amplification of SFH-YME2 cassette and checking for
	deletion
YME2-SF	CGGGCCTATACAGGAAGTAGTATTTGTAAAAGTAA
	ACCATGTTCGTACGCTGCAGGTCGAC
YME2-SR	CATATGTACAGAAAAGCAGAATAGTAAGTTTTGTTT
	CATCACCACTAGTGGATCTGATATC
YME2-F	TGCTCCTCTGAATCGTCTCCCTCGATAT
YME2-R	CITACCITCCITAGATATTTTTCATGCTG
K2	ATTTTAATCAAATGTTAGCG
<u>K3</u>	TGTACGGCGACAGTCACATC

4.3 Discussion

Fungi assimilate benzoate and phenylalanine via 4-hydroxybenzoate, through the β-adipate pathway (Fujii, et al., 1997; McNamee and Durham, 1985; van den Brink, et al., 2000). While prototrophic S. cerevisiae strains can use tyrosine and 4hydroxybenzoate as sole carbon source, they are not able to use phenylalanine or benzoate (Figure 4.2a, b; Figure 4.3a, b). It appears therefore that S. cerevisiae lacks the benzoate-4-hydroxylase needed for assimilation of the latter two compounds. Thus, by selecting for S. cerevisiae growth on benzoate plates can constitute a simple strategy for the complementation cloning of benzoate-4-hydroxylase genes. The cloning of a homologue of the S. cerevisiae mitochondrial protein Yme2p/Rna12p by this strategy was most unexpected, as all the previously-studied fungal benzoate-4-hydroxylases are microsomal P450s (Fujii, et al., 1997; McNamee and Durham, 1985; van den Brink, et al., 2000). Of these, the benzoate-inducible benzoate-4-hydroxylase of Aspergillus niger has been particularly well studied (van den Brink, et al., 2000). It appears therefore that ZbYme2p may confer a mitochondrial monooxygenase function with benzoate-4hydroxylase activity, both in Z. bailii and, when expressed heterologously, in S. cerevisiae. This function may have been lost during the course of evolution in the case of the corresponding S. cerevisiae protein Yme2p. Clearly ZbYme2p is important for the catabolism of benzoate by Z. bailii (Figures 4.6a, b, c, d) and, at least when expressed as a functional GFP fusion in S. cerevisiae, is mitochondrial in location. Further studies must be done to investigate the full catabolic potential conferred by ZbYme2p. Since it allows S. cerevisiae to catabolise sorbate (not shown), it may therefore confer a broadfunction mono-oxygenase.

Prior to this work there had been indications that mitochondrial activities could influence weak acid resistance, at least in *S. cerevisiae*. Thus the presence of weak acid preservatives dramatically enhances the endogenous production of superoxide free radicals by the mitochondrial respiratory chain. This is shown by the *increases* in weak acid resistance with loss of respiratory chain function in *S. cerevisiae* (Cheng and Piper, 1994) and the corresponding *decreases* in resistance with loss of superoxide dismutase activities (Piper, 1999). Especially marked is the increased sensitivity to weak acids with the loss of the mitochondrial Sod2 (Piper, 1999). Previously, the loss of the native

mitochondrial Yme2p in S. cerevisiae had been shown to cause; (i) a high rate of DNA escape from the mitochondria to the nucleus, (ii) an inability to grow on nonfermentable carbon sources at 30°C in cells of yme1 genetic background, and (iii) an ability to suppress the cold-sensitive growth phenotype of yme1 strains at 14°C (Hanekamp and Thorsness, 1996; Thorsness and Fox, 1993). This study revealed that yme1-1,yme2-1 S. cerevisiae cells are also unusually sensitive to weak acid stress, a sensitivity reversed by expression of the heterologous ZbYME2 gene in these cells (Figure 4.12a, b). Reversal of respiratory growth and weak acid sensitivity in yme1, yme2 cells by ZbYME2 expression is independent of the S. cerevisiae native YME1 and YME2. Why then it needs deletions of both these genes in S. cerevisiae to create above phenotypes?

YME2 can be added to the growing list of genes identified as contributing to weak acid resistance in S. cerevisiae (Holyoak, et al., 1999; Holyoak, et al., 2000; Piper, et al., 2000; Piper, 1999; Piper, et al., 1998). Its Z. bailii homologue, ZbYME2, is shown here to contribute to weak acid resistance in Z. bailii and to decrease the rate of mtDNA escape in yme1-1,yme2-2 S. cerevisiae cells (Figure 4.13).

Chapter 5. Multicopy suppressors of the weak organic acid sensitive $\Delta pdr12$ mutant of S. cerevisiae

5.1 Introduction

The importance of the induction of a weak acid efflux pump for weak acid resistance by yeasts was a contentious issue for several years (Warth, 1977; Cole and Keenan, 1987). This has now been resolved by identification of an ATP binding cassette (ABC) transporter Pdr12, essential for the adaptation of *S. cerevisiae* to weak acid stress. *S. cerevisiae* $\Delta pdr12$ cells are hypersensitive at low pH to the food preservatives sorbate, benzoate and propionate, as well as high acetate levels (Piper, *et al.*, 1998).

Earlier work by (Warth, 1989) had established that adaptation of Z. bailii to grow in the presence of 1mM benzoate caused cells to maintain an intracellular versus extracellular distribution of benzoate that is not in equilibrium. A plasma membrane protein of approximately the same size as the Pdr12 transporter is induced in Z. bailii during adaptation to growth at pH 4.5 in the presence of sorbate, but its induction is much weaker than for Pdr12 of S. cerevisiae (Figure 5.1). However, it can be hypothesised that a Pdr12 like activity is a significant contributor to Z. bailii weak acid resistance.

The aim of this investigation was to isolate S. cerevisiae and Z. bailii genes that could suppress the weak acid sensitivity of the $\Delta pdr12$ mutant. Libraries of genomic DNA fragments from Z. bailii and S. cerevisiae in pRS415 (Section 2.7.2) were transformed into the $\Delta pdr12$ S. cerevisiae mutant and transformants displaying reversion of the weak acid sensitive phenotype selected. This led to the isolation of a S. cerevisiae gene (YPL246c) and two Z. bailii DNA fragments (a YOR114w homologue; ZbYOR114 and a part of the gene for apocytochrome b) that all acted as multicopy suppressors of $\Delta pdr12$ weak acid sensitivity.

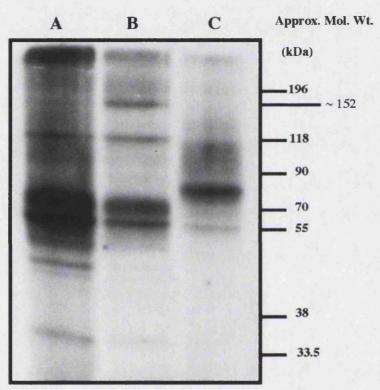


Figure 5.1. Plasma membrane proteins of Z. bailii cells [S] pulse lablled in vivo for 2 h. A) Control cells grown YPD pH4.5 minus sorbate, B) Cells grown on YPD pH4.5 plus 1mM sorbate overnight at 30°C, C) Cells grown on YPD pH 4.5 plus 9mM sorbate for 2 h at 30°C. (This gel was prepared by Dr. C. Ortiz-Calderon).

5.2 Results

5.2.1 Isolation of S. cerevisiae genes that act as suppressors of the weak organic acid sensitivity of $\Delta pdr12$ S. cerevisiae

A library of genomic DNA fragments from S. cerevisiae strain FY 1679-28C was constructed in pRS415 as described in section 2.7.2. This library was transformed into S. cerevisae YYM19 and approximately 25,000 transformants obtained by selection for leucine prototrophy. These transformants were replica plated onto SD pH 4.5 plates containing histidine, uracil, tryptophan, and 1.5-2.0mM sorbate, a level of weak acid stress that normally inhibits the growth of $\Delta pdr12$ cells (Piper, et al., 1998). Plasmid DNA was isolated from one sorbate resistant colony, and its ability to re-confer resistance confirmed by re-transformation of S. cerevisiae strain YYM19. The S. cerevisiae DNA fragment insert of this plasmid was sequenced with BigDyeTM terminator cycle sequencing ready reaction kit (PE Applied Biosystems) using universal primers T3/T7, which anneal to either side of the pRS415 multiple cloning site (Sikorski and Hieter, 1989). Using the S. cerevisiae genome database (http://genomewww.stanford.edu/) the DNA insert (Figure 5.2) was identified as a fragment of chromosome XVI, containing two ORFs of unknown function; YPL247c and YPL246c. It was suspected that only one of these ORFs is a true suppressor of the $\Delta pdr12$ weak acid sensitivity.

Screening of libraries of genomic DNA from Z. bailii inserted in pRS415 also led to isolation of a homologue of S. cerevisiae YOR114w (ZbYOR114) acted as a suppressor of the $\Delta pdr12$ phenotype (see 5.2.6). Therefore YOR114 of S. cerevisiae was also tested for its ability to act as a multicopy suppressor (see 5.2.3 and 5.2.7).

5.2.2 The effects of deletion of YOR114w, YPL247c and YPL246c on the weak acid sensitivity of S. cerevisiae

In order to probe whether YOR114w, YPL247c or YPL246c contribute to weak acid resistance in S. cerevisiae, $\Delta yor114w$, $\Delta ypl247c$ and $\Delta ypl246c$ deletant strains in CEN.PK2 genetic background (Table 2.2) were obtained from the Euroscarf collection.

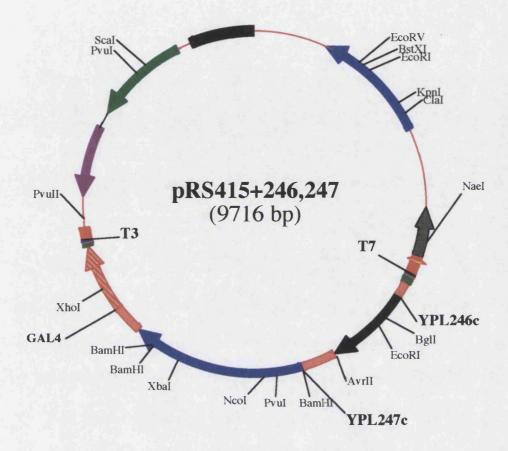


Figure 5.2. Schematic diagram of a pRS415 derived plasmid contained a S. cerevisiae DNA insert that acted as a multicopy suppressor of S. cerevisiae $\Delta pdr12$ weak acid sensitivity.

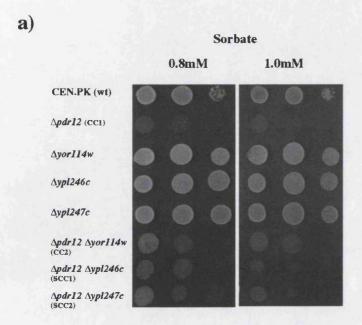
The PDR12 gene was also deleted in the $\Delta yor114w$ strain and its CEN.PK2 parent, so as to generate strains CC1 and CC2 (Table 2.2). Deletion of PDR12 coding sequences was performed using PCR-generated SFH kanMX4 cassettes with primers PDR12-SFH-F/R (Güldener, et al., 1996) (Table 5.1). Deletions were confirmed by colony PCR (Ling, et al., 1995), using primers P1 (Table 5.1) together with either P3 or K3; and P4 together with either P2 or K2 (Table 5.1). Strains SCC1 and SCC2 (Table 2.2) were meiotic products of the diploids formed by mating the $\Delta pdr12$ and $\Delta ypl246c$ strains, respectively.

Figure 5.3 shows the resultant 8 strains growing on pH4.5 YPD containing 0.8mM and 1.0mM sorbate (a), or 2.0mM and 2.5mM benzoate (b). The $\Delta yor114w$, $\Delta ypl247c$ and $\Delta ypl246c$ strains could grow in the presence of up to 5mM sorbic acid (data not shown). Also, deletion of the PDR12 gene in these strains produced mutants that had sensitivity to sorbate or benzoate that was indistinguishable from that of $\Delta pdr12$ cells (Figure 5.3a and b). It appears that the loss of YOR114w, YPL246c and YPL247c is not having any appreciable influences on the weak acid resistance of wild type and $\Delta pdr12$ cells.

A $\Delta yor114w$ $\Delta ypl246c$ strain (SCC3; Table 2.2) was formed by crossing $\Delta yor114w$ and $\Delta ypl246c$; also a $\Delta yor114w$ $\Delta ypl247c$ strain (SCC4; Table 2.2) by crossing $\Delta yor114w$ and $\Delta ypl247c$. Figure 5.4 shows these strains plus $\Delta yor114w$ and $\Delta yor114w$ $\Delta pdr12$ cells growing on pH4.5 YPD containing 1.0mM sorbate and 2.0mM benzoate. Growth of the double deletants was not affected by the presence of sorbate or benzoate at low pH. Figure 5.4 therefore demonstrates that double deletion of S. cerevisiae genes that act as $\Delta pdr12$ multicopy suppressors does not produce a weak acid sensitive phenotypes.

5.2.3 The effects of over-expression of YOR114w, YPL247c and YPL246c on the weak acid sensitivity of S. cerevisiae

In order to confirm that YOR114w expression partially reverse the weak acid sensitivity associated with loss of pdr12, the S. cerevisiae YOR114w coding region was



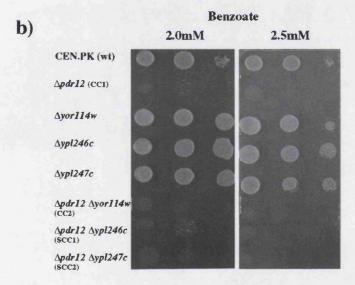
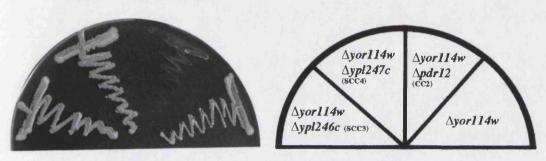


Figure 5.3. Growth of S. cerevisiae CEN.PK2 (wild type) and isogenic $\Delta pdr12$, $\Delta yor114w$; $\Delta ypl246c$; $\Delta ypl247c$ and $\Delta pdr12$ $\Delta yor114w$; $\Delta pdr12$ $\Delta ypl246c$ and $\Delta pdr12$ $\Delta ypl247$ mutants under conditions of weak acid stress. Cell suspensions of OD600 = 0.025, as well as 1:10 and 1:100 serial dilutions were spotted onto pH4.5 YPD plates with the indicated concentrations of a) sorbate or b) benzoate. The plates were photographed after 3 days at 30°C.

a) 1.0mM sorbate YPD pH4.5



b) 2.0mM benzoate YPD pH4.5



Figure 5.4. Growth of S. cerevisiae $\Delta yor114w$, with and without $\Delta ypl246c$, $\Delta ypl247c$ and $\Delta pdr12$ deletions, was monitored on sorbate and benzoate plates. Cells were streaked onto pH4.5 YPD plates with a) 1.0 mM sorbate and b) 2.0mM benzoate. The plates were photographed after 3 days incubation at 30°C.

cloned into the GFP fusion expression vectors pGFP-CFUS and pGFP-NFUS (Niedenthal, et al., 1996). This has two advantages; first it results in Yorl14p over-expression controlled by the MET25 promoter in S. cerevisiae and second, it allows GFP to be linked to either the C (pGFP-CFUS) or the N terminus of S. cerevisiae YOR114w coding region.

YOR114w was amplified from S. cerevisiae genomic DNA using primers SyorgfNF/SyorgfNR and SyorgfCF/SyorgfCR (Table 5.1) and the PCR products digested with HindIII/XhoI and XbaI/HindIII respectively. These fragments were then ligated into HindIII/XhoI and XbaI/HindIII digested pGFP-CFUS and pGFP-NFUS respectively (Niedenthal, et al., 1996) (Figure 5.5a, b). The resulting plasmids (pGFP-C-YOR114, pGFP-N-YOR114) and controls (pGFP-CFUS and pGFP-NFUS) were then transformed into S. cerevisiae strain CC1 (Δpdr12). Figure 5.6 shows growth of the resulting transformants on pH4.5 SD medium in the presence of 2.0mM sorbate, with and without methionine. Absence of methionine should cause over-expression of the GFP-tagged YOR114w, but only the pGFP-C-YOR114 construct led to suppression of the sorbate-sensitivity of S. cerevisiae Δpdr12 cells. This suggested that an N-terminal GFP addition to Yor114p may abolish function of the latter protein. To determine the subcellular localisation of the GFP-tagged Yor114p, the Δpdr12 cells transformed with pGFP-C-YOR114 were examined by microscopy (Niedenthal, et al., 1996). However only very weak GFP staining was detected (Figure 5.7).

To obtain galactose inducible Yor114p expression, the pYES2YOR114 construct was made (Figure 5.8). The YOR114w coding region was PCR amplified with primers TAGP1/TAGP2(GLU) (Table 5.1); the PCR product being digested with BamHI/EcoRI and ligated to BamHI/EcoRI digested pYES2 (Figure 5.8). This construct (pYES2YOR114) and the control vector (pYES2) were then transformed into S. cerevisiae CEN-PK2 and CC1 ($\Delta pdr12$). Figure 5.9 shows growth of the resulting transformants on pH4.5 glucose or galactose media in the presence of 1.0mM sorbate. The sorbate sensitivity of $\Delta pdr12$ cells (strain CC1) was substantially suppressed with over-expression of YOR114w on galactose media. This confirms that YOR114w over-expression can suppress the weak acid sensitivity of the $\Delta pdr12$ S. cerevisiae mutant.

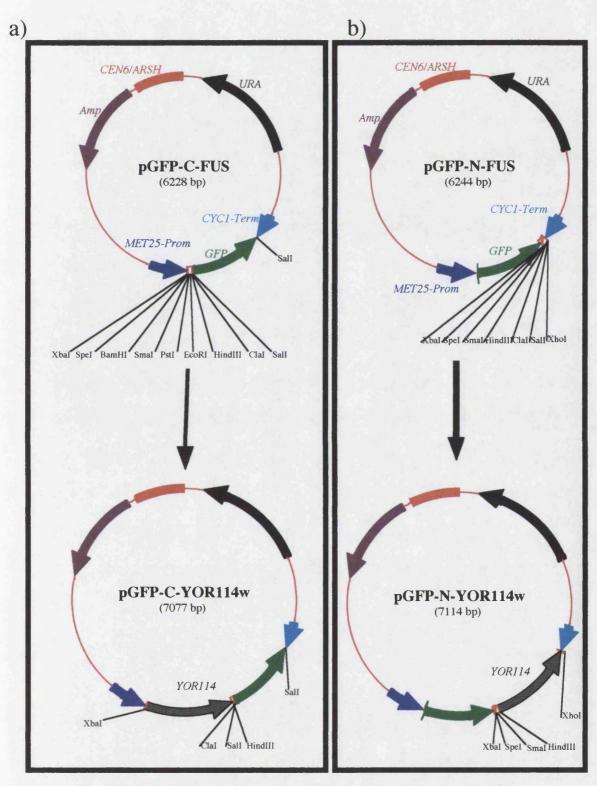


Figure 5.5. Diagrammatic representation of the construction of vectors for expressing fusion of *GFP* to the a) C-terminus of *S. cerevisiae YOR114w* (pGFP-C-YOR114) or b) to the N-terminus of *YOR114w* (pGFP-N-YOR114).

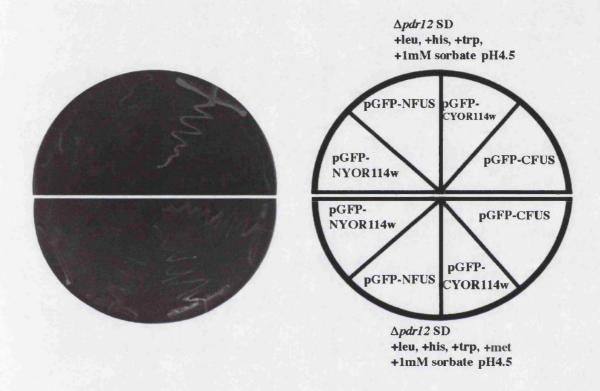


Figure 5.6. Overexpression of GFP gene fusions to the C- and the N-termini of the Yor114p in S. cerevisiae $\Delta pdr12$ strain. Only cells tranformed with the pGFP-C-YOR114w contruct were able to grow on SD media at pH4.5 plus 2.0mM sorbate, when the GFP fusion were induced with lack of methionine.

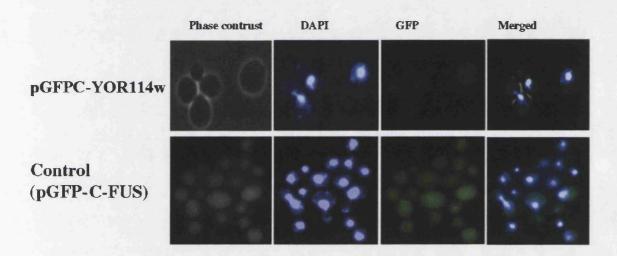


Figure 5.7. Subcellular localization of GFP-tagged Yorl14p. Δpdr12 cells containing the pGFP-C-YOR114w vector and the control (pGFP-C-FUS) constructs were grown in pH4.5 SD with appropriate amino acids minus methionine plus 2.0mM sorbate. Cell were visualized microscopically by phase contrast and nuclear DNA stain (DAPI). The Yorl14p-GFP was not visualised.

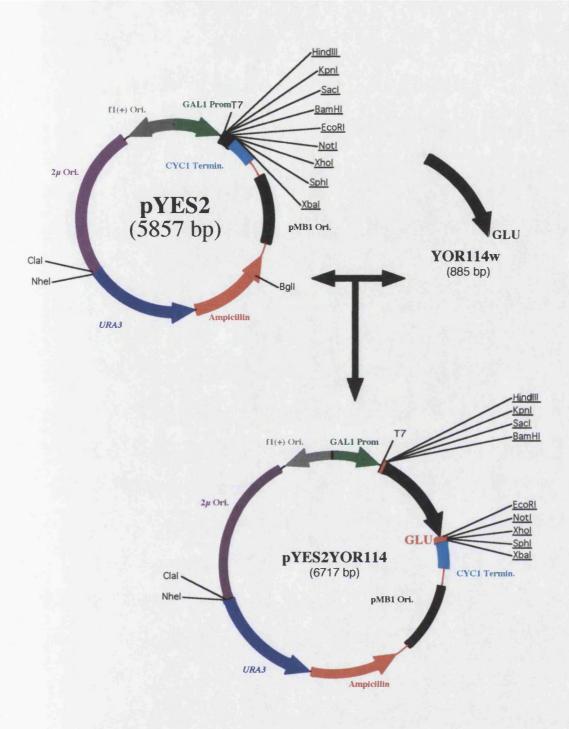


Figure 5.8. Schematic diagram of the construction of pYES2YOR114.

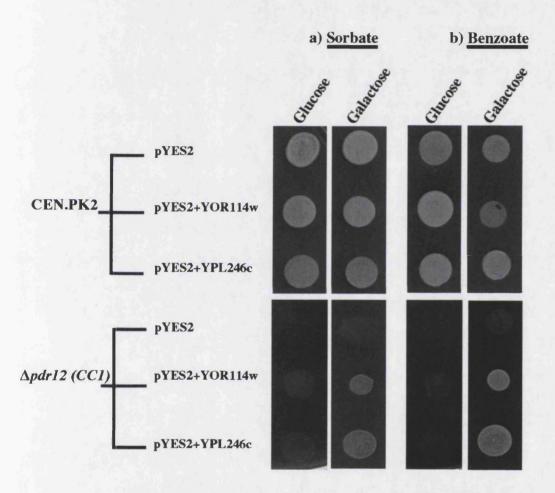


Figure 5.9. Over-expression of YOR114w and YPL246c in S. cerevisiae $\Delta pdr12$. The pYES2 vector was used as a control. Transformants were grown on glucose or galactose pH4.5 minimal media plus a) 2mM sorbate or b) 4mM benzoate for 3d at 30°C.

The isogenic wild type strain CEN.PK2 did not show any difference in its growth on sorbate-containing plates when YOR114w was over-expressed (Figure 5.9).

Another S. cerevisiae DNA fragment that suppressed weak acid sensitivity of $\Delta pdr12$ cells contained both the YPL247c and YPL246c ORFs (Figure 5.2). Since only one of these genes might act as a suppressor of $\Delta pdr12$ weak acid sensitivity, the YPL247c and YPL246c coding regions were individually cloned into vector pYES2 (Figure 5.10), so as to allow galactose-inducible Ypl247p or Ypl246p expression in S. cerevisiae. The YPL246c and YPL247c coding regions were amplified using primers 247F/247RGLU and 246F/246RGLU respectively (Table 5.1). These PCR products were digested with HindIII/XhoI and then ligated to HindIII/XhoI digested pYES2. The resulting plasmids (pYES2YPL247 and pYES2YPL246; Figure 5.10) were then transformed into S. cerevisiae CC1 ($\Delta pdr12$) (Table 2.1). The sorbate sensitivity of the CC1 ($\Delta pdr12$) strain was substantially suppressed with over-expression of YPL246c (Figure 5.9) but not YPL247c (data not shown). This shows that it is YPL246c that acts as a multicopy suppressor of the weak acid sensitivity of $\Delta pdr12$ S. cerevisiae. The wild type strain (CEN.PK2) did not show any difference in its growth on sorbate-containing plates when either YPL246c or YPL247c was over-expressed on galactose (not shown)

5.2.4 In vivo and subcellular localisation of Yor114p in S. cerevisiae

YOR114w potentially encodes a 35kDa basic, hydrophilic protein (Kyte and Doolittle, 1982) (Figure 5.11). During construction of the vector pYES2YOR114, the GLU-GLU epitope tag (EFMPME) was added to the C-terminus of the YOR114w coding region, so as to allow detection of Yor114p with an anti-Glu-tag monoclonal antibody (BabCo., Covance Research Products, U.S.A). It was found that the Glu-tagged Yor114p was present in the crude membrane fraction, mainly co-purifying with plasma membranes (Figure 5.12). The fact that the protein is largely hydrophilic suggests that it is not an integral membrane protein. However, it may be membrane-associated. According to PathCalling® at (http://portal.curagen.com) no protein has been found to interact with Yor114p. Also amino acid sequence analysis using PSI-BLAST (Position Specific Iterated BLAST) at (http://www.ncbi.nlm.nih.gov/), or PRODOM at

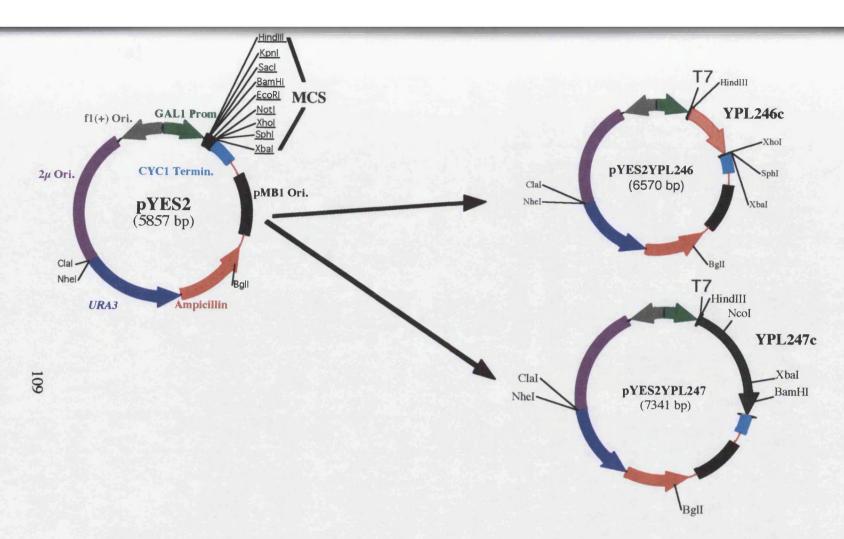


Figure 5.10. Schematic representation of the construction of vectors pYES2YPL246 and pYES2YPL247.

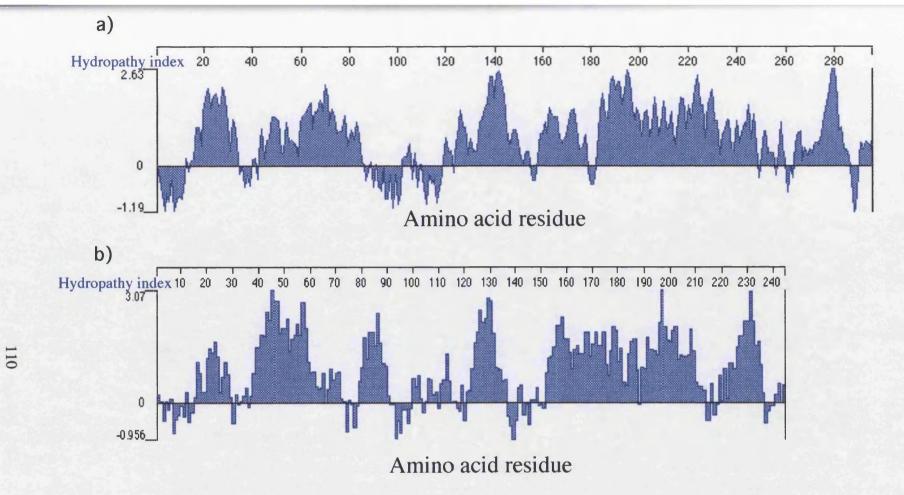


Figure 5.11. Hydropathy plot of the a) S. cerevisiae and b) Z. bailii Yor 114 proteins. The plot was obtained after the algorithm of Kyte and Doolittle (Kyte and Doolittle, 1982).

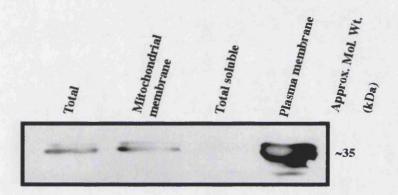


Figure 5.12. Western blot analysis of Glu-tagged Yor114p in extracts from $\Delta pdr12$ cells. Proteins of total cell extracts, mitochondrial, total soluble, and plasma membrane fractions (approximately $10\mu g$ protein per lane) were immunoblotted and probed using an anti-Glu-tag monoclonal antibody.

(<u>http://protein.toulouse.inra.fr/prodom.html</u>) did not show sequence homology to any known protein and did not identify a putative trans-membrane (TM) domain.

5.2.5 In vivo and subcellular localisation of YPL246c in S. cerevisiae

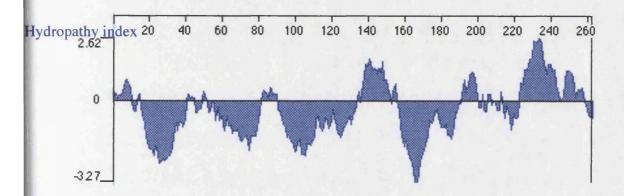
S. cerevisiae YPL246c potentially encodes a 29.5 kDa basic protein with a hydrophobic hydropathy profile (Kyte and Doolittle, 1982), (Figure 5.13). PSI-Blast (http://www.ncbi.nlm.nih.gov/) indicates that Ypl246p has a weak amino acid sequence identity (24%) with the TFC3/YAL001C gene in S. cerevisiae and also to the mouse proteinase activated receptor 2. Two-hybrid results from PathCalling® at (http://portal.curagen.com) indicate interaction of Ypl246p with (a) Vam7p (a regulator of vacuolar morphogenesis), (b) Ypt1p (involved in the secretion pathway at the ER-to-Golgi step), (c) Yhr105p (a protein of unknown function).

During the construction of pYES2YPL246 (Figure 5.10) a GLU-GLU epitope tag (EFMPME) was added to the C-terminus of the YPL246c coding region, so as to allow detection of the protein with the anti-Glu-tag monoclonal antibody. Western blot analysis indicated a plasma membrane localisation (Figure 5.14).

5.2.6 Isolation of Z. bailii DNA fragments that act as suppressors of the weak organic acid sensitivity of $\Delta pdr12$ S. cerevisiae

Libraries of genomic DNA fragments from Z. bailii strains 1427 and 563 inserted in pRS415 (constructed as in section 2.7.2) were transformed into S. cerevisae YYM19 by selection for leucine prototrophy as in section 3.2.1. Approximately 25,000 transformants were replica plated onto SD pH 4.5 plates containing histidine, uracil, tryptophan, and 1.5-2.0mM sorbate. Two colonies from the Z. bailii 563 library grew on these plates. Plasmids DNA were isolated from these two colonies and their ability to reconfer resistance confirmed by re-transformation into S. cerevisiae strain YYM19.

The Z. bailii DNA inserts in these two plasmids were sequenced using primers in Table 5.1. These sequences were analysed using ORF-finder and PSI-Blast at (http://www.ncbi.nlm.nih.gov/) in order to find sequence homology to any known genes. The single ORF (ZbYOR114-Part) within one of these fragments showed strong homology to the N-terminal part of S. cerevisiae YOR114w (Figure 5.11a), an ORF on



Amino acid residue

Figure 5.13. Hydropathy plot of the *S. cerevisiae* Ypl246p. The plot was obtained after the algorithm of Kyte and Doolittle (Kyte and Doolittle, 1982).

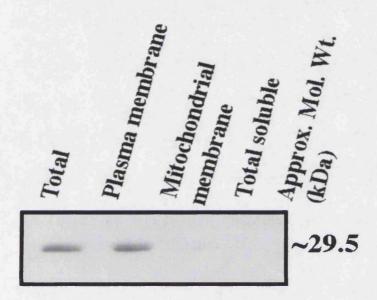


Figure 5.14. Western blot analysis of the Glu-tagged Ypl246p in extracts from $\Delta pdr12$ cells. Proteins of total cell extracts, mitochondrial, total soluble, and plasma membrane fractions (approximately $10\mu g$ protein per lane) were immunoblotted and probed using an anti-Glu-tag monoclonal antibody.

Chromosome XV of S. cerevisiae, whose function has still to be identified (see 5.2.2). This insert also contained 167bp upstream the start codon (Figure 5.15a), but the absence of a stop codon indicated that the translation of this ORF in the S. cerevisiae Δpdr12 transformant probably terminates within adjacent pRS415 vector sequences. On the basis of the complete S. cerevisiae YOR114w sequence and this ZbYOR114-(Part) sequence, primers ZYF1 and ZYR1 (Table 5.1) were designed and used to amplify a larger fragment from Z. bailii 563 genomic DNA. Sequencing of this fragment provided the sequence of the remainder of this Z. bailii ORF, ZbYOR114 (Figure 5.16a). ZbYOR114 has a 50% amino acid sequence similarity to YOR114w of S. cerevisiae (Figure 5.16b).

The other Z bailii DNA fragment that acted as a multicopy suppressor of $\Delta pdr12$ also contained a small ORF, Zbcytb167 (Figure 5.15b and 5.17a). This had 87% similarity to an internal portion of the coding region of the S. cerevisiae mtDNA-encoded gene for apocytochrome b (COXB). Nevertheless, it was considerably smaller than the latter (Figure 5.17b), therefore it may be a fragment of mtDNA that "escaped" to the nucleus (see 5.2.8).

5.2.7 Over-expression and sub-cellular localisation of ZbYOR114 in the weak acid sensitive S. cerevisiae $\Delta pdr12$

ZbYOR114 encodes a 30 kDa basic protein which, although smaller than its S. cerevisiae homologue Yor114p, has a very similar hydropathy profile (Kyte and Doolittle, 1982), (Figure 5.11b). In order to see whether the function of ZbYor114p is conserved in S. cerevisiae, two different versions of this protein were over-expressed in Δpdr12 S. cerevisiae.

The ZbYOR114 ORF was amplified by PCR from Z. bailii 563 genomic DNA using primers ZYORGLUF/R (Table 5.1). The product was digested with HindIII/XhoI and then ligated into HindIII/XhoI digested pYES2, to produce a vector (pYES2ZbYOR114, Figure 5.18) for galactose-inducible expression of ZbYor114p with a C-terminus GLU-GLU epitope (EFMPME) in S. cerevisiae. This pYES2ZbYOR114 construct and control pYES2 were then transformed into S. cerevisiae Δpdr12. Over-





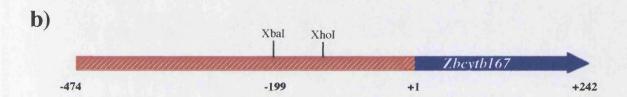


Figure 5.15. Schematic diagrams of the DNA fragments isolated from Z. bailii that acted as multicopy suppressors of S. cerevisiae $\Delta pdr12$ weak acid sensitivity. a) ZbYOR114-(Part), and b) Zbcytb167.

Figure 5.16a) Nucleotide sequence of ZbYOR114 and 166bp upstream DNA from Z. bailiistrain 563. Arrows represent primers used to sequence this region. b) Amino acid sequence alignment of Yor114p from S. cerevisiae and Z. bailii

Figure 5.17a) Nucleotide sequence of the fragment of apocytochrome b from b. So b and b are shown. This fragment is 716 bp long with an ORF of 80 amino acid. b) Amino acid sequence alignment with b acid. b amino acid sequence alignment with b acid.

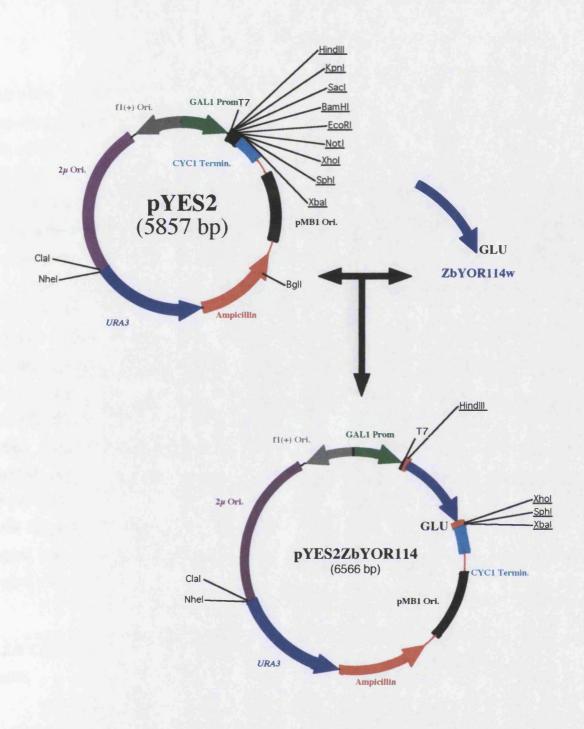


Figure 5.18. Schematic representation of pYES2ZbYOR114 construction.

expression of ZbYOR114 on galactose suppressed the weak acid sensitivity of S. cerevisiae $\Delta pdr12$ (Figure 5.19). The transformants were also used to investigate the sub-cellular localisation of ZbYor114p expressed in S. cerevisiae. It was found that the Glu-tagged ZbYor114p was present in the crude membrane fraction, and mainly copurified with plasma membranes (Figure 5.20). This result is similar to that previously obtained for the native S. cerevisiae Yor114p (Figure 5.12) and indicates that these two proteins may both act at the plasma membrane.

Two GFP-tagged versions of the ZbYor114p were over-expressed under the control of the MET25 promoter. The ZbYOR114 ORF was amplified by PCR from combinations genomic DNA with primer ZYORGFNF/ZYORGFNF ZYORGFCF/ZYORGFCF (Table 5.1) and the products digested with HindIII/XhoI or Xbal/HindIII respectively. These fragments were then ligated into HindIII/XhoI or Xbal/HindIII-digested pGFP-CFUS or pGFP-NFUS respectively (Niedenthal, et al., 1996) (Figure 5.21). The resulting constructs (pGFP-N-ZbYOR114, pGFP-C-ZbYOR114) and controls minus inserts (pGFP-CFUS and pGFP-NFUS) were then transformed into S. cerevisiae $\Delta pdr12$. The resultant transformants were not able to grow on pH4.5 SD containing 2.0mM sorbate (Figure 5.22), indicating that ZbYor114 tagged with GFP may be non-functional in S. cerevisiae, unlike the native Yor114p tagged with GFP (Section 5.2.3).

5.2.8 Cloning and sequencing of the mitochondrial gene for apocytochrome b in Z. bailii.

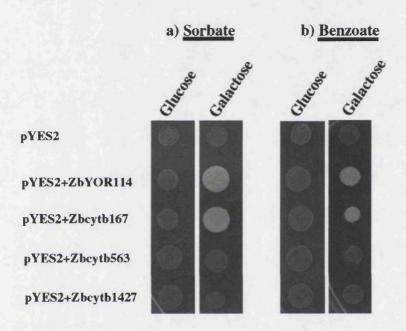


Figure 5.19. Overexpression of ZbYOR114, Zbcytb167, Zbcytb563, Zbcytb1427 in S. $cerevisiae \Delta pdr12$. Transformants were grown on glucose or galactose pH4.5 minimal media plus a) 2.0mM sorbate or b) 3.0mM benzoate for 3d at 30°C.



Figure 5.20. Western blot analysis of the Glu-tagged Zbyor114p in extracts from $\Delta pdr12$ cells. Proteins of total cell extracts, mitochondrial, total soluble, and plasma membrane fractions (approximately $10\mu g$ protein per lane) were immunoblotted and probed using an anti-Glu-tag monoclonal antibody.

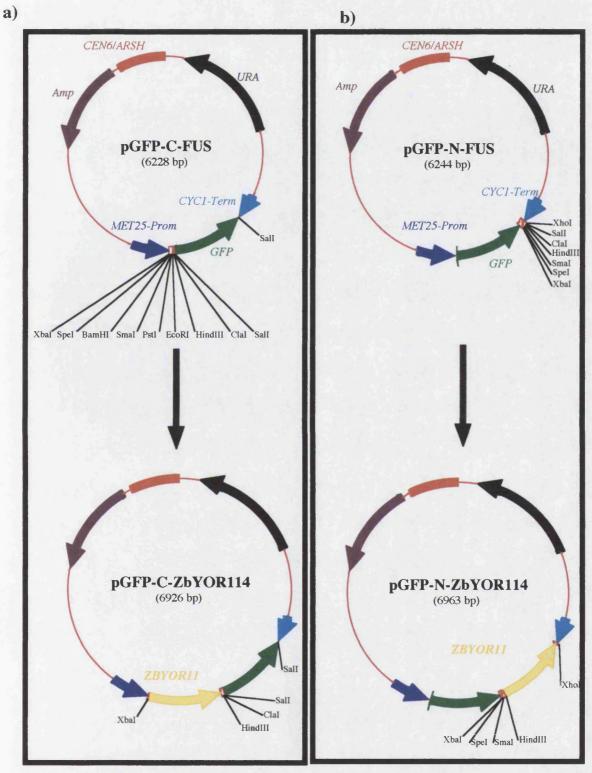


Figure 5.21. Diagrammatic representation of the construction of vectors for expressing fusion of *GFP* to the a) C terminus of *ZbYOR114* (pGFP-C-ZbYOR114) or b) to the N-terminus of *ZbYOR114* (pGFP-N-ZbYOR114).

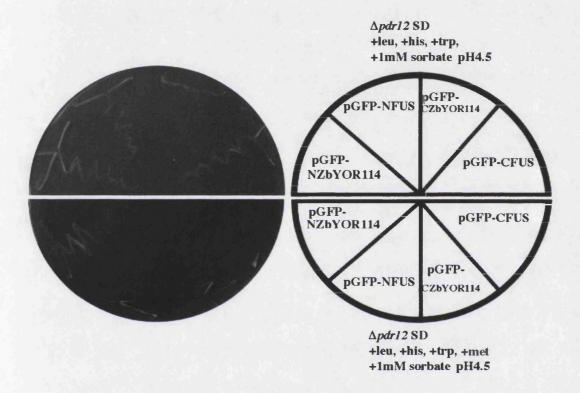


Figure 5.22. Over-expression of GFP fusions to the C- or the N-termini of ZbYor114p in S. cerevisiae $\Delta pdr12$. Transformants were grown on SD pH4.5 plus 2.0mM sorbate. They did not revert the weak acid sensitivity of the $\Delta pdr12$ strain.

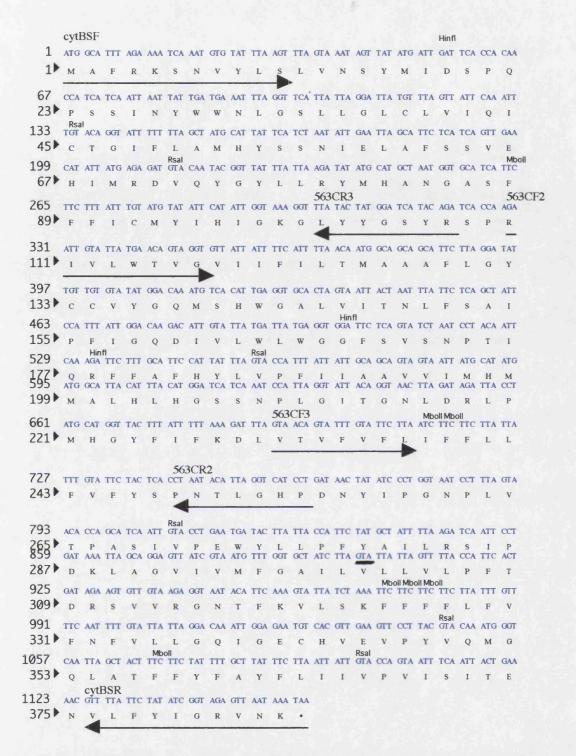


Figure 5.23. Sequences of the complete apocytochrome *b* coding region of *Z. bailii*. The amino acid sequences are given on the bottom strand and relevant restriction sites are shown. Arrows represent the primers used in the sequencing strategy.

encoding products with 88% and 85% amino acid sequence similarity respectively with the apocytochrome b of S. cerevisiae (Figure 5.24) and K. lactis. The codon usage in Zbcytb was very similar to that of other mitochondrial genes with mostly U or A in the third position. There were two unusual features. All threonines were coded by AC(A/U), and all arginines by AGA. Also all tryptophan codons were UGA and not UGG.

Zbcytb167 has 100% amino acid sequence similarity to the middle part of the Z. bailii apocytochrome b (Figure 5.24). Southern blot analysis showed that the 5' non-coding region (-199 to -474bp) of Zbcytb167 (Figure 5.15b) hybridised only to the total genomic DNA of Z. bailii and not to mtDNA (Figure 5.25a, b). Zbcytb167 is therefore a fragment of mtDNA that has "escaped" to the nucleus, where it may be expressed as a minigene. It is unlikely that fragments of mtDNA, inserted in pRS415, could act as multicopy suppressors of $\Delta pdr12$, since these would not have the recognition sequences for RNA polymerase II directed transcription in the nucleus. Zbcytb 167 full fragment (716bp) was amplified by PCR using primers 167F/R (Figure 5.25c). This result demonstrate the Zbcytb 167 fragment colinearity with cellular DNA.

5.2.9 The effect of attempted nucleus over-expression of apocytochrome b of Z. bailii on the weak acid sensitivity of S. cerevisiae

To determine whether the small Zbcytb167 ORF or the complete mitochondrial apocytochrome b gene of Z. bailii, or both of these sequences are multicopy suppressors of weak acid sensitivity in $\Delta pdr12$, these sequences were amplified by PCR using primers 167F/167RGLU and ZcytbF/ZcytbR respectively (Table 5.1). The resulting PCR products were then digested with HindIII/XbaI or HindIII/XhoI respectively and ligated to HindIII/XbaI and HindIII/XhoI digested pYES2. The resultant constructs (pYES2-167GLU, pYES2-167GLU, pYES2-

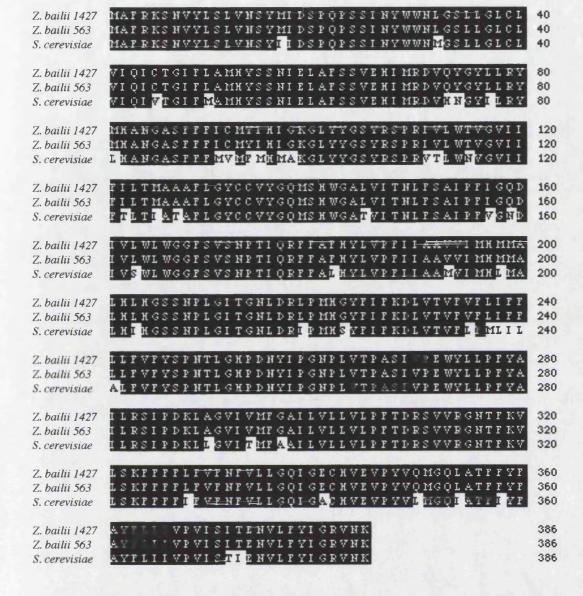


Figure 5.24. Amino acid sequence alignment between apocytochrome b of S. cerevisiae and Z. bailii The one-letter amino acid code is used.

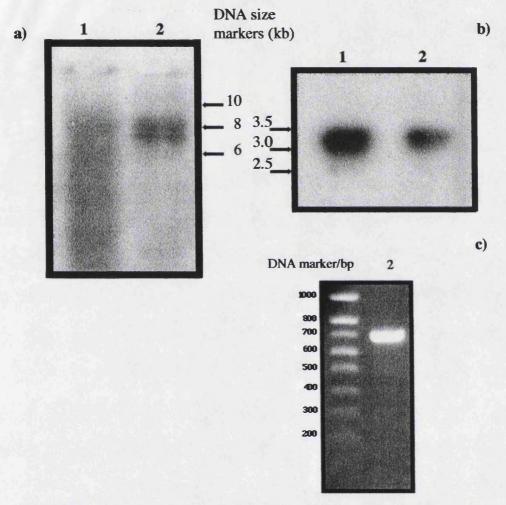


Figure 5.25. Detection of Zbcytb167 in Z. bailii total genomic DNA and its absence in the mtDNA. Using a) 5' non-coding region (-199 to -474bp) of Zbcytb and b) Zbcytb ORF, DNA blot hybridisation was performed on 1) purified mtDNA and 2) genomic and mt DNA from Z. bailii 563 digested with XbaI. c) PCR amplification of Zbcytb167 (fragment) using Z. bailii 563 total cellular DNA (nuclear plus mt) as template.

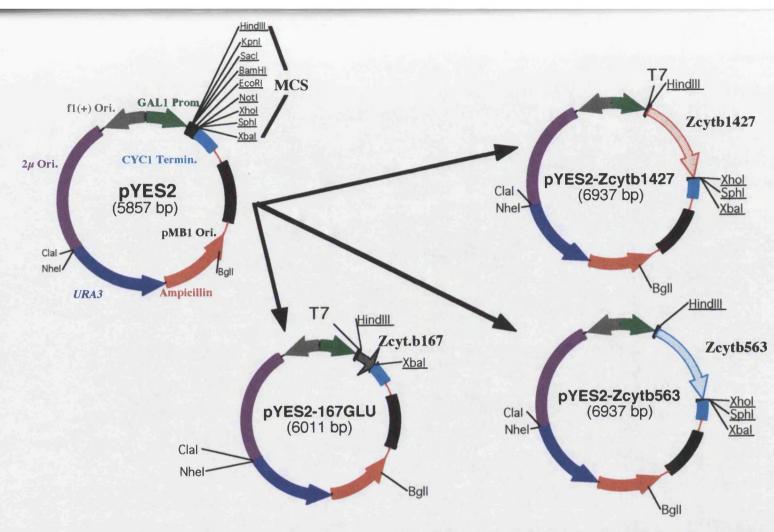


Figure 5.26. Schematic representation of construction of pYES2-167GLU, pYES2- Zcytb1427 and pYES2- Zcytb563 for galactose inducible expression of Zbcyt b167 and full-length Zbcyt b in S. cerevisiae.

b is normally mtDNA-encoded. The apocytochrome b of Z. bailii 1427 and 563 contains 7 TGA (stop) codons (Figure 5.23). This in turn has effected their over-expression in $\Delta pdr12$ S. cerevisiae.

5.2.10 Cytochrome absorption spectra of mitochondrial particles from $\Delta pdr12$ cells expressing the Zbcytb167 ORF.

The absorption spectra of the reduced cytochromes of S. $cerevisiae \Delta pdr12$ cells expressing Zbcytb167 from Zbcytb167-pRS415 and pYES2-167GLU were measured using a previously described method (Edderkaoui, et~al., 1997). Over-expression of the Zbcytb167 fragment under its native promoter did not affect the cytochrome b peak at 560 nm when compared to the control (Figure 5.27a). A similar result was also observed for over-expression of Zbcytb167 under GAL1-promoter control (Figure 5.27b). This indicates that Zbcytb167p expression does not affect the haem composition of the bc_1 complex.

5.2.11 Subcellular localisation of Zbcytb167 using GFP as a marker

Zbcytb167 encodes a 7.8 kDa basic protein with a hydropathy profile consistant with a single transmembrane domain (Figure 5.28a, b). Construction of the pYES2-167GLU vector gave rise to linkage of the Glu-Glu epitope tag (EFMPME) to the C terminus of Zbcytb167p expressed in S. cerevisiae. This protein could not be detected with an anti-Glu-tag monoclonal antibody, due to its small size. Therefore, two versions of the Zbcytb167p, with GFP fused at the N- and C-terminus, were over-expressed in S. cerevisiae YMM19 (Niedenthal, et al., 1996).

The Zbcytb167 ORF was amplified using primers 167gfpNF/167gfpNR and 167gfpCF/167gfpCR, respectively (Table 5.1). These PCR products were digested with HindIII/ XhoI or XbaI/ HindIII and cloned into HindIII/ XhoI cleaved pGFP-N-FUS or XbaI/ HindIII cleaved pGFP-C-FUS respectively (Figure 5.29). The resulting pGFP-N-Zbcytb167 and pGFP-C-Zbcytb167 constructs were transformed into $\Delta pdr12$ S. cerevisiae. Transformants were then grown on pH4.5 SD plates plus 2.0mM sorbate but

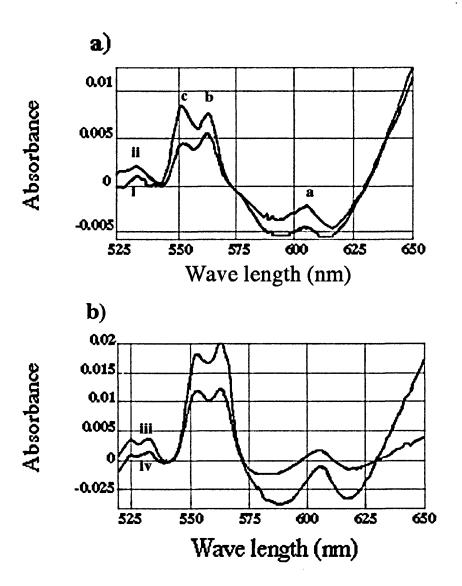


Figure 5.27. Absorption spectra of cytochromes in a) S. cerevisiae Δpdr12 transformed with i) Zbcytb167-pRS415, ii) pRS415 (control) and b) S. cerevisiae Δpdr12 transformed with iii) pYES2-167GLU and iv) pYES2 (control). Cells were suspended in 50% glycerol and a small amount of sodium sulfite was added to the suspension to convert cytochromes to their reduced forms (Edderkaoui, et al., 1997). Peaks corresponding to cytochromes a, b and c are indicated.

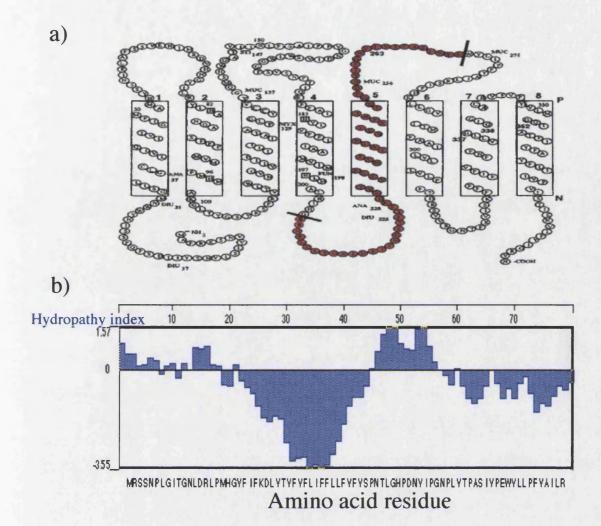


Figure 5.28. a) Cytochrome b folding showing the prediction of eight spanning α helices in the mitochondrial inner membrane. The amino acid sequence corresponding to Zbcytb167 is in red. ANA (antimycin A), DIU (diuron), STI (stigmatellin), FUN (funiculosin), MYX (myxothiazol) and MUC (mucidin) indicate the amino acids involved in resistance to these the inhibitors. N and P indicate the electronegative and electropositive sides of the membrane (Edderkaoui, et al., 1997). b) Hydropathy plot of the Zbcyt b167 polypeptide. The plot was obtained after the algorithm of Kyte and Doolittle (Kyte and Doolittle, 1982).

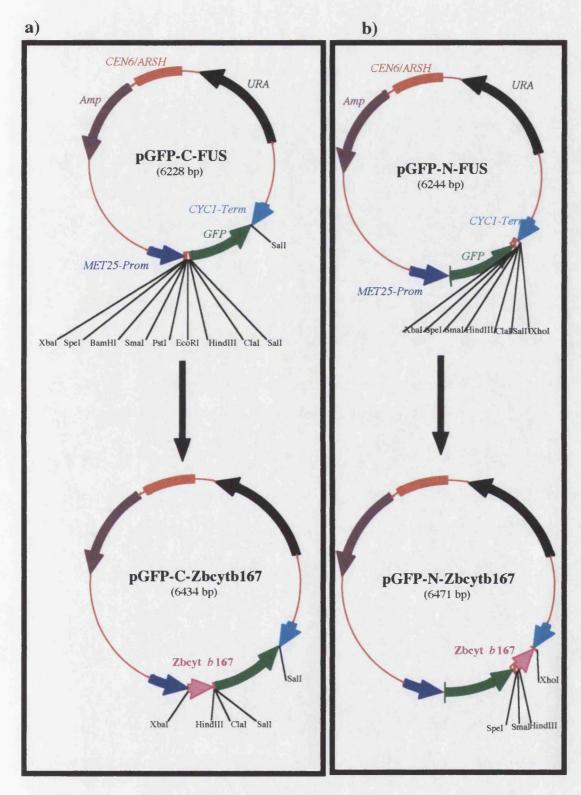


Figure 5.29. Diagrammatic representation of the construction of vectors linking GFP to the a) C-terminus of Zbctyb167 (pGFP-C-Zbcytb167) or b) the N-terminus (pGFP-N-Zbcytb167).

without methionine in order to induce high expression of Zbcytb167 from the MET25 promoter. Only cells that were transformed with the pGFP-C-Zbcytb167 were able to grow on pH4.5 SD plates plus 2.0mM sorbate plates (Figure 5.30), indicating that only Zbcytb167p tagged with GFP at the C-terminus suppressed the Δpdr12 phenotype. These cells were prepared for microscopy as described previously (Niedenthal, et al., 1996) and GFP-tagged Zbcytb167p was detected in the cytoplasm and in a component adjacent to the nucleus that may be the Golgi (Figure 5.31).

5.2.12 The ρ^- S. cerevisiae $\Delta pdr12$ mutant expressing the Zbcytb167 gene are weak acid sensitive

Respiratory-deficient (ρ) derivatives of S. cerevisiae $\Delta pdr12$ expressing the Zbcytb167 gene were obtained by overnight growth on liquid YPD medium at 37°C and then confirmed as respiration-deficient from their total incapacity to grow on respiratory carbon sources. They were unable to grow on pH4.5 SD plates with 2mM sorbate (Figure 5.32) even though they retained the capacity for growth on glucose SD plates lacking sorbate and, therefore, still maintained the plasmid. This indicates that respiratory chain function is needed for Zbcytb167p over-expression to suppress the weak acid sensitivity of $\Delta pdr12$ cells. It is unlikely though that Zbcytb167p is a part of the bc_1 complex in mitochondria. It is more likely that its expression ameliorates the effects of the severe oxidative stress experienced by weak acid stressed $\Delta pdr12$ S. cerevisiae (Piper, 1999), an oxidative stress largely generated by the mitochondrial respiratory chain. With loss of respiratory chain function, this oxidative stress is also lost and the cells became more weak acid resistant.

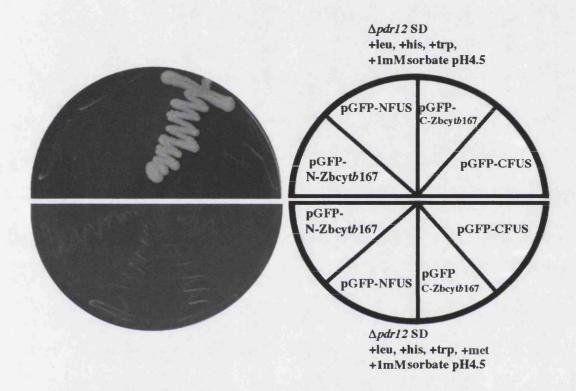


Figure 5.30. Overexpression of GFP fusions to the C- and the N-termini of the Zbcytb167p in *S. cerevisiae* Δ*pdr12*. Only cells transformed with the pGFP-C-Zbcytb167 construct were able to grow on SD pH4.5 plus 2.0mM sorbate for 3d at 30°C.

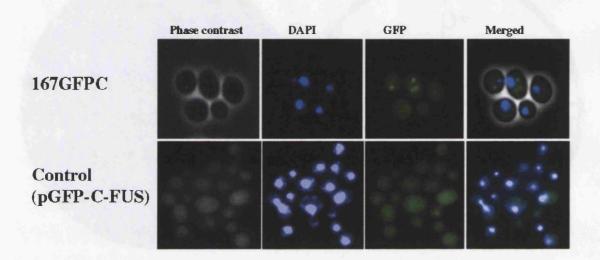


Figure 5.31. Subcellular localization of GFP-tagged Zbcytb167. Δpdr12 cells containing the167GFPC and the control (pGFP-C-FUS) constructs were grown in SD media pH4.5 with appropriate amino acids without methionine plus 2.0mM sorbate for 3dat 30°C. Cells were visualised microscopically by phase contrast and nuclear DNA stain (DAPI).

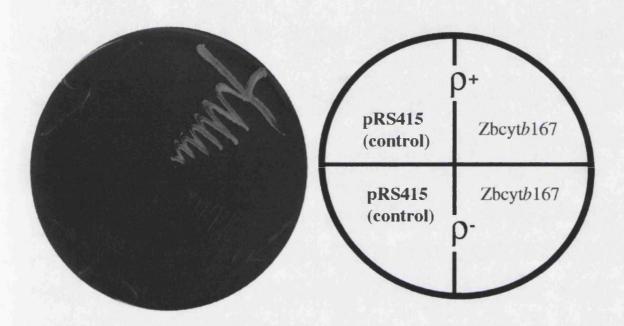


Figure 5.32. A respiratory-deficient (ρ -) derivative of *S. cerevisiae* $\Delta pdr12$ expressing Zbcytb167 was unable to grow on SD pH4.5 plus 2.0mM sorbate. The (ρ +) cells expressing Zbcytb167 retained their capacity for grow on this media. Plate was photographed after 3d at 30°C

Table 5.1. PCR primers. DNA sequence encoding Glu-Glu epitope tags are represented in bold.

Oligonucleotide	Sequence and usage (restriction sites underlined)
	Amplification of SFH-PDRKAN cassette and checking of deletion
PDR12-SFH-F	GGTTTACAGATTTATTGTTATTGTTCTTATTAATAAAAAATTCG
	TACGCTGCAGGTCGAC
PDR12-SFH-R	TGTGTTAAACCACGAAATACAAATATATTTGCTTGCTTGTCCA
	CTAGTGGATCTGATATC
P1	TGATACAAATCTCTAGCAGCCTGA
P2	CTTCAACCAAGGAAACACGCTTACGTT
P3	AGTGTGGTGTTTCTGAAAATCCAGCTGAAT
P4	TGACGACTGTAGAAGTATGCAAATCAT
K2	TGTACGGGCGACAGTCACATC
K3	ATTTTAATCAAATGTTAGCG
	Construction of pYES2YOR114
TAGP1	AAACATTCGA <mark>GGATCC</mark> ATGAAGGCTACTTTACTG
TAGP2(GLU)	TAGTGAAGTGCTTTCGGATCCTTATTCCATTGGCATGAATTC
` ,	ACCTGGACCTTTCTTTTTAGTAGGTACGTCAT
	Construction of pGFP-N-YOR114
SyorgfNF(HindIII)	GGGGGAAGCTTATGAAGGCTACTTTACTGTTGAAG
SyorgfNR(XhoI)	GGGGGCTCGAGTCATTTCTTTTTAGTAGGTACGT
	Construction of pGFP-C-YOR114
SyorgfCF(XbaI)	GGGGGTCTAGAATGAAGGCTACTTTACTGTTGAAG
SyorgfCR(HindIII)	GGGGG <u>AAGCTT</u> TTTCTTTTTTAGTAGGTACGT
	Construction of pYES2YPL247
247F (HindIII)	GGGGGAAGCTTATGGATCCCTTTCACAATGGCAATAA
247RGLU (XhoI)	GGGGGCTCGAGCTATTCCATTGGCATGAATTCAACTGGAC
247RGES (MIST)	CGGCAGTCCCTAGGACAGTGCC
	Construction of pYES2YPL246
246F (HindIII)	GGGGGAAGCTTATGAATTGGAAGTCGTACGTTTTT CC
246RGLU (XhoI)	GGGGGCTCGAGCTATTCCATTGGCATGAATTCAACTGGAC
	CAAGGACGCGGACGTTTTGAAAC
	Z. bailii NCYC 563 ZbYOR114 sequencing.
T3	AATTAACCCTCACTAAAGGG
T7	GTAATACGACTCACTATAGGGC
ZYF1	GATCACTATTTCATAATAAATAATACTAAT
ZYF2	GACAAGTTATTAAGCATGCC
ZYR1	TCATTTCTTTTTAGTAGTACTCTA
ZYR2	AACTCCTCAGTGTCATGGTATTCT
ZYR3	GGAATGCAGTACTAGGTAAG
2110	Construction of pYES2ZbYOR114
ZYORGLUF(HindIII)	GGGGGGAAGCTTATGCGTTGCACATATGCCATCAGG
	GGGGGGCTCGAGTCATTCCATTGCCATCAGG GGGGGCTCGAGTCATTCCATTGGCATGAATTCACCTGGACCT
ZYORGLUR(XhoI)	
	TTCTTTTAGTAGTACTCTA
ZVODGENIE/I##III)	Construction of pGFP-N-ZbYOR114
ZYORGEND(Yhol)	GGGGGGAAGCTTATGCGTTGCACATATGCCATCAGG
ZYORGFNR(XhoI)	GGGGGGCTCGAGTCATTTCTTTTTAGTAGTACTCTA
TVODOTOTOTO N	Construction of pGFP-C-ZbYOR114
ZYORGFCF(XbaI)	GGGGGG <u>TCTAGA</u> ATGCGTTGCACATATGCCATCAGG
ZYORGFCR(HindIII)	GGGGG <u>AAGCTT</u> TTTCTTTTAGTAGTACTCTA
	Z. bailii NCYC 1427 apocytochrome b sequencing
cytBSF	ATGGCATTTAGAAAATCAAATGTGTATTTAAGT
1427CF2	CTATGGATCATACAGATCAC

1427CF3	CCTAATACATTAGGTCATCCT
cytBSR	TTATITATTAACTCTACCGATATAGAATAAAAC
1427CR2	CCAAACATTACGATAACTCCTGC
1427CR3	ATACATACAAATAAAGAAGAA
	Z. bailii NCYC 563 apocytochrome b sequencing
cytBSF	ATGGCATTTAGAAAATCAAATGTGTATTTAAGT
563CF2	GAATTGTATTATGAACAGTAGGTG
563CF3	GTAACAGTATTTGTATTCTTA
cytBSR	TTATTTATTAACTCTACCGATATAGAATAAAAC
563CR2	AGGATGACCTAATGTATTAG
563CR3	TCTGTATGATCCATAGTATAA
	Construction of pYES2-167GLU vectors
167F (HindIII)	GGG <u>AAGCTT</u> ATGCGATCATCAAATCCATT
167RGLU (<i>Xba</i> I)	GGGGGG <u>TCTAGA</u> TTATTCCATTGGCATGAATTCACCTGGAC
	CTTCAGGTACAATTGATGCTGGTG
	Construction of pYES2-Zbcytb1427 and pYES2- Zbcytb563 vectors
ZcytbF(HindIII)	CCCCCAAGCTTATGGCATTTAGAAAATCAAAT GTG
•	TATTTAAGT
ZcytbR(XhoI)	CCCCCC <u>CTCGAG</u> TTATTTATTAACTCTACCGATATAGAATAAA
	AC
	Construction of pGFP-N-Zbcytb167
167gfpNF(<i>Hind</i> III)	GGGGGGAAGCTT ATGCGATCAAATCCATTAGGTATT
167gfpNR (XhoI)	GGGGG <u>CTCGAG</u> TCATTCAGGTACAATTGATGCTGGTGT
	Construction of pGFP-C-Zbcytb167
167gfpCF(XbaI)	GGGGG <u>TCTAGA</u> ATGCGATCATCAAATCCATTAGGTATT
167gfpCR(HindIII)	GGGGGG <u>AAGCTT</u> TTCAGGTACAATTGATGCTGGTGTTAC
167E'	CATCHWAY A TOTAL A CO

167F' GATCTTTTTTTATGTTAGG 167R' GATCTTAAAATAGCATAGA

5.3 Discussion

Pdr12 is essential for the adaptation of *S. cerevisiae* to grow under weak acid stress, since $\Delta pdr12$ mutants are hypersensitive to sorbate, benzoate and propionate at low pH (Piper, et al., 1998). Initially, the aim of this study was to isolate a *PDR12* homologue from *Z. bailii*, by screening its genomic library for sequences that reverts the weak acid sensitivity of the *S. cerevisiae* $\Delta pdr12$ mutant. This objective was not achieved. Instead, this genetic screen led to the isolation of a *Z. bailii YOR114w* homologue (*ZbYOR114*) and a gene that encodes part of apocytochrome b (*Zbcytb167*). Both genes act as multicopy suppressors of the weak organic acid sensitivity of $\Delta pdr12$ cells. Similar screening was also done using a *S. cerevisiae* genomic library, yielding two *S. cerevisiae* ORFs of unknown function (*YOR114w* and *YPL246c*).

Inactivation of YOR114w or YPL246c in S. cerevisiae did not create a weak acid sensitive mutant. Their products both appear to be associated with the plasma membrane (Figure 5.12, 5.14), but they have no sequence homology to known proteins. The results

from PathCalling® at (http://portal.curagen.com) showed interaction of Ypl246p with (a) Vam7p (b) Ypt1p, and (c) Yhr105p. Further work on the significance of these protein interactions may reveal a function for YPL246p. Also, it is conceivable that Yor114p or Ypl246p assist in extrusion of weak acids at low pH, therefore compensating for the lack of Pdr12p in S. cerevisiae.

ZbYor114p is smaller than its homologue in S. cerevisiae (Yor114p) but still retains 50% amino acid sequence similarity with the latter (Figure 5.16b). Since ZbYor114p has a very similar hydropathy plot to Yor114p (Figure 5.11) and is also located in the plasma membrane (Figure 5.13), one can assume that both proteins have similar functions. Deletion of ZbYOR114 in Z. bailii might possibly create a mutant with a hypersensitivity to weak acids at low pH, (unlike loss of the corresponding protein in S. cerevisiae (Figure 5.3a, b)).

A small, evidently nuclear, gene that encodes a small part of the apocytochrome b of Z. bailii (Zbcytb167) also acted as a multicopy suppressor of the pdr12 weak acid sensitive mutant. Cytochrome absorption spectra of mitochondrial particles from cells expressing this Zbcytb167 (Figure 5.27a, b), and also immunoblot analysis, indicated that this protein is not a part of the bc_1 complex in mitochondria. Previous work by (Piper, 1999) has shown that weak acid food preservatives are both pro-oxidants and mutagenic toward the mitochondrial genome in aerobically maintained yeast. The $\rho^- S$. cerevisiae $\Delta pdr12$ cells over-expressing the Zbcytb167 gene are weak acid sensitive. Therefore, it can be concluded that the ability of Zbcytb167p to act as a multicopy suppressor requires a functional mitochondrial respiratory chain. Further work on Zbcytb167p localisation may reveal how it confers weak acid resistance to the S. cerevisiae $\Delta pdr 12$ mutant. Electron leakage at the ubisemiquinone and bc_1 complex step of electron transport is the major source of endogenous productions of superoxide free radicals (Kowaltowski and Vercesi, 1999), a free radical production dramatically enhanced in weak acid stressed S. cerevisiae. The growth inhibition in pH4.5 sorbatestressed $\Delta pdr12$ cells is largely due to this superoxide radical production, as it is substantially reversed with the loss of superoxide dismutases (Piper, 1999). Thus one can surmise that sequestration of acid anions by its binding to Zbcytb167 may reduce endogenous oxidative stress in sorbate-treated $\Delta pdr12$ cells.

Chapter 6. Discussion

6.1 Introduction

For many years, all the research on weak acid resistance in Z. bailii concentrated on physiological aspects. Such studies have shown that yeasts retain an inverse correlation between the rates of benzoate uptake and the resistance to propionate (Warth, 1989). Also, they indicated that Z. bailii is better able to maintain intracellular pH homeostasis in the presence of high concentration of acetate than S. cerevisiae (Arneborg, et al., 2000; Fernandes, et al., 1999). However, the molecular mechanisms of how Z. bailii acquires high weak acid resistance have not been addressed.

In contrast, the mechanism of weak acid adaptation in *S. cerevisiae* has been recently studied at the molecular level (Piper, *et al.*, 1998). This was assisted by its well-developed genetic manipulations and also completion of the genome sequence in 1996. Pdr12 was discovered to be a major determinant conferring sorbate, benzoate and acetate resistance to *S. cerevisiae* (Piper, *et al.*, 1998). This thesis describes for the first time that similar molecular techniques have been applied in *Z. bailii*. Genes that may be involved in weak acid adaptation and the *ZbYME2* gene needed for preservative degradation of this yeast were isolated (Chapters 3 and 5). Also it was shown for the first time that inactivation of these genes is feasible, using transformation and gene deletion procedures originally developed for *S. cerevisiae* (Chapter 3).

6.2 Potential genes involved in weak acid resistance in Z. bailii

ZbYME2, ZbYOR114, and Zbcytb167 are Z. bailii genes isolated by functional complementation in S. cerevisiae. ZbYme2p was found to be important not only for benzoate metabolism but also weak acid adaptation and low temperature respiratory growth in Z. bailii (sections 3.2.7 and 4.2.3). The zbyme2::kanMX4/zbyme2::hphMX4 homozygous mutant was unable to grow on YPD plates pH4.5, containing 8mM benzoate or 6mM sorbate (Figure 4.6b, c). This mutant was also respiratory growth deficient at 15°C. ZbYme2p expressed as a GFP fusion in S. cerevisiae was located in the mitochondria (Figure 4.8), and the action of ZbYme2p in benzoate metabolism

requires respiratory chain function (Figure 4.15). The sequence of ZbYme2p did not reveal any potential haem binding sites. Expression of ZbYme2p in S. cerevisiae confers benzoate utilisation and also suppresses a number of phenotypes associated with yme1, yme2 S. cerevisiae cells (Section 4.2.5). There are important questions need to be addressed here: a) does ZbYme2p form a complex with another protein in Z. bailii or S. cerevisiae mitochondria in order to provide mono-oxygenase function that allows the cells to catabolise benzoate? b) Why can ZbYme2p confer an additional function (benzoate catabolism) not conferred by the S. cerevisiae Yme2p? (Has the latter protein lost this function in evolution?) and c) Why should Yme1p function in S. cerevisiae appear to need Yme2p whereas the benzoate catabolism by ZbYme2p does not?

Future work can reveal whether ZbYme2p is part of a protein complex. This can be achieved by constructing a 6-His tag ZbYme2p and using nickel nitrilotriacetic beads to isolate any protein that interacts with ZbYme2p. The degradation of benzoate in Aspergillus niger (van den Brink, et al., 2000) and in Rodotorula glutinis was observed by enzyme assay or by HPLC analysis. Similar experiments could be applied to Z. bailii or to S. cerevisiae cells expressing ZbYME2.

Previous works have indicated that other mitochondrial activities could influence weak acid resistance, at least in *S. cerevisiae* (Cheng and Piper, 1994; Piper, 1999). The presence of weak acid preservatives can dramatically enhance the endogenous production of superoxide free radicals by the mitochondrial respiratory chain. Also loss of mitochondrial Sod2 in *S. cerevisiae* increased sensitivity of the cells to weak acids at low pH (Piper, 1999).

S. cerevisiae Yme1p is a member of the AAA family of ATPases and it determines the stability of components of the inner mitochondrial membrane encoded by mtDNA, including cytochrome oxidase subunit II (Kominsky and Thorsness, 2000). Its mutation with loss of YME2 function results in several phenotypes (see Chapter 4) (Hanekamp and Thorsness, 1996; Thorsness and Fox, 1993). In this study, it was shown for the first time that theyme1-1,yme2-1 S. cerevisiae mutant is unusually sensitive to weak acid stress. This phenotype was suppressed by expression of the heterologous ZbYME2 gene. ZbYME2 however confers a function (benzoate, sorbate and

phenylalanine degradation) that is not conferred in S. cerevisiae. This additional function in S. cerevisiae does not depend on the native Yme1/2p.

In human spastic paraplegia impairment is caused by mutations in paraplegin (Casari, et al., 1998). This nuclear-encoded mitochondrial metalloprotease is highly homologous to the yeast mitochondrial ATPases, AFG3, RCA1, and YME1. These have proteolytic and chaperon-like activities at the inner-mitochondrial membrane (Langer, 2000). It would be interesting to see if ZbYme2p could confer a novel mono-oxygenase function in mammalian mitochondria.

Expression of a fragment of apocytochrome b of Z. bailii reverted the weak acid sensitivity of S. cerevisiae $\Delta pdr12$ mutant (Figure 5.19). This suppression needed mitochondrial respiratory chain function but it is unlikely that this fragment alters respiratory chain function. The ubiquinol cytochrome c oxidoreductase (also called the bc_1 complex) is an oligomeric transmembrane complex that catalyses the reduction of the c-type cytochrome of the mitochondrial respiratory chain. It contains at least 3 catalytic subunits: cytochrome b, the Fe-S protein and cytochrome c_1 (Edderkaoui, et al., 1997). In S. cerevisiae two variants of the cytochrome b gene have been described. The so-called short form has three exons and two introns and the long form has six exons and five introns (Nobrega and Tzagoloff, 1980). However K. lactis (Brunner and Coria, 1989) and Z. bailii (Figure 5.23) have a continuous cytochrome b gene with no introns. The introns can be totally lost from the S. cerevisiae gene without loss of function. Furthermore, only apocytochrome b is coded by the mitochondrial genome while other subunits of the complex are of nuclear inheritance (Edderkaoui, et al., 1997). During assembly of the cytochrome bc_1 complex, apocytochrome b is incorporated in a subcomplex, which it comprises 6 of 9 subunits (Rödel, 1997). Two spectrally distinguishable haems of different redox potentials, the b_{562} and b_{565} in yeast are noncovalently bound to the apocytochrome b (Rödel, 1997).

The Zbcytb167 could be an "escaped" fragment of mtDNA that is now expressed as a nuclear minigene in Z. bailii. The PCR amplification of Zbcytb167 fragment from Z. bailii total cellular DNA (Figure 5.25c) proofs that the insert of the original clone has colinear sequence in mitochondrial DNA.

6.3 S. cerevisiae genes that act as multicopy suppressors of $\Delta pdr12$

Two ORFs of unknown function, YPL246c and YOR114w, can both act as multicopy suppressors of the weak acid sensitivity of S. cerevisiae Δpdr12. Both proteins appeared to be located in the plasma membrane (Figure 5.12 and 5.14) but they do not have sequence homology to any known proteins. Also, deletion of these genes in S. cerevisiae did not produce weak acid sensitive mutants (Figure 5.3). One can only assume that these two proteins are therefore compensates for the lack of Pdr12 in S. cerevisiae by weak acid stressed cells, possibly by reducing intracellular anion levels. The YPL246c sequence was used in non-homology based function prediction at (www.doe-mbi.ucla.edu/people/marcotte/yeast.html) and the results showed the overexpression of YMR088c. This protein has a homology to a multidrug resistance protein. The future investigation of the S. cerevisiae Δymr088c strain can reveal whether this mutant is weak acid sensitive.

6.4 Towards the genetics of Z. bailii

The S. cerevisiae-E. coli shuttle vectors Ycplac111 and YEplac181 (Gietz and Sugino, 1988), containing a kanMX4 gene insert can replicate in Z. bailii and allow the cells to be selected on G418 media (data not shown). This could be the basis of expressing Cre (chapter 3) or epitope tagged genes in Z. bailii. Inducible S. cerevisiae promoters such as GAL1 or MET25 may not be functional in Z. bailii. It is more likely that sequences from K. lactis would show authentic regulation in this yeast, since K. lactis is more closely related to Z. bailii than S. cerevisiae. This work has demonstrated targeted gene deletion (Chapter 3). Future work could also be done to produce auxotrophic Z. bailii mutants. This would not be a time consuming process since genes involved in amino acid biosynthesis in Z. bailii (such as ZbURA3 (GenBank A/C AF279259) and ZbTRP1 (GenBank A/C AF279262)) are now available (Chapter 3). Deletions of these genes in Z. bailii could be the new beginning in genetic studies of this yeast (Figure 6.1).

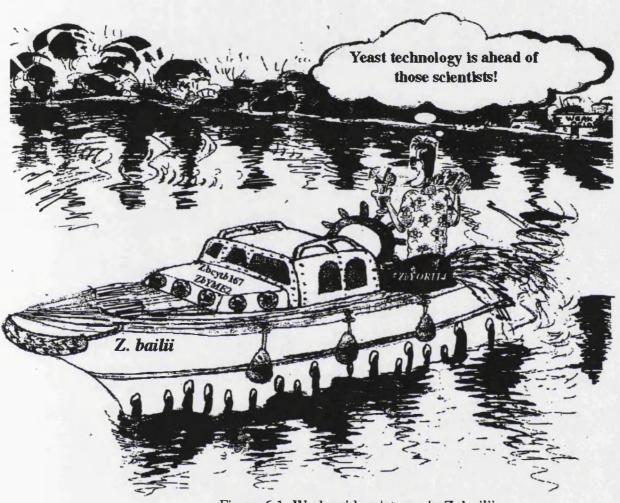


Figure 6.1. Weak acid resistance in Z. bailii.

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