RESPONSES OF YEASTS TO HYPO-OSMOTIC STRESS

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not been previously in its entirety or in part been submitted at any other university for a degree.

GERALD KAYINGO Date 28/11/2000

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PREFACE

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately and written according to the style of the journal to which the manuscript was or will be submitted.

Chapter 2: Microbial water and glycerol channels.

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Trends in Microbiology 2000, **8**: 33-38.
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Chapter 3: Growth, conservation, and release of osmolytes by yeasts during hypo-osmotic stress *Manuscript in preparation.*

Chapter 4: Effect of ergosterol on survival and glycerol release from *Saccharomyces cerevisiae* cells after osmotic downshock .
Parts of this chapter have been *submitted for publication.*

Chapter 5: An investigation of the possible existence of homologues of *FPS1*, a glycerol facilitator of *Saccharomyces cerevisiae*, in the osmotolerant yeasts *Zygosaccharomyces rouxii* and *Pichia sorbitophila.*
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Kluwer academic/Plenum Publishers. Pages 393-404.

Chapter 6: Isolation and characterisation of the TIM10 homologue from the yeast *Pichia sorbitophila*: A putative component of the mitochondrial protein import system. Published in *Yeast 2000,* **16**:589-596.

Chapter 7: Characterisation of a putative glycerol facilitator in the fission yeast *Schizosaccharomyces pombe* *Manuscript in preparation.*

CONTENTS

CHAPTER 1

Introduction

Yeasts are found in diverse habitats where conditions such as temperature and water availability vary considerably and often impose severe stress on growth. However, yeast cells are endowed with adaptive mechanisms that sense and respond to environmental changes in order to protect the cell and ensure cellular activity. Over the last two decades, there has been considerable interest in understanding the response of yeast cells to hyper-osmotic stress. This thesis examines the yeast osmotic stress response with special emphasis on hypo-osmotic stress, where less attention has been given.

The osmotic stress response in yeast

What is osmotic stress?

Various terms are currently used to describe the amount of thermodynamically available water in the environment of an organism, namely water potential, osmotic potential, osmotic pressure, water activity (a_w), osmolarity and turgor pressure (Brown, 1978). Water activity, one of the most commonly used parameter, refers to the mole fraction of water in a solution whereas turgor pressure refers to the difference between internal and external osmotic pressure maintained by the cell envelope. Changes in solute concentration automatically affect the osmolarity of the medium and leads to concomitant flux of water in or out of the cell. This osmotic flow of water might cause a physiological burden and affect the normal functioning of the cell. Therefore, osmotic stress loosely refers to the adverse effect of increased or reduced a_w on cell metabolism and integrity. Attfield (1998) broadly considers osmotic stress as the exertion of an external osmotic pressure greater or lower than those allowing optimal cell metabolism or growth. An organism that can tolerate high external osmotic stress is thus considered osmotolerant whereas a series of events that occurs when a cell is exposed to osmotic stress constitute the osmotic stress response (Blomberg and Adler, 1992). The yeast osmotic stress response occurs in phases. The immediate (seconds) and short-term responses (minutes) are largely physiological and involve functions of vacuoles, membrane proteins, modification of membrane composition and metabolic changes (Attfield, 1998). The longer-term responses (hours) involve signalling, expression of genes and proteins required for protection, and continuation of cellular activity (Albertyn *et al.*, 1994a).

Physiological and morphological changes in response to osmotic stress

When yeast cells are exposed to hyper-osmotic stress, water osmotically moves across the cell membrane to the external media. Eventually, the cells shrink, lose turgor and polarity. Their cytoskeleton becomes severely impaired and growth ceases (Fig. 1). If this is allowed to continue, the cell will eventually die. By accumulating osmotically active solutes (osmolytes), water is retrevieved and retained within the cell and an osmotic equilibrium can be established with its external environment. It has been observed that once an osmotic equilibrium has been achieved, following the osmotic shock, the cells partially restore their volume, reform their cytoskeleton and growth resumes (Albertyn *et al.*, 1994a, Brewster and Gustin, 1994).

Exposure of cells to a rapid decrease in external osmolarity (hypo-osmotic shock) results in a massive inflow of water and cell swelling. Consequently, the turgor pressure increases and if allowed to continue for too long, the cell may rupture. Although little is known about the recovery process, it appears that cells rapidly dispose of the accumulated osmolytes thereby restoring the osmotic equilibrium (Fig. 1).

Osmolytes are generally small molecules that are compatible with the cell macromolecular structure and metabolic activity (Brown and Simpson, 1972; Brown, 1978; Yancey *et al.*, 1982). The main solutes accumulated in yeast exposed to osmotic stress are polyhydroxy alcohols (polyols) such as glycerol, D-arabitol, D-mannitol, and *meso*-erythritol (Spencer and Spencer, 1978, van Eck *et al.*, 1993). Glycerol is the major osmolyte accumulated during osmotic stress in the less tolerant yeasts *S. cerevisiae,* and *Schizosaccharomyces pombe* as well as osmotolerant yeasts Z*ygosaccharomyces rouxii* (Edgley and Brown, 1983; Reed *et al.*, 1987) and *Debaryomyces hansenii* (Nobre and Costa, 1985) as well as in various filamentous fungi (Luard, 1982).

Synthesis, accumulation and transport of osmolytes

During osmotic stress conditions, yeasts synthesise and accumulate high amounts of glycerol intracellularly as the membrane permeability for glycerol decreases. Furthermore, the activity of the key enzymes in glycerol synthesis and the expression of the corresponding genes such as *GPD1* are up-regulated by the high osmolarity glycerol (HOG) signal transduction pathway (Albertyn *et al.*, 1994b). Glycerol is synthesised from the glycolytic intermediate, dihydroxyacetone phosphate via glycerol-3-phosphate.

The reactions are catalysed by glycerol-3-phosphate dehydrogenase (Gpd1p, Gpd2p) and glycerol-3-phosphate phosphatase (Gpp1, Gpp2p) (Albertyn *et al.*, 1994b; Norbeck and Blomberg, 1996; Ansell *et al.*, 1997). Although the alternative pathway which involves dihydroxyacetone (DHA) does not appear to be osmotically very significant in *S. cerevisiae* (Albertyn *et al.*, 1994a), it is utilised for glycerol formation in *S. pombe* (Gancedo *et al.*, 1968) and possibly in *Z. rouxii* (Van Zyl *et al.*, 1991). A secondary solute, arabitol is synthesised in *Z. rouxii* via two pathways, the non-oxidative pentose pathway and the oxidative pathway (Ingram and Wood, 1965). In the oxidative pentose pathway, glucose-6-phosphate is converted to ribulose-5-phosphate. In contrast, ribulose-5-phosphate is formed from fructose-6-phosphate via the non-oxidative pathway. Ribulose-5-phosphate is dephosphorylated by an acid phosphatase to ribulose. Arabitol is formed when ribulose is reduced by a ribulose dehydrogenase.

Upon hyper-osmotic stress, yeast cells respond by not only increasing glycerol production but also its accumulation. Consequently, yeasts have developed other mechanisms to maintain a high osmolyte level in response to osmotic stress. These include a reduction in dissimilation, reduction in the osmolyte leakage across the membrane, conservation and increased retention, as well as regulating glycerol transport across the plasma membrane (Edgley and Brown, 1983; Prior and Hohmann, 1997; Attfield, 1998).

Osmolyte transport in yeast has only been studied extensively with glycerol, and little is known on how other polyols such as arabitol, mannitol, or erythritol cross the plasma membrane. The movement of glycerol across yeast cell membranes occurs via active transport, by channel mediated diffusion and by passive diffusion. The extent to which glycerol permeates the cell may then be influenced by the membrane lipid composition (Watanabe and Takakuwa, 1987). It has been observed that the mode of transport differ between yeasts (Lages *et al.*, 1999) and may differs according the carbon source (Sutherland *et al.*, 1997). Osmotolerant yeasts generally use an osmotically active transport system to accumulate high amounts of glycerol whereas in the less tolerant *S. cerevisiae*, glycerol conservation appears to be mainly controlled by a glycerol facilitator Fps1p (Tamas *et al.*, 1999; Luyten *et al.*, 1995). The occurrence of glycerol facilitators in other yeasts has not been reported and was investigated in this study.

Signal transduction mechanisms controlling the osmotic stress response in yeast

Currently, two signalling pathways have been implicated in the osmotic stress response of yeast (Banuett, 1998; Gustin *et al.*, 1998). The high osmolarity glycerol (HOG) response pathway consists of two putative membrane sensors Sln1p and Sho1p that recognise the external osmolarity and trigger a mitogen activated kinase (MAP) cascade with Hog1p as the sole terminal MAP kinase (Brewster *et al.*, 1993; Maeda *et al.*, 1994; Maeda *et al.*, 1995). An increase in osmolarity stimulates the phosphorylation of Hog1p in a matter of seconds (Brewster *et al.*, 1993). Upon activation, the Hog1p accumulates in the nucleus and induce transcription of osmo-responsive genes such as those involved in glycerol biosynthesis (Albertyn *et al.*, 1994b; Rep *et al.*, 2000).

The signalling pathways by which cells sense and respond to hypo-osmotic shocks are not well known but the protein kinase C (PKC) MAP kinase cascade appears to be involved. It has been shown that hypotonic shock increases tyrosine phosphorylation of Mpk1/Slt2, the ultimate kinase of the PKC pathway (Davenport *et al.*, 1995). Mutants in the yeast PKC pathway are very sensitive to hypo-osmotic shock and lyses in medium without osmotic stabilizers (Lee and Levin, 1992).

In conclusion, osmotic adaptation in yeast is a complex process involving physiological and metabolic shifts, signalling and induction of gene expression, protein and membrane modifications. However, the production, accumulation and transport of osmolytes appear to be the central mechanism underlying osmotolerance.

Aims of the study

1) To study the pattern and kinetics of osmolyte export during hypo-osmotic stress in various yeasts as well as the influence of membrane compostion (ergosterol) in the survival and glycerol release from *S. cerevisiae* cells after osmotic downshock.

2) To investigate the occurrence of genes encoding the osmolyte exporters in the osmotolerant yeasts *Zygosaccharomyces rouxii* and *Pichia sorpitophila* using molecular techniques.

3) Functional analysis of a putative glycerol facilitator in the fission yeast *Schizosaccharomyces pombe*.

4) An *in silicio* analysis of microbial water and glycerol channels of the MIP family.

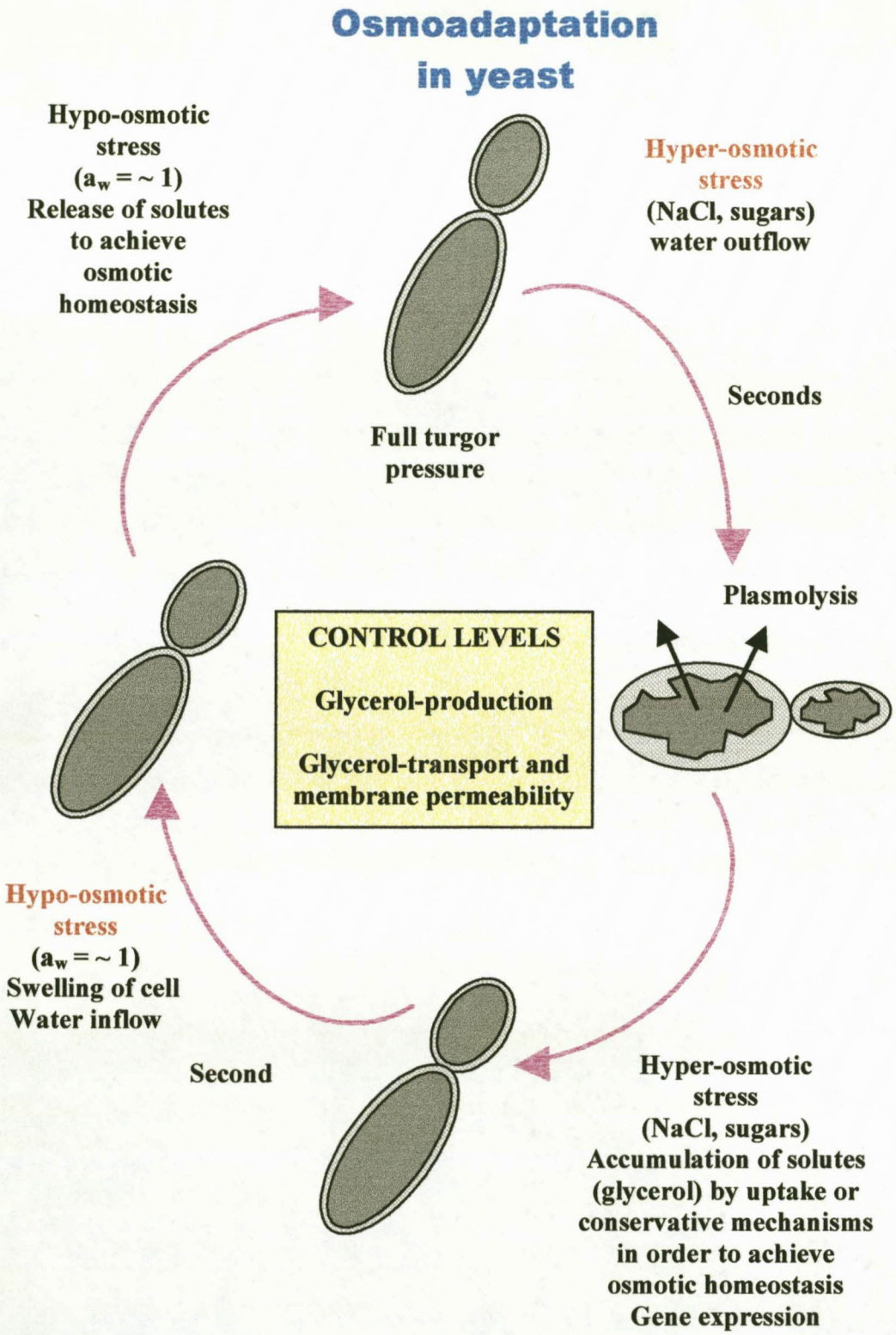

Figure 1. Osmoadaptation in yeast (adapted from Hohmann, 1997).

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CHAPTER 2

Microbial water channels and glycerol facilitators

Abstract

The recent sequencing of a variety of Archeal, Bacterial, Fungal and Protozoan genomes has revealed a wealth of novel Major Intrinsic Protein (MIP) family members. Most microorganisms possess between one and four MIP channels, namely glycerol facilitators and aquaporins. Bacterial glycerol facilitators appear to be involved in the catabolism of glycerol and other closely-related compounds, and their genes are co-expressed with those encoding enzymes in such catabolic pathways. The yeast glycerol facilitator Fps1p has been shown to be involved in osmoregulation by controlling the cellular content of glycerol, the compatible solute in yeast. In fact, Fps1p is the only known eukaryotic solute exporter and its transport function is controlled by osmolarity changes. Microbial water channels appear to be important in osmoregulation but their precise physiological role and the conditions under which aquaporin-mediated rapid water movement is important are not very well defined. Expression data suggest that some aquaporins in eukaryotic microbes may be involved in developmental processes such as spore formation and germination. Overall, the study of microbial MIP channels has the potential to provide novel information both for structure-function analysis and for a clearer understanding of microbial metabolism and osmoregulation. Future research will be aimed at better defining the physiological role of these proteins.

I. Introduction

In contrast to the majority of cells from multicellular organisms, microbial cells are in direct contact with a highly variable environment. Hence, bacteria, fungi, algae and protozoa must be able to respond to a wealth of widely-varying conditions. For instance, fungi such as yeasts tolerate pH values from about 3–8 and many bacteria are productive over a range of more than 30°C. In particular, microorganisms can live and proliferate at variable water activities and under different nutritional conditions. This inevitably requires the ability to adjust transport processes for the uptake and/or efflux of water, osmolytes, nutrients and metabolic end products.

Transmembrane transport in unicellular microorganisms is mediated by different systems that are classified according to their mode of function into channels, pores, facilitators, carriers, porters or pumps (Nikaido and Saier, 1992; Saier, 1994; André, 1995; Saier, 1998; Paulsen *et al.*, 1998a; Paulsen *et al.*, 1998b; Saier *et al.*, 1999). Pores and channels allow free passage of solutes across the membrane while carriers and porters possess specific binding sites via which the solute traverses the membrane. Whereas proteins that catalyze facilitated diffusion (facilitators) do not involve energy coupling and therefore cannot operate against a substrate concentration, pumps couple metabolic energy during active transport. Most transport proteins so-far studied fall into relatively few families, which are characterized by conserved motifs and/or similar topology. For instance, the major facilitator super family (MFS) comprises a huge number of proteins for the uptake of sugars, amino acids, ions and other compounds (Pao *et al.*, 1998). Facilitated transport by these proteins can be coupled as symport or antiport to a proton gradient, thereby allowing transport against a substrate concentration gradient. Another major class of transport proteins are the ATP binding cassette (ABC) transporters, which use the energy derived from ATP hydrolysis for active transport of many different substrates into or out of the cell (van Veen and Konings, 1997; van Veen and Konings, 1998). All microbial genomes sequenced so far contain genes encoding many MFS and ABC transporters: the genome of the Gram negative bacterium *Escherichia coli* encodes some 70 MFS transporters and 80 ABC transporters (Blattner *et al.*, 1997), that of the Gram-positive bacterium *Bacillus subtilis* 81 MSF transporters and 77 ABC transporters (Kunst *et al.*, 1997) and that of the yeast *Saccharomyces cerevisiae* 78 MFS transporters and 22 ABC transporters (Paulsen *et al.*, 1998b).

Small molecules such as water and glycerol can passively cross the plasma membrane. However, it appears that different membranes exhibit very different permeability for water and glycerol accounting for the different rates of passive diffusion observed in organisms. In fact, it has been known for many years that the permeability of specialized biological membranes for water is much higher than that of artificial lipid bilayers. This observation implied the possible involvement of channels that facilitate water flux across cell membranes (Koefoed-Johnson and Ussing, 1953; Paganelli and Solomon, 1957; Macey and Farmer, 1970; Macey, 1984; Finkelstein, 1987; Wayne and Tazawa, 1990). However, the molecular justificaton of this view remained elusive until the discovery of the aquaporin family of transmembrane water channels, first in mammals, subsequently in plants and finally in microorganisms (Preston *et al.*, 1992; Maurel *et al.*, 1993; Calamita *et al.*, 1995). Similarly, the occurrence of glycerol facilitators in bacteria was proposed nearly thirty years ago (Sanno *et al.*, 1968; Richey and Lin, 1972; Heller *et al.*, 1980) and was confirmed by the cloning of *glpF*, a gene encoding the *Escherichia coli* glycerol facilitator (Sweet *et al.*, 1990). Subsequent comparative sequence analyses revealed significant homology between glycerol facilitators and water channels (Baker and Saier, 1990). They were found to be related to the bovine lens major intrinsic protein (Gorin *et al.*, 1984) from which the family name MIP is derived.

To date, more than 200 MIP family members have been identified and their role in solute and water transport has been established both *in vitro* and *in vivo*, as described in detail in other chapters of this volume. As for the microbial MFS and ABC transporters mentioned above, higher organisms possess an amazing number of MIP channel isoforms expressed in different subcellular compartments and tissues, under different environmental conditions or during different developmental stages. For example, more than 30 genes encoding MIP channels have been reported in the model plant *Arabidopsis thaliana* (Kjellbom et al., 1999) and 10 have been described in humans (Borgnia et al., 1999). Furthermore, nine can be recognized in the nematode *Caenorhabditis elegans* genome data base (http://www.sanger.ac.uk/Projects/C_elegans/Genomic_Sequence.shtml).

Most functional studies suggest that MIP channels mediate water flux across cell membranes. In plants and animals, MIP channels appear to play a role in osmoregulation at the cellular and/or organismal level. For example, plant aquaporins are involved in stress responses and in developmental processes and their mammalian homologs display a wide variety of roles in physiology and are consequently involved in several clinical disorders.

All these aspects are described in detail elsewhere in this volume and have been subject of recent reviews (Borgnia *et al.*, 1999; Kjellbom *et al.*, 1999).

Like other major transport protein families, MIP channels are also widespread among microbes but the number of genes encoding MIP channels per microbial genome is not more than four. However, many new MIP channel genes continue to be identified during the sequencing of microbial genomes (see www.tigr.org/tdb/mdb/mdb.html). Table 1 lists the 76 MIP channels that we have located in various databases by the end of March 2000 (the table does not include the sequence of the *Thermus flavus* glpF (Darbon *et al.*, 1999), which does not appear in the databases). These proteins are found in 52 different species belonging to Archea, Bacteria, Fungi and Protozoa. Microorganisms constitute the biggest resource of MIP channel sequences in terms of species number. This is especially interesting for structure-function analysis because many different sequences with similar or identical function are available for comparison (Heymann and Engel, 2000).

Relatively little attention, however, has yet been given to the physiological role of microbial MIP channels. In fact, functional and physiological studies are largely restricted to MIP channels from the bacterium *E. coli* and the yeast *S. cerevisiae*. This chapter attempts to summarize the available information on microbial aquaporins and glycerol facilitators with emphasis on their evolutionary relationships, molecular properties, patterns of gene expression and their physiological roles. It is anticipated that studies on microbial aquaporins will provide novel insights into the structure and function of MIP channels as well as their physiological roles. Microbial MIP channels thus provide a suitable model for understanding the role of these proteins in cellular water relations since the underlying concepts of cellular osmoregulation are conserved from bacteria to humans (Yancey *et al.*, 1982; Wiggins, 1990; Blomberg and Adler, 1992; Wood, 1999).

Table 1: Microbial MIP family channel proteins

Organism	Classification and habitat	Genome sequence	Sequence source	Gene or Clone or Cosmid	Accession number	Phylogenetic subfamily/ predicted function	Gene context (operon)	Protein size (aa)
Eukaryota								
Aspergillus nidulans	Fungus, ascomycete; saprophyte	ongoing	GenBank/EBI	C5f02a1.r1[a]	AA783486	ND	none	118[b]
Botrytis cinerea	Fungus, ascomycete; plant, pathogen	partial	www.genoscope.cns.fr	CNS01A8X	AL112633	2 / GlpF	none	239[b]
Candida albicans	Fungus, yeast; human pathogen	ongoing	sequence-www.stanford.edu	stanford 5476	Contig4-2389	1/ AQP	none	273
Dictyostelium discoideum	Protozoan, saprophyte; soil	ongoing	www.sanger.ac.uk	*wacA*	U68246	1 / AQP	none	277
			www.sanger.ac.uk	*aqpA*	AB032841	1 / AQP	none	279
Neurospora crassa	Fungus, ascomycete; saprophyte	ongoing	GenBank/EBI	NCSM1G3T3[a]	AI392589	2 / AQP	none	195[b]
Saccharomyces cerevisiae	Fungus, yeast; fruits and flowers; model and industrial organism	complete	www.proteome.com	*FPS1*[c]	P23900	ND/GlpF[e]	none	669
			www.proteome.com	*YFL054*	P43549	2 / GlpF	none	646
			GenBank/EBI	*AQY1-1*[d]	AAC69713	1 / AQP[e]	none	327
			GenBank/EBI	*AQY1-2*[d]	P53386	1 / AQP	none	305
			GenBank/EBI	*AQY2*[d]	AAD25168	1 / AQP	none	289
Schizosaccharomyces pombe	Fungus, yeast; fruits and flowers; model organism	ongoing	www.sanger.ac.uk	SPAC977	CAB69639	2 / GlpF	none	598

Trypanosoma brucei	Protozoan; human pathogen; sleeping sickness	ongoing	www.tigr.org	RPCI93	AQ641778	2 / GlpF	none	160[b]
Gram-positive Bacteria								
Bacillus anthracis	Pathogen; anthrax	ongoing	www.tigr.org	*aqpZ*	gba 92	1/AQP	ND	221
			www.tigr.org		gba 1391	3/AGP	ND	273
Bacillus subtilis	Saprophyte; model and industrial organism	complete	www.pasteur.fr	*glpF*	P18156	3/GlpF[e] (AGP)	operon	274
Caulobacter crescentus	Freshwater; model organism; bacterial differentiation	ongoing	www.tigr.org	*aqp*	gcc 515	1/AQP	none	81[b]
			www.tigr.org		gcc 439	1/ND	ND	100[b]
Clostridium acetobutylicum	Anaerobe, industrial organism	complete	www.genomecorp.com		AE001437	3/AGP	none	242
Clostridium perfringens	Pathogen; protein-rich foods; soil		GenBank/EBI	*glpF*	X86492	3/AGP	ND	148[b]
Corynebacterium diphteriae	Pathogen; diphtheria	ongoing	www.sanger.ac.uk		Contig423	3/AGP	operon	227[b]
Deinococcus radiodurans	Natural habitat unknown; resistant to radiation	complete	www.tigr.org	*glpF*	8796	2/GlpF	ND	271
Enterococcus faecalis	Small intestine; urinary tract; pathogen; endocarditis	ongoing	www.tigr.org		gef 6204	3/AGP	*glpF/glpO*	239
			www.tigr.org	*glpF*	gef 6176	ND/GlpF	*glpF*/PTS	236
			www.tigr.org	*aqpZ*	gef 6403	1/AQP	none	233
Lactococcus lactis	Saprophyte, anaerobic, industrial organism	complete	GenBank/EBI	*ydp1*	P22094	3/AGP[e]	none	289
Staphylococcus aureus	Pathogen (food poisoning), skin; meat and dairy products	ongoing	www.tigr.org		4410	3/AGP	operon	272

Streptococcus pneumoniae	Pathogen of the respiratory tract; pneumonia	ongoing	www.tigr.org	*glpF*	U12567	3/GlpF	operon	233
			www.tigr.org		sp 42	3/AGP	none	289
			www.tigr.org	*aqpZ*	sp 16	1/AQP	none	269[b]
Streptococcus pyogenes	Pathogen of the respiratory tract; scarlet fever	ongoing	www.genome.ou.edu		Contig115	3/AGP	operon	233
			www.genome.ou.edu		Contig104	3/AGP	ND	282
Streptomyces coelicolor	Soil and aquatic; producer of antibiotics	ongoing	GenBank/EBI	*gylA*	P19255	3/AGP	operon	80[b]
Thermotoga maritima	Extreme thermophile, marine, hydrothermal vents	complete	GenBank/EBI	*glpF*	AAD36499	3/AGP	operon	234
Gram-negative Bacteria								
Borrelia burgdorferi	Anaerobe; pathogen; lyme disease	complete	GenBank/EBI	*glpF*	AAC66629	2/GlpF	operon	254
Bordetella bronchiseptica	Pathogen; respiratory diseases	ongoing	www.sanger.ac.uk		Contig 2552	1/AQP	ND	236
Brucella melitensis	Goat pathogen and parasite; milk, meat, soil	ongoing	GenBank/EBI	*aqpZ*	AAF36396	1/AQP	ND	228
Chlorobium tepidum	Anaerobe; thermophilic green sulfur bacterium; phototroph	ongoing	www.tigr.org	*aqp*	gct 5	1/AQP	ND	268
Escherichia coli	Facultative anaerobe; mammalian colon; model organism	complete	GenBank/EBI	*glpF*	P11244	2/GlpF[e]	operon	281
			GenBank/EBI	*aqpZ*	U38664	1/AQP[e]	none	231
Haemophilus influenzae	Pathogen; upper respiratory tract	complete	GenBank/EBI	*glpF*	P44826	2/GlpF	operon	264
			GenBank/EBI	*glpF*	U32782	3/AGP	none	213[b]

Klebsiella pneumoniae	Pathogen; respiratory tract	ongoing	genome.wustl.edu	*aqpZ*	Contig1030	1/AQP	ND	155[b]
			genome.wustl.edu	*aqp*	Contig1071	1/AQP	ND	180[b]
			genome.wustl.edu	*glpF*	Contig848	2/GlpF	operon	267
			genome.wustl.edu	*glpF*	Contig757	2/GlpF	operon	293
Mycoplasma capricolum	Goat pathogen; contagious caprine pleuropneumonia		Genbank/EBI	*glpF*	Z33098	3/GlpF	operon	89[b]
Mycoplasma gallisepticum	Fowl pathogen; anaerobe		GenBank/EBI	*glpF*	P52280	3/GlpF	ND	205[b]
Mycoplasma genitalium	Pathogen; urinary tract	complete	GenBank/EBI	*glpF*	P47279	ND/GlpF	*GlpF/ThyK*	258
Mycoplasma pneumoniae	Pathogen; mucous membrane	complete	GenBank/EBI	*glpF*	P75071	ND/GlpF	*GlpF/ThyK*	264
Pasteurella multicoda	Fowl pathogen; pasteurellosis	ongoing	www.cbc.umn.edu	*glpF*	Contig82	2/GlpF	operon	261
Plesiomonas shigelloides	Pathogen; food and water borne diarrhoea		GenBank/EBI	*ORF10P*	AB025970	1/AQP	cluster	233
Pseudomonas aeruginosa	Pathogen of the gastro intestinal tract; soil	ongoing	www.genome.washington.edu	*aqpZ*	Contig54	1/AQP	none	308
			GenBank/EBI	*glpF*	Q51389	2/GlpF[e]	operon	279
Pseudomonas putida	Soil	ongoing	www.tigr.org	*glpF*	all 2259	2/GlpF	ND	147[b]
			www.tigr.org	*glpF*	all 2406	2/GlpF	ND	162[b]
Pseudomonas tolaasii	Soil		GenBank/EBI	*glpF*	AB015973	2/GlpF	operon	285
Salmonella enterica serovar *typhim.*	Pathogen; gut		GenBank/EBI	*pduF*	AF026270	2/PduF	operon	264

Salmonella typhi	Pathogen of the gut; typhoid fever; aquatic	ongoing	www.sanger.ac.uk	*pduF*	Contig 443	2/PduF	ND	264
			www.sanger.ac.uk	*glpF*	Contig460	2/GlpF	ND	279
			www.sanger.ac.uk	*pduF*	Contig 417	2/PduF	ND	269
Salmonella typhimurium	Pathogen of the gut; typhus; aquatic	ongoing	genome.wustl.edu	*pduF*	P37451	2/PduF[e]	operon	264
			genome.wustl.edu	*glpF*	Contig79	2/GlpF	ND	288
Shewanella putrefaciens	Soil and aquatic; food spoilage	ongoing	www.tigr.org	*aqpZ*	4323	1/AQP	none	231
Shigella flexneri	Pathogen of the gut; dysentery		GenBank/EBI	*glpF*	P31140	2/GlpF[e]	operon	281
			GenBank/EBI	*aqpZ*	AAC12651	1/AQP	none	231
Shigella sonnei	Pathogen of the gut; enteritis	ongoing	GenBank/EBI	*ORF10S*	BAA85070	1/AQP	cluster (plasmid)	25[b]
Sinorhizobium meliloti	Root nodules; nitrogen fixation	ongoing	cmgm.stanford.edu	Stanford 382	423050H01	ND	ND	204[b]
Synechococcus sp.PCC7942	Phototroph; aquatic		GenBank/EBI	*smpX*	D43774	ND	*smpX/pacS*	269
Synechocystis sp.PCC6803	Phototroph; aquatic	complete	GenBank/EBI	*aqpZ*	BAA17863	1/AQP	none	247
Thiobacillus ferrooxidans	Chemolithotroph; soil; aquatic	ongoing	www.tigr.org	TIGR 920	3498	ND	ND	215[b]
Vibrio cholerae	Pathogen of the gut; cholera; aquatic; soil	ongoing	www.tigr.org	*glpF*	asm814	ND/GlpF		261
			www.tigr.org	*glpF*	1741	2/GlpF	operon	285
Yersinia pestis	Pathogen; plague	ongoing	www.sanger.ac.uk	*glpF*	Contig630	2/GlpF	operon	282

Archaea								
Archaeoglobus fulgidus	Anaerobe; thermophilic	complete	GenBank/EBI	*glpF (aqp?)*	AAB89820	1/AQP	none	246
Methanobacterium thermoautotrophicum	Anaerobe, extreme enviroments	complete	GenBank/EBI	*aqp*	AAB84602	1/AQP	none	246

Key: ND, not determined; GlpF, glycerol facilitator/transporter; PduF, propanediol facilitator; AQP, aquaporin; AGP, aquaglyceroporin; aa, number of amino acids. [a]Deduced from mRNA sequence. [b]Incomplete sequence obtained from GenBank. [c]One or both 'NPA' motifs have a different sequence. [d] Polymorphic form . [e]Function confirmed experimentally.

Completed genomes lacking MIP channels: *Methanococcus janaschii, Helicobacter pylori, Aquifex aeolius, Pyrococcus horikoshi, Mycobacterium tuberculosis, Treponema pallidum, Rickettsia prowazekii, Chlamydia trachomatis, Chlamydia pneumoniae, Aeropyrum pernix, Neisseria meningitidis.*

II. Microbial aquaporins and glycerol facilitators

A. Classification of microbial MIP channels into subfamilies

MIP channels have historically been divided into two major subgroups, 1) the aquaporins *sensu strictu*, which are specifically permeable only to water and 2) the glycerol facilitators, which are permeable to water, glycerol, and to varying degrees, other small solutes (Park and Saier, 1996; Agre *et al.*, 1998; Froger *et al.*, 1998).

In addition to substrate specificity, phylogenetic and sequence analyses (Froger *et al.*, 1998; Heymann and Engel, 2000) have revealed that certain conserved residues are distinct between putative aquaporins and glycerol facilitators. These signature residues can be used for classification (Froger *et al.*, 1998; Heymann and Engel, 2000) even when functional studies have not been conducted.

Whereas most MIP channels from plants and animals are classified as water channels, glycerol facilitators account for the majority of MIPs in microorganisms. However, functional studies have only been performed on the glycerol facilitators, GlpF, from *Escherichia coli* (Heller *et al.*, 1980; Sweet *et al.*, 1990) and Fps1p from the yeast *Saccharomyces cerevisiae* (Luyten *et al.*, 1995; Sutherland *et al.*, 1997). Hence classification of glycerol facilitators is based mainly on sequence comparison and operon organization. Although the term glycerol facilitator is well established, we believe that it is somewhat misleading since the *Escherichia coli* and yeast proteins have also been shown to transport a range of other polyols and related compounds (Heller *et al.*, 1980; Sanders *et al.*, 1997; Sutherland *et al.*, 1997; Karlgren and Hohmann, 2000). Furthermore, even though phylogenetic analysis (Fig. 1) illustrates that microbial MIP channels can be classified as aquaporins (Fig. 1, subfamily 1) or glycerol facilitators, the latter group appears to be split further into two subfamilies (Fig. 1, subfamilies 2 and 3). Subfamily 1 comprises the functionally characterized water channels AqpZ from *Escherichia coli* (Calamita *et al.*, 1995), wacA from *Dictyostelium discoideum* (Flick *et al.*, 1997) and Aqy1p from *Saccharomyces cerevisiae* (Bonhivers *et al.*, 1998). The classification of all other proteins in this subfamily is based on sequence similarity only. Although the MIP channel from the Archea, *Archeaoglobus fulgidus*, has been classified as a glycerol facilitator in the databases (www.tigr.org/tdb/mdb/mdb.html), it clusters with aquaporins (Fig. 1) and shows the residues characteristic of water channels (Froger *et al.*, 1998). Hence, its initial classification may be incorrect.

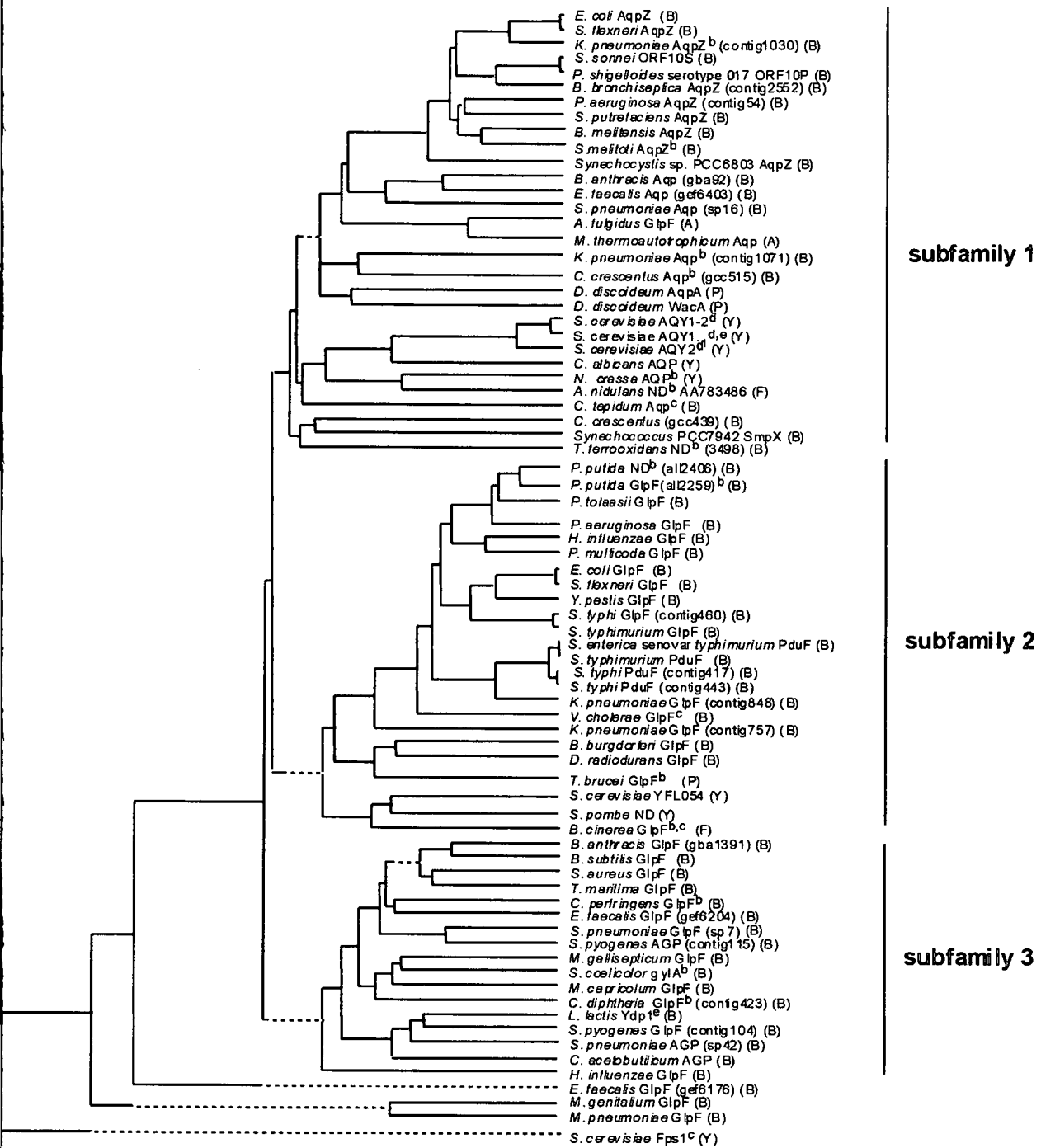

Figure 1. Phylogenetic tree of microbial MIP channels.
Phylogenetic analysis of the MIP channels listed in Table 1.
A, archeaobacterium; B, bacterium; F, fungus; P, protozoan; Y, yeast

Subfamily 2 comprises the glycerol facilitators of *Escherichia coli* (Sweet *et al.*, 1990) and *Pseudomonas aeruginosa* (Schweizer *et al.*, 1997) as well as the propanediol facilitator from *Salmonella typhimurium* (Walter *et al.*, 1997). The transport specificities of the latter two proteins have not been determined experimentally, but the genes are respectively part of the well-characterized *glp* operon in *Pseudomonas aeruginosa* (Schweizer *et al.*, 1997) which is required for glycerol catabolism, and the *pdu* operon in *Salmonella typhimurium* (Fig. 2) which is required for propanediol utilization (Walter *et al.*, 1997).

The third subfamily contains the *Lactobacillus lactis* glycerol facilitator, which has been shown to transport both water and glycerol (Froger *et al.*, 2000). Whether this is a general feature of the third subfamily is unknown and hence conclusions about functionality can only be speculative. In order to encompass the possible roles of these proteins in transporting water *and* glycerol, the term aquaglyceroporin (AGP) has been suggested for them.

Some microbial MIP channels do not appear to fall into any of the three subfamilies, such as the putative glycerol facilitators from *Enterococcus faecalis*, *Mycobacterium genitalium*, *Mycobacterium pneumoniae* and Fps1p from *Saccharomyces cerevisiae*, which has a number of unusual features (see further). This may reflect functional specialization as is apparent for Fps1p, which functions mainly as an export channel.

B. Distribution of MIP channels in microorganisms

Table 1 lists the known MIP channels that were found in a total of 23 complete microbial genome sequences by the end of March 2000, as well as those from ongoing sequencing projects. The data from the completed microbial genomes allows some conclusions to be drawn on the distribution of MIP channels in microorganisms. For example, there are apparently some organisms that lack MIP channels altogether, such as the Archaea *Methanococcus jannaschii* and *Pyrococcus horikoshii* and the Bacteria *Aquifex aeolicus*, *Helicobacter pyroli*, *Mycobacterium tuberculosis*, *Treponema pallidum*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Rickettsia prowazekii* and *Campylobacter jejuni*. The majority of these microbes are either animal pathogens or deep-sea dwellers. It is plausible that in such habitats microbes might not experience stressful osmolarity changes that would require MIP channel mediated-water/solute flux. Interestingly most microorganisms lacking a glycerol facilitator gene also do not possess a glycerol kinase gene suggesting that these organisms might not utilize glycerol as a carbon source or as a metabolic precursor.

Of course it is possible that these organisms have other currently undefined mechanisms for water or solute transport.

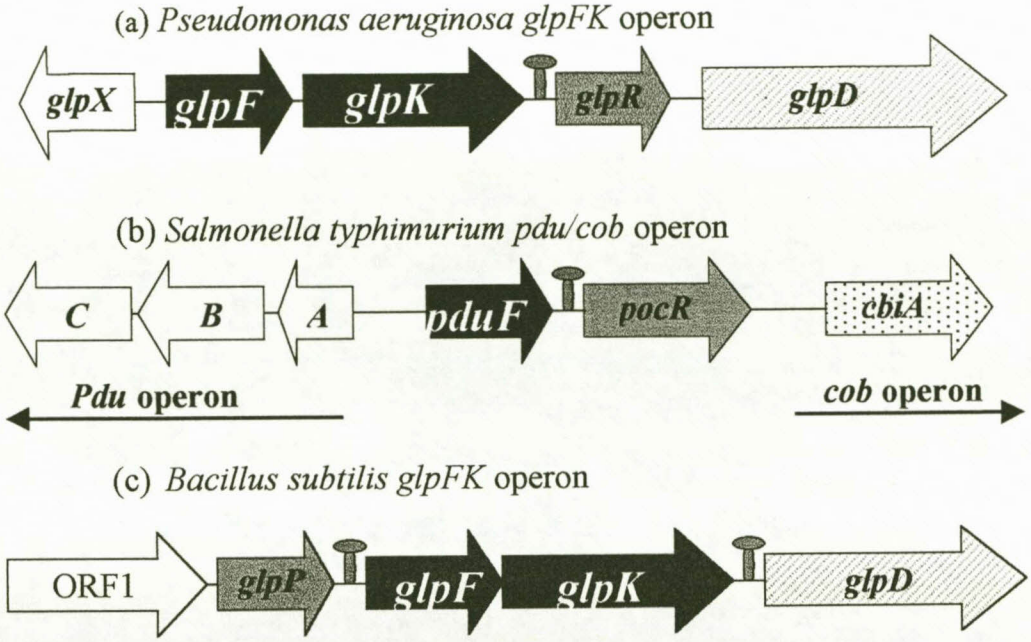

Figure. 2. Operon structure

Operon organization of glycerol and propanediol facilitator genes in different bacteria.

a) Operon organization of the *glpFK*-containing region of the *Pseudomonas aeruginosa* chromosome. The genes encoding the glycerol facilitator and glycerol kinase are indicated as *glpF* and *glpK* respectively; *glpX* and *glpR* encode regulatory proteins and *glpD sn*-glycerol-3-phosphate dehydrogenase (Schweizer *et al.*, 1997).

b) *Salmonella typhimurium pdu/cob* operon containing the *pduF* gene. The *pdu* operon controls the degradation of propanediol whereas the *cob* operon controls the synthesis of cobalamin, which is required for propanediol catabolism. The region between the two operons encodes two proteins, the propanediol facilitator PduF and PocR, a regulatory protein, which mediates the induction of the *pdu/cob* operon by propanediol (Chen *et al.*, 1994; Chen *et al.*, 1995). The letters A, B and C designate the first three genes in the *pdu* operon. The *pduA* gene encodes a hydrophobic protein with high similarity to the carboxysome-forming proteins of several photosynthetic bacteria whereas *pduB* and *pduC* encode proteins of unknown function. The arrows indicate the direction of gene transcription.

c) The *Bacillus subtilis glpPFKD* region containing genes essential for growth on glycerol or glycerol 3-phosphate. The genes encoding a glycerol facilitator and a glycerol kinase are indicated as *glpF* and *glpK* respectively. The *glpP* gene encodes a regulatory protein whereas *glpD* encodes a glycerol 3-phosphate dehydrogenase. The four genes represent three separate transcription units (*glpP*, *glpFK*, *glpD*) and the activities of glpFK and glpD are controlled by *glpP*, the phosphoenolpyruvate:sugar phosphotransferase system (PTS) and glucose repression (Beijer *et al.*, 1993). Inverted repeats are indicated by hairpin symbols.

In general, it appears that most bacteria whose genomes have been fully sequenced possess a glycerol facilitator homolog that is part of the same operon as the gene for glycerol kinase: an indication of a role in glycerol metabolism, as we discuss below. In addition, several genomes contain a second MIP channel, which may be an aquaporin homolog, such as in *Escherichia coli*, *Pseudomonas aeruginosa* or *Shigella flexneri*. The second MIP channel may also be (i) an additional glycerol facilitator as in *Pseudomonas putida*, (ii) a propanediol facilitator, as in *Salmonella typhimurium* or (iii) a glycerol facilitator from a different subfamily, as in *Bacillus anthracis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. Some bacteria appear to have more then two MIP channels, often one from each subfamily as found in *Enterococcus faecalis* and *Streptococus pneumoniae* or from at least two different subfamilies as in *Klebsiella pneumoniae* and *Salmonella typhi*. The presence of more than one MIP channel from the same subfamily is found only in very few cases, such as in *Klebsiella pneumoniae* (two putative, quite distinct water channels and two closely related subfamily 2 members), *Pseudomonas putida* (two closely related subfamily 2 glycerol facilitators) and *Salmonella typhi* (two highly similar, putative propanediol facilitators). With the possible exception of the two *Klebsiella pneumoniae* water channels, two proteins from the same subfamily are likely to be the result of a recent gene duplication event and may fulfill the same physiological role in that microorganism. In general, however there appears to be a tendency to maintain only one (if any) MIP channel per subfamily in a given organism. This distribution pattern supports the idea that the members of the different subfamilies may indeed exhibit different functions. This is further corroborated by the finding that in bacteria which only have a single MIP channel, the protein is in most cases found in subfamily 3, whose members may transport both water and glycerol (Froger *et al.*, 2000). Hence, these proteins may fulfill functions as water channels *and* glycerol facilitators. Whether this is of any physiological relevance has yet to be addressed.

From the four sequenced Archeal genomes, only two encode a MIP channel and in both instances it is a putative water channel. Hence, from this limited information it appears that glycerol is either not utilized by these organisms or that alternative uptake systems are required.

The only eukaryotic microorganism for which a complete genome sequence is available is that of *Saccharomyces cerevisiae*. This genome encodes four MIP channels (André, 1995): two aquaporin homologs, 86% identical to each other, and two related glycerol facilitator homologs.

The functions of only one of the aquaporins and one of the glycerol facilitators have been confirmed (Luyten *et al.*, 1995; Bonhivers *et al.*, 1998). Strikingly, most laboratory yeast strains seem to have mutations that inactivate both aquaporin genes and even industrial strains and yeasts isolated from Nature appear to have mutated versions of the *AQY2* gene (Laizé *et al.*, 2000). So far, only one laboratory yeast strain, Σ1278, a derivative from an industrial isolate, has been found to have two complete aquaporin genes. *AQY1* from Σ1278 differs from that of other laboratory strains by three amino acids and, due to a frame-shift mutation, the entire carboxy-terminus is different. Two of the amino acid substitutions seem to be responsible for functional alteration in most laboratory strains (Bonhivers et al., 1998). *AQY2* in all laboratory strains investigated so far contains an 11bp deletion in the center of the gene leading to a premature translational stop. Various different alleles for *AQY2* have also been found in laboratory and industrial strains as well as in natural isolates (Laizé *et al.*, 2000). Although strain Σ1278 has a complete open reading frame for *AQY2*, it has not been possible to confirm in the *Xenopus* oocyte system whether *AQY2* actually encodes a functional aquaporin (Laizé *et al.*, 2000). With regard to other eukaryotic microorganisms, two aquaporins have been found in the slime mold, *Dictyostelium discoideum*; the function of one of these, WacA, has been confirmed experimentally (Flick *et al.*, 1997). Similarity searches reveal putative aquaporins in the pathogenic yeast *Candida albicans* and the filamentous ascomycetes, *Aspergillus nidulans* and *Neurospora crassa*.

Glycerol facilitators such as that found in the parasitic protozoan *Trypanasoma brucei*, are also well represented in eukaryotic microorganisms. In particular, Fps1p from *Saccharomyces cerevisiae* has been well characterized as a glycerol and polyol facilitator and will be discussed later (Luyten *et al.*, 1995; Sutherland *et al.*, 1997; Tamás *et al.*, 1999). This organism has a second open reading frame encoding a putative glycerol facilitator, *YFL054c*. Fps1p and Yfl054p are 32% identical in their six transmembrane domain cores. Like Fps1p, the protein encoded by *YFL054c* has an approximately 300 residue amino-terminal extension. Unfortunately, analysis of strains deleted for *YFL054* have not yet lead to the elucidation of the protein's function (Tamás, 1999). Recently, glycerol facilitators have been recognized in the fission yeast *Schizosaccharomyces pombe* and in the plant pathogenic ascomycete *Botrytis cinerea*. Strikingly, sequence comparison suggests that these are homologs of *Saccharomyces cerevisiae* Yfl054p. Yfl054p and the homolog from *Schizosaccharomyces pombe* are 76% identical within the transmembrane core and share 30% identity even within their extensions (Tamás and Hohmann, unpublished data).

However, the extensions of these two proteins and that of Fps1p appear totally unrelated. Hence Yfl054p may be the founding protein of a glycerol facilitator subfamily in fungi.

C. Origin of microbial MIP channels

It is generally believed that the MIP family emerged as a result of an intragenic duplication event, which probably took place 2.5 to 3 billion years ago (Wistow *et al.*, 1991; Pao *et al.*, 1998; Heymann and Engel, 2000). It has been postulated that a single gene arose in prokaryotes shortly before the emergence of eukaryotes and that subsequent gene duplication and divergence resulted in the various MIP family genes. However, the occurrence of both highly similar and dissimilar MIP channels in a single organism suggests that some MIP proteins did not arise via gene duplication, but rather have been acquired horizontally from other organisms (Park and Saier, 1996). For instance, the G+C content of *Saccharomyces cerevisiae AQY1* and *FPS1* is 50% and 43% respectively, whereas the overall G+C content of *Saccharomyces cerevisiae* is 40%. This deviation lends support to the notion that *AQY1* could have been acquired horizontally and that the second highly similar yeast aquaporin gene (86% identity at protein level) was the result of a subsequent duplication event.

III. Transport properties and channel selectivity of microbial MIP channels

The transport properties of MIP channels can be studied in a number of ways including the use of the heterologous *Xenopus laevis* oocyte system or the osmotic swelling of whole cells, spheroplasts or membrane vesicles to determine water or solute transport (Hohmann *et al.*, 2000). Most available data from these types of measurements indicate that transport is very rapid, has low activation energy and can be sensitive to mercury compounds, such as $HgCl_2$, if a cysteine residue lines the pore.

The transport properties of microbial MIP channels have been well characterized in just a few cases and in most instances functional studies have been performed in *Xenopus laevis* oocytes. It is reasonable to assume that in such a heterologous system, a different lipid environment will affect transport function or specificity (Truniger and Boos, 1993) and this could explain conflicting data obtained in some cases. The most informative transport coefficients such as osmotic water permeability (hydraulic conductivity), solute permeability and reflection coefficient have been determined for only a limited number of channels.

For example, the *Escherichia coli* glycerol channel, GlpF, transports polyols, glyceraldehyde, glycine and urea (Heller *et al.*, 1980) but little or no water (Maurel *et al.*, 1994; Calamita *et al.*, 1995). Consistent with a pore-type mechanism, glycerol transport via GlpF has a low activation energy (Ea = 4.5 kcal/mol) and is non-saturable (Maurel *et al.*, 1994). GlpF-mediated glycerol uptake is also sensitive to the membrane lipid composition (Truniger and Boos, 1993). Similar transport properties are expected for other microbial glycerol channels since the molecular architecture of bacterial glycerol uptake systems is apparently highly conserved. With regard to microbial aquaporins, expression of the prokaryotic aquaporin gene, *Escherichia coli aqpZ*, in *Xenopus laevis* oocytes results in a 15-fold increase in osmotic water permeability, but negligible solute transport. The observed water transport has a low activation energy (Ea = 3.8 kcal/mol) and is insensitive to $HgCl_2$ (Calamita *et al.*, 1995).

The water permeability of the putative *Saccharomyces cerevisiae* aquaporin encoded by *AQY1*, from both wild type and laboratory strains, has been evaluated in *Xenopus laevis* oocytes. Only oocytes expressing *AQY1* from the strain Σ1278, which is closely related to industrial isolates, exhibit an increase in water permeability (Bonhivers *et al.*, 1998). Transport assays using yeast membranes and yeast vesicles also lead to similar observations (Coury *et al.*, 1999). In contrast, data is not yet available for *AQY2*, since it could not be functionally expressed in oocytes (Laizé *et al.*, 1999; Laizé *et al.*, 2000).

The transport characteristics of the glycerol exporter, Fps1p, are similar to those of *Escherichia coli* GlpF although Fps1p is known to be a regulated channel (Tamás *et al.*, 1999), which we discuss later. In contrast to most other MIP channels, the transport properties of Fps1p can be studied homologously in *Saccharomyces cerevisiae*. At least three groups have also tried to functionally express Fps1p in oocytes, but this has been unsuccessful to date. Fps1p transports glycerol, erythritol and xylitol and probably other polyols (Luyten *et al.*, 1995; Sutherland *et al.*, 1997 and Karlgren *et al.*, 2000). Sorbitol and mannitol seem to be transported only with very low efficiency if at all (Karlgren and Hohmann, unpublished data) and water does not seem to be transported (Coury *et al.*, 1999). Like GlpF (Sanders *et al.*, 1997), Fps1p also seems to transport antimonite (Wysocki, unpublished data), probably because the hydrated form of this ion resembles a polyol. These observations are consistent with poor substrate specificity, which is probably determined by pore size and interactions between the polyol and residues lining the channel.

Although the open reading frame *YFL054* is predicted to encode a glycerol facilitator, it has not yet been possible to determine its substrate specificity.

The mechanisms dictating the commonly observed water/solute channel selectivity described above remain poorly understood. The amino acid content and length of the predicted loop region of MIP channels may play a role in determining specificity. Froger and colleagues have proposed that the molecular basis of substrate selectivity is determined by key amino acids (Froger *et al.*, 1998) as the substitution of two amino acids can switch the selectivity of an insect aquaporin to a glycerol channel in the *Xenopus* oocyte system (Lagree *et al.*, 1999). It is possible that these residues influence channel pore size, since this factor is also likely to be key to a complete understanding of selectivity. For example, it appears from structural data at 4.5Å that the pore of human AQP1 is large enough to allow the passage of water, but too small for solutes such as glycerol (Mitsuoka *et al.*, 1999). However, size alone cannot explain the fact that microbial glycerol facilitators such *Saccharomyces cerevisiae* Fps1p and *Escherichia coli* GlpF transport glycerol and not water. More studies on microorganisms are thus needed to clearly elucidate the factors governing channel selectivity and determine its significance in *vivo*.

IV. From primary to quaternary structure in microbial MIPs

A. Structure- function analysis of microbial MIP channels

From an analysis of their amino acid sequences, all MIP channels are predicted to have six transmembrane domains, and to share highly conserved residues. This is no less true of the microbial branch of the family. As for all MIPs, the most notable of the conserved residues are present in the presumed channel-forming loops (Heymann *et al.*, 1998), B and E, and comprise the family's signature sequences, Ser–Gly–X–His–X–Asn–Pro–Ala–Val–Thr and Asn–Pro–Ala–Arg, respectively, the so-called 'NPA boxes' being underlined. However, striking differences can be observed between the sequences of microbial MIPs both within these signature motifs and at the termini. This is well illustrated in the case of the glycerol facilitator, Fps1p, from *Saccharomyces cerevisiae*. Although Fps1p is clearly related to bacterial glycerol facilitators such as GlpF from *Escherichia coli* (31% identity within the core of six transmembrane domains), it is – so far – unique in the MIP family for a number of reasons (Hohmann *et al.*, 2000).

For example, neither of the family's signature NPA boxes is fully preserved, being Asn-Pro-Ser (NPS) and Asn-Leu-Ala (NLA), respectively. In fact, only four additional microbial MIP sequences contain motifs other than NPA, and it is apparent that NPA is preserved in loop B but not in loop E in most of these cases. In *Enterococcus faecalis*, the presumed glycerol facilitator (gef 6176) contains an NQA motif in loop E, in *Chlorobium tepidum*, the putative aquaporin (gct 5) has an NPV motif (Hohmann *et al.*, 2000) and the *Botrytis cinerea* putative glycerol facilitator contains an NPS. The only deviation from NPA in loop B of a microbial MIP occurs in the *Salmonella typhimurium* (contig 1308) which has an NLA motif. Unfortunately, these MIPs are incompletely characterized and thus their transport characteristics cannot be used to aid our understanding of the role of these atypical features. This is of particular relevance to a general understanding of MIP channels since their generic NPA motifs are believed to be integral to the formation of a continuous solute channel, the so-called 'hourglass' (Jung et al., 1994). One possible functional consequence of these atypical motifs is that they influence MIP channel transport properties, resulting in transport specialization. Recently, it has been suggested that loop B in particular may be involved in the determination of transport direction following a comparison of the glycerol transport properties of Fps1p and *Escherichia coli* GlpF. Physiologically, these proteins are a glycerol exporter and an uptake facilitator, respectively. Comparison of mutants where the NPA motifs were 'restored' in Fps1p with those where GlpF was made more Fps1p-like by mutating NPA to NPS and/or NLA, indicated that the NPS of loop B may be important in influencing Fps1p's export characteristics (Bill et al., 2000).

In addition to a deviation from the family's signature motifs in the channel-forming loops, Fps1p further distinguishes itself from most other microbial MIPs by having long amino- and carboxy-terminal hydrophilic extensions. This results in a protein of 669 amino acids, compared with 281 amino acids for GlpF and other typical family members. As mentioned above, the putative second *Saccharomyces cerevisiae* glycerol facilitator, Yfl054p, as well as the similar *Schizosaccharomyces pombe* protein, also have long amino-terminal extensions unrelated to that of Fps1p.

Standard secondary structure predictions suggest that MIPs are rich in α-helical segments. This is yet to be confirmed experimentally since to date, low-resolution structural data (at 4.5Å) are only available for human AQP1 (Mitsuoka *et al.*, 1999). Even though the most divergent members of the MIP family are less than 20% identical (Park and Saier, 1996), it is expected that the gross structural features apparent in AQP1 will also be present in other

water and glycerol channels. For example, AQP1 is functionally homotetrameric; this quaternary structure is thus anticipated for all other MIPs. A study of the crystal organization of MIP and *Escherichia coli* AqpZ and GlpF confirms the close overall structural relationship between MIP channels (Hasler *et al.*, 1998; Ringler *et al.*, 1999) although it has been proposed recently that glycerol channels could be functionally monomeric (Lagree *et al.*, 1999).

V. Physiological roles

As we have already mentioned, the physiological role of microbial MIP channels has been mostly studied in the bacteria *E. coli, P. aeruginosa, T. flavus,* and *S. typhimurium,* in the yeast *S. cerevisiae,* and in the protozoan *D. discoideum*. In *E. coli, S. cerevisiae* and *D. discoideum,* the genes for microbial MIP channels have been deleted and the phenotype of the mutant strains compared to wild type strains. Studies on the expression of genes encoding MIP channels as well as on the location of bacterial genes in operons have provided additional information on their physiological roles. In general, the function of microbial MIP channels is in osmoregulation, metabolism — via the uptake of glycerol or related compounds as sources of carbon and energy— or disposal of metabolic end products.

A. Glycerol facilitators in the uptake of substrates

Although glycerol and other uncharged small molecules are able to move across microbial membranes by simple diffusion, there is currently sufficient evidence to suggest that MIP channel proteins facilitate the uptake of these solutes. In bacteria, the organization of genes in operons, which are co-expressed and hence co-regulated, usually points to their function in a common pathway. Hence, knowledge of the function and regulation of a single gene in a bacterial operon allows the function of other genes in the same operon to be predicted. Such a relationship is not observed in eukaryotic microorganisms.

Genes encoding glycerol facilitators are commonly part of the *glp* operon in both Gram-positive and Gram-negative bacteria (Table 1, Fig. 2) The *glp* operon comprises *glpF*, encoding the facilitator, *glpK* encoding a glycerol kinase, *glpD* encoding a glycerol-3-phosphate dehydrogenase and two genes, *glpX* and *glpR*, which presumably encode regulators of the operon (Schweizer *et al.*, 1997).

The glycerol kinase appears to be closely associated with the facilitator resulting in glycerol phosphorylation during uptake which prevents re-export of glycerol (Voegele *et al.*, 1993). Exceptions to this operon organization have been found in a number of bacteria such as *Lactococcus lactis* (P22094), *Streptococcus pneumoniae* (SP42), *Corynebacterium acetobutylicum* (AE001437) and *Haemophilus influenzae* (U32782) where putative glycerol facilitator genes do not form part of the glycerol operon, suggesting that these MIP family proteins might also have functions other than glycerol uptake (Park and Saier, 1996).

Escherichia coli mutants lacking *glpF* grow poorly on low glycerol concentrations presumably due to insufficient glycerol permeating the cell (Voegele *et al.*, 1993). Hence it has been proposed that the facilitator is required for efficient uptake of glycerol, especially at low concentrations. The *glpF* mutant also shows altered kinetics for glycerol phosphorylation and the fact that free glycerol is undetectable in wild type cells utilizing glycerol supports the conclusion that transport and phosphorylation of glycerol are closely coupled (Voegele *et al.*, 1993). Substantial glycerol transport by passive diffusion through the lipid bilayer is also apparent since a *glpF* mutant does not show a glycerol-negative phenotype at high glycerol concentrations (Voegele *et al.*, 1993). Recently, a gene encoding a glycerol facilitator in *Pseudomonas aeruginosa* has been cloned and a chromosomal Δ*glpFK* mutant isolated (Schweizer *et al.*, 1997). This mutant, which lacks both the facilitator and the glycerol kinase, does not grow on medium containing glycerol as the sole carbon source and does not transport glycerol.

The *Salmonella typhimurium pdu* operon is required for the catabolism of 1,2-propanediol. The *pdu* operon (Fig. 2) is closely linked to the *cob* operon, which controls the synthesis of adenosyl-cobalamin (vitamin B12), a cofactor required for the catabolism of propanediol. The region between the *pdu* and *cob* operons encodes two proteins, PduF, the putative propanediol transporter, and PocR, a regulatory protein that mediates the induction of the *pdu/cob* operon by propanediol (Chen *et al.*, 1994; Chen *et al.*, 1995). The transport characteristics of PduF have not been determined experimentally but it is reasonable to assume that this protein is involved in the uptake of 1,2-propanediol, a compound closely related to glycerol. It is not yet known whether PduF can transport glycerol in addition to 1,2-propanediol or, indeed, whether other GlpFs can transport 1,2-propanediol. However, in addition to PduF, *Salmonella typhimurium* has a gene encoding a glycerol facilitator that forms part of a *glpFK* operon. This suggests, in fact, that the organism possesses two facilitators, one for catabolism of propanediol and another for glycerol uptake.

Whether members of the MIP channel protein family from eukaryotic microorganisms play a role in the uptake of solutes such as glycerol is less clear. Extensive analysis of the glycerol transport characteristics of yeast wild type and *fps1Δ* mutants has demonstrated that this protein can transport glycerol in both directions (Luyten *et al.*, 1995; Lages and Lucas, 1997; Sutherland *et al.*, 1997; Tamás *et al.*, 1999). However, mutants lacking Fps1p, Yfl054p or the double mutant lacking both putative yeast glycerol facilitators, do not show a defect in glycerol utilization (Tamás et al., 1999; Tamás and Hohmann, unpublished data). Since the yeast plasma membrane seems to be relatively impermeable to glycerol (Luyten *et al.*, 1995; Tamás *et al.*, 1999), the existence of uptake proteins involved in glycerol catabolism has been suggested. In addition, it has been demonstrated that yeast cells have at least one system for the active uptake of glycerol (Van Zyl *et al.*, 1990; Lages and Lucas, 1997; Lages *et al.*, 1999). It has also been shown that yeast mutants unable to produce any glycerol themselves, and which therefore do not grow on medium containing NaCl (see below), can be rescued by as little as 5mM glycerol in the growth medium, indicative of an active uptake system mediating accumulation of glycerol against a concentration gradient (Holst *et al.*. 2000). This effect has been used to identify a yeast gene, *GUP1*, which is required for rescue of the glycerol-negative mutant by low concentrations of glycerol. Gup1p is a membrane protein that either takes up glycerol or at least controls glycerol uptake, a process that appears to be closely coupled to glycerol phosphorylation. This phosphorylation is catalyzed by the glycerol kinase Gut1p. Gup1p is not a MIP channel (Holst *et al.*, 2000).

Surprisingly, deletion of the genes encoding the glycerol facilitators in *Escherichia coli* and *Saccharomyces cerevisiae* results in diminished passive diffusion of glycerol and altered cellular lipid composition (Truniger and Boos, 1993; Sutherland *et al.*, 1997). The reason for this observation is not clear, but it is possible that glycerol uptake and phosphorylation via the facilitator/kinase system provides glycerol-3-phosphate for phospholipid metabolism. There are several pathways that lead to the synthesis of glycerophospholipids, and the balance between different precursors seems to be critical for phospholipid biosynthesis (Daum *et al.*, 1998). In this context it is of interest that the regulation of expression of the yeast glycerol kinase gene, *GUT1*, was recently reported to be not only controlled by glucose repression and glycerol induction but also by the same regulators that control genes encoding enzymes involved in phospholipid metabolism (Grauslund *et al.*, 1999). In conclusion it appears as there may be a connection between glycerol transport and phosphorylation on the one hand and cellular phospholipid metabolism on the other.

VI. Microbial MIP channels in osmoregulation

A. Microbial aquaporins

Most available information on the physiological roles of microbial aquaporins is derived from studies on *E. coli* AqpZ, *S. cerevisiae* Aqy1p, and *D. discoideum* WacA. AqpZ has been shown to have a direct role in the way that *Escherichia coli* adjusts cell turgor within the range needed for growth and survival (Calamita *et al.*, 1998). This function has been demonstrated by comparing *Escherichia coli* cells carrying a null mutation in the *aqpZ* gene with their parental wild-type strain. Disruption of *aqpZ* is not lethal, but the viability of cells in which *aqpZ* has been knocked out is strikingly reduced when they are grown at low osmolarity. On the contrary, no significant changes in cell viability are observed when these cells are grown in high osmolarity medium. The reduced growth observed in hypo-osmotic conditions can be rescued by transforming the knockout strain with a plasmid bearing a functional *aqpZ* gene. Overall, these data are consistent with the results of regulatory studies which show a marked increase of the *aqpZ* transcription rate when *Escherichia coli* is grown in low osmolarity medium and a reduced expression in high osmolarity medium (Calamita *et al.*, 1998). Although questions on the physiological necessity of a water channel during prolonged hypo-osmotic stress remain to be answered, this finding clearly indicates involvement of AqpZ in prokaryotic osmoadaptation. In fact, AqpZ seems to be required both during the long-term osmoregulatory response triggered by hypo-osmotic stress and the short-term responses that occur suddenly after changes in the extra-cellular osmolarity. Although further investigation is required to elucidate the precise role of AqpZ, especially during the osmotic response to hypo-osmotic stress, it is likely that involvement in osmoregulation is a general feature of microbial aquaporins (Booth and Louis, 1999).

Escherichia coli wild type and *aqpZ* mutant strains have been used in cryoelectron microscopy studies to demonstrate, *in vivo*, the ability of AqpZ to mediate rapid outward- and inward-directed water fluxes triggered by sudden up- and down-shifts of the extracellular osmolarity, respectively (Delamarche *et al.*, 1999). Besides demonstrating the functional expression of AqpZ in *Escherichia coli*, these studies indicate that AqpZ transports water in both directions, a property that has been also reported for many mammalian aquaporins (Meinild *et al.*, 1998). A role for AqpZ in mediating the bulk water uptake needed for cell expansion during rapid growth is suggested both by its maximal expression at the mid-logarithmic phase of growth and the reduced viability characterizing the *Escherichia coli aqpZ* mutant grown at 39°C, a temperature where the growth rate is highest.

However, this function apparently contrasts with the assumption that sufficient water for cell division may be absorbed by simple diffusion across the cytoplasmic membrane during its 20–30 minute generation time (Haines, 1994). Additional studies are therefore required to better elucidate the physiological relevance of AqpZ expression during the exponential growth phase of *Escherichia coli*.

Interestingly, an AqpZ-like protein seems to be necessary for the expression of certain surface antigens (Kopecko *et al.*, 1980), which in turn appear to be one of the requirements for pathogenic bacteria to invade epithelial cells. In fact, it has been observed that the *Shigella sonnei* ORF10, an open reading frame with striking sequence similarity to the *aqpZ* coding region, is part of a gene cluster composed of ten contiguous ORFs located in a plasmid (pHH201) encoding the form I antigen. It has been found that deletions of ORF10 and/or any of the other nine cluster ORFs eliminate form I antigen expression of *Shigella sonnei* (Houng and Venkatesan, 1998). An identical gene cluster including an *aqpZ*-like coding region (ORF10p) is also found in the pathogenic species *Plesiomonas shigelloides* (serotype 017) where in association with other genes it leads to the expression of a cell surface O-antigen (Chida *et al.*, 2000). A possible role for AqpZ in the virulence mechanisms of pathogenic bacteria is an exceedingly appealing hypothesis, which deserves investigation. We note, however, that several pathogenic organisms lack MIP channels alltogether and hence the importance of aquaporins in virulence, if any, must be restricted.

Although a role in osmoregulation seems likely for the *Saccharomyces cerevisiae* aquaporin Aqy1p, surprisingly, the related null mutant yeast strain tolerates osmotic changes better than the wild-type strain under laboratory conditions (Bonhivers *et al.*, 1998). In these experiments wild type and mutant cells were co-cultivated and repeatedly osmotically shocked. The wild type cells did not survive this treatment and were consequently depleted in the culture, whereas the *aqy1* mutant cells survived. This evidence together with the observation that the *AQY1* gene product appears to be non-functional in many laboratory strains (Bonhivers *et al.*, 1998; Laizé *et al.*, 2000) led to the suggestion that functional aquaporins might have been lost in strains maintained under laboratory growth conditions (Bonhivers *et al.*, 1998). However, the interpretation of these observations is complicated by the fact that the *AQY1* gene is very poorly expressed during vegetative growth, conditions under which the above-mentioned phenotype was determined.

Expression of *AQY1* is, however, strongly stimulated when diploid yeast cells enter sporulation and hence *AQY1* is clearly a developmentally-regulated yeast gene, similar to *Dictyostelium discoideum wacA* (see below; Chu et al., 1998; Laizé and Hohmann, unpublished data). Key questions currently under study are the precise localization of Aqy1p and whether the protein has any role during sporulation, spore maturation or spore germination.

A potential role in mediating the extrusion of water during prespore cell encapsulation has been suggested for the *Dictyostelium discoideum* WacA aquaporin (Flick *et al.*, 1997). Although the *wacA* gene is expressed only in prespore cells, it has been observed that disruption of the gene does not lead to any apparent alterations in prespore cells or their ability to germinate or respond to osmotic stresses. However, the lack of an apparent phenotype could possibly be explained by the presence of unknown alternative aquaporins or the fact that the *Dictyostelium wacA* mutant was not exposed to the selective challenges of its natural habitat, the soil. According to Cotter (Cotter and Raper, 1968), germinating spores of *Dictyostelium* take up water very rapidly unlike prespore cells, which extrude water during encapsulation. Such rapid water fluxes imply the involvement of aquaporins. A similar argument may be used to support the involvement of Aqy1p in yeast sporulation/spore germination. Further research is required to address this intriguing question.

As outlined above, yeast *AQY2* seems to be mutated in most yeast strains (Laizé *et al.*, 2000). This is a rather unusual scenario and consequently one might speculate that there is some selective pressure against maintaining this gene. The *AQY2* ORF is complete in strain Σ1278, which consequently can express both *AQY1* and *AQY2* (Laizé *et al.*, 2000). In this strain *AQY2* is expressed during vegetative growth and expression is stimulated after a hyperosmotic shock. This observation suggests that Aqy2p might be involved in the uptake of water during the recovery of cells from osmotic upshock. However, at present the subcellular localization of Aqy2p has not been determined and hence the protein could perhaps be located in an intracellular compartment controlling water fluxes within the cell. In fact, the two yeast aquaporins are most similar to the tonoplast aquaporins from plants and hence Aqy2p could, in fact, be a vacuolar protein. So far, attempts to assign a phenotype to cells deleted for the *AQY2* gene have been unsuccessful (Laizé and Hohmann, unpublished data).

The unresolved issues discussed in the preceding paragraphs clearly illustrate that no well-defined physiological role can yet be assigned to any microbial aquaporin. The identification and characterization of additional aquaporins in genetically tractable systems will provide further insight into the role of water transport in microbial water relations and in osmoregulation in general. Indeed, such studies certainly deserve a more widespread interest from microbiologists.

B. The yeast osmolyte system: control of glycerol metabolism

A common strategy in osmoadaptation is the accumulation of compatible solutes (Yancey *et al.*, 1982). A range of quite different compounds are employed as compatible solutes by microorganisms, such as polyols (glycerol, D-arabitol, D-mannitol and *meso*-erythritol) in fungi (Spencer and Spencer, 1978; Yancey *et al.*, 1982) and potassium ions, trehalose and amino acids or their derivatives in Bacteria and Archea (Measures, 1975; da Costa *et al.*, 1998). The yeast *Saccharomyces cerevisiae* employs glycerol for this purpose (Brown, 1978; Brown and Edgley, 1980; Blomberg and Adler, 1992). This is surprising, because glycerol is known to diffuse through lipid bilayers and hence one might expect that yeast cells could lose the glycerol they produce under hyperosmotic stress. However, this does not appear to be the case to any significant extent since the yeast plasma membrane is relatively impermeable to glycerol (Luyten *et al.*, 1995; Sutherland *et al.*, 1997; Tamás *et al.*, 1999). In fact, there is evidence that yeast cells can actively control the permeability of their plasma membrane to glycerol under osmotic stress, perhaps by altering the lipid composition (Sutherland *et al.*, 1997).

Glycerol is produced in two steps from the glycolytic intermediate dihydroxy acetone phosphate (Fig. 3; Blomberg and Adler, 1992) catalyzed by the enzymes glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphatase (Gpp), respectively. Both enzymes have two isoforms whose expression is differentially regulated (Norbeck *et al.*, 1996; Ansell *et al.*, 1997). The expression of *GPD1* and *GPP2* is strongly induced by hyperosmotic stress (Albertyn *et al.*, 1994; Norbeck *et al.*, 1996) and hence these two proteins appear to account for most of glycerol production capacity under osmotic stress. The expression of *GPP1*, which is more strongly expressed under normal growth conditions than *GPP2*, is also somewhat induced under osmotic stress (Rep *et al.*, 2000).

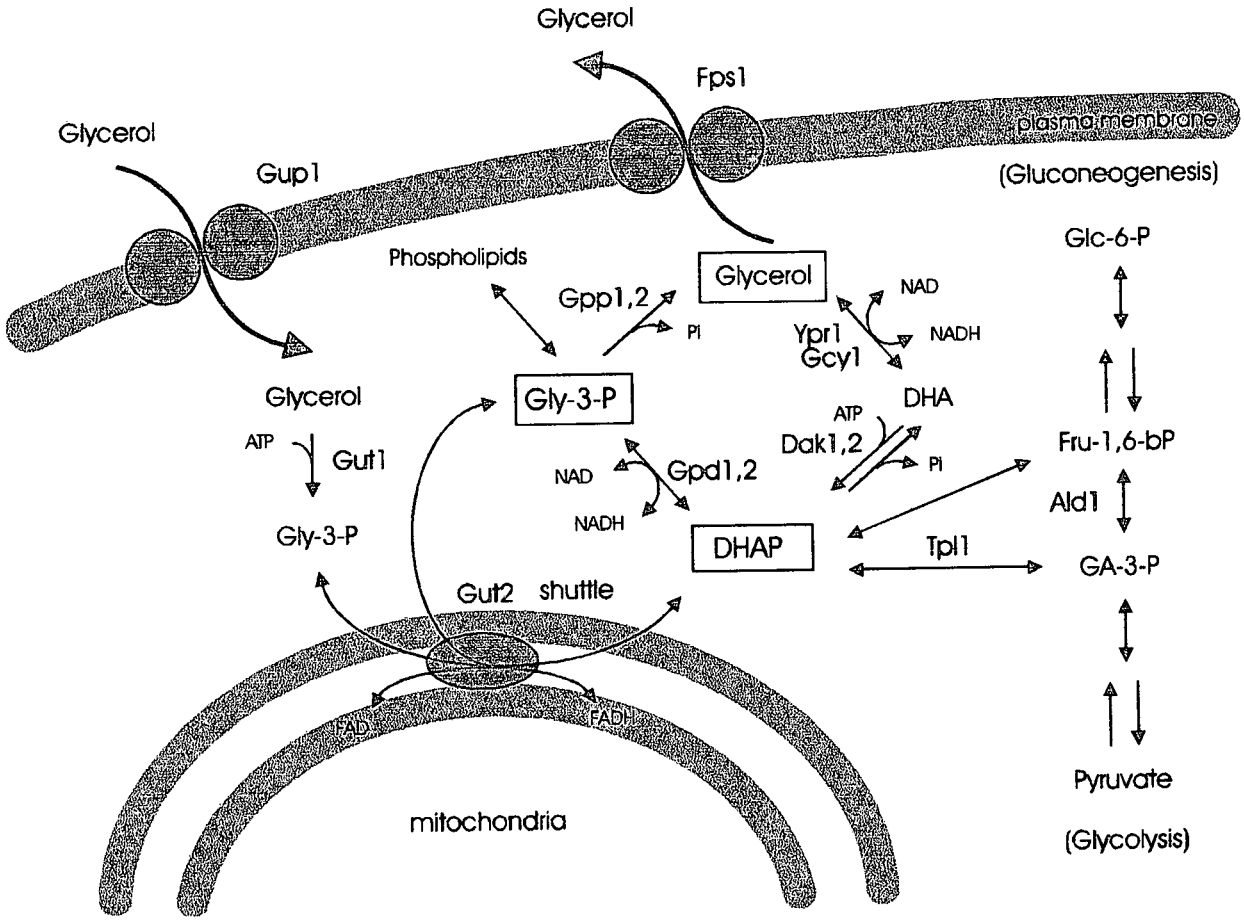

Figure. 3. Yeast glycerol metabolism

Schematic overview of yeast glycerol metabolism. Glycerol catabolism starts with uptake, presumably through Gup1p. Glycerol is phosphorylated to glycerol-3-phosphate, Gly-3-P, by a glycerol kinase, Gut1p, and oxidized by an FAD-dependent, mitochondrial glycerol-3-phosphate dehydrogenase to dihydroxyacetonephosphate, DHAP, a glycolytic intermediate. For glycerol production DHAP is converted to Gly -3-P by an NADH-dependent, cytosolic glycerol-3-phosphate dehydrogenase, Gpd1p or Gpd2p, and subsequently dephosphorylated by glycerol-3-phosphatase, Gpp1p or Gpp2p, to glycerol. Glycerol is either accumulated within the cell or exported through the osmoregulated glycerol facilitator Fps1p, a MIP channel. DHAP and glycerol can also be interconverted via dihydroxyacetone, DHA, but the relevance of this pathway in *Saccharomyces cerevisiae* is unclear. The actions of the FAD-dependent Gut1p and the NADH-dependent Gpd1p/Gpd2p provide a shuttle for electrons into the mitochondrial electron transport chain (adapted from Hohmann, 1997).

GPD2 and *GPP1* expression are stimulated under anaerobic conditions (Ansell *et al.*, 1997) since in the absence of oxygen, glycerol production is essential for the reoxidation of NADH to NAD, which is normally performed by the respiratory chain (Van Dijken and Scheffers, 1986; Hohmann, 1997).

The mechanisms that control the induction of *GPD1* and *GPP2* under osmotic stress are being studied extensively. The High Osmolarity Glycerol (HOG) response pathway plays a central, though not exclusive, role in the induction of *GPD1* and *GPP2* (Rep *et al.*, 1999a; Rep *et al.*, 1999b; Rep *et al.*, 2000). This pathway is a prototypical MAP (Mitogen Activated Protein) kinase cascade (Fig. 4), as found in all eukaryotes. Recent transcriptome analysis indicates that this pathway controls the expression of more than 100 yeast genes upon osmotic shock (Rep *et al.*, 2000) and that it mediates its effects via different transcription factors such as Hot1p, Msn1p, Msn2p, Msn4p and Sko1p. Of these, Hot1p and Msn1p are involved in controlling the glycerol biosynthesis genes (Rep *et al.*, 1999b).

Upon osmotic shock yeast cells rapidly stimulate the production of glycerol and substantial levels of glycerol are built up in the cell within a few hours. Concentrations of up to 1M of glycerol have been reported (Blomberg and Adler, 1992). In their natural environment, yeast cells are frequently exposed to high osmolarity, especially to high sugar concentrations. Equally common is exposure to hypo-osmotic shock, for instance during rainfall. Under these conditions the cell has to rapidly dispose of accumulated glycerol in order to diminish turgor pressure. Hence, yeast has developed an efficient system to export the majority of its accumulated glycerol within a few minutes through the MIP channel, Fps1p.

C. The Fps1p solute exporter

The *FPS1* gene was originally isolated as a multicopy suppressor of a growth defect on fermentable sugars, such as glucose, of a mutant with defective feedback control of glycolysis (Van Aelst *et al.*, 1991). Subsequently it was shown that this growth defect could be partially corrected by overproduction of glycerol (Luyten *et al.*, 1995). As outlined above, Fps1p is an unusual MIP channel (Fig. 5). Its 'NPA' motifs are not fully conserved, being NPS and NLA in loops B and E, respectively and its A loop being unusually long. In addition, Fps1p is 669 amino acids long due to amino- and carboxy-terminal cytosolic extensions. Apart from two other fungal proteins, only the *Drosophila* BIB (Big Brain) protein has such long extensions. However, Fps1p's extensions do not show any sequence similarity to other proteins.

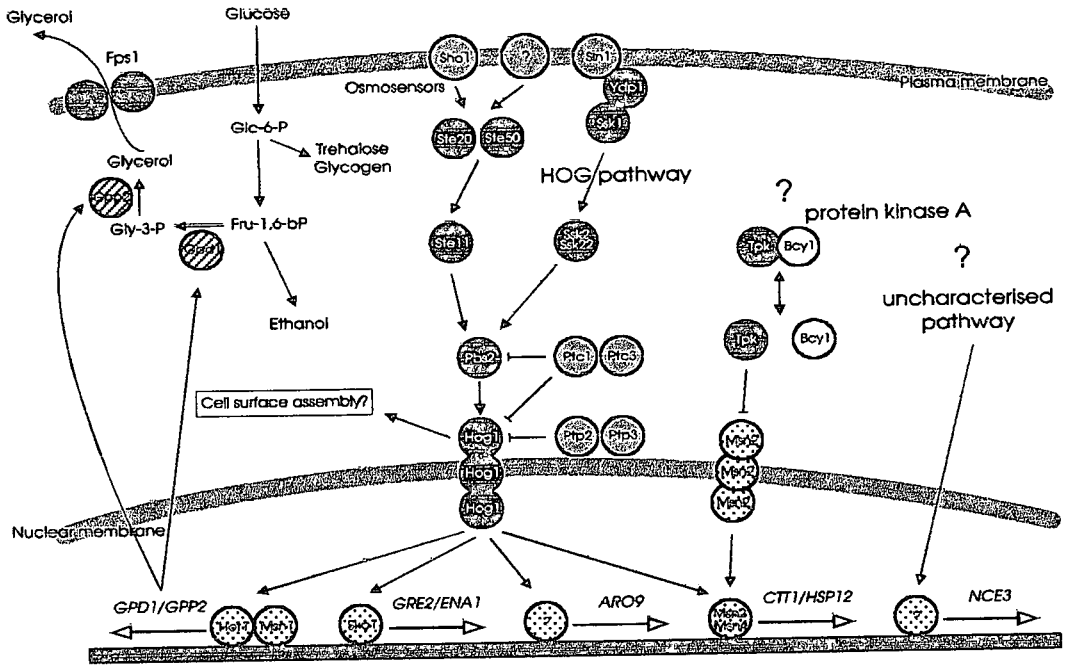

Figure 4. Signaling upon osmotic shock in yeast

Schematic overview of signaling upon an osmotic shock in *Saccharomyces cerevisiae*. Central to the response is the High Osmolarity Glycerol (HOG) pathway. Osmotic shock is sensed by at least two putative transmembrane osmosensors; one of those, Sln1p-Ypd1p-Ssk1p forms a phosphorelay system similar to bacterial two-component systems. The signal is then transmitted through a MAP kinase cascade eventually to Hog1p, which is translocated to the nucleus. Hog1p controls and/or interacts with different transcription factors to stimulate expression of more than 100 genes. Examples are the glycerol biosynthesis genes *GPD1* and *GPP1*, the gene for the sodium pump *ENA1*, the gene *GRE2* whose product may be involved in detoxification of oxygen radicals, *ARO9*, which encodes in enzyme in amino acid metabolism, *CTT1*, which encodes a catalase and *HSP12*, which encodes a heat shock protein of unknown function. *NCE3* encodes carbonic anhydrase and an unknown signaling pathway mediates its induction by osmotic stress. The transcription factors Msn2p and Msn4p mediate a general stress response and their subcellular localization is controlled by protein kinase A. The scheme on the left hand side depicts glycerol metabolism. Grey: osmosensors; black with white text: protein kinases; gray fountain fill: protein phosphatases; shaded: enzymes; dotted fill: transcription factors.

Fps1p has been demonstrated by direct transport assays with radiolabelled glycerol to mediate transport of glycerol into and out of the yeast cell (Luyten *et al.*, 1995; Sutherland *et al.*, 1997; Tamás *et al.*, 1999). The glycerol facilitator from *Escherichia coli*, GlpF, when expressed in yeast, can replace Fps1p's glycerol transport function lending further support to the role of Fps1p as a glycerol transporter (Luyten *et al.*, 1995; Sutherland *et al.*, 1997; Tamás *et al.*, 1999). The phenotype associated with deletion of Fps1p clearly classifies this protein as a glycerol exporter, namely the inability to grow under anaerobic conditions and its sensitivity to hypo-osmotic shock (Tamás *et al.*, 1999). As indicated above, yeast cells produce glycerol when grown in the absence of oxygen for redox balancing (Ansell *et al.*, 1997). In cells lacking Fps1p, glycerol accumulates inside the cell and inhibits growth, presumably because it leads to an osmotic imbalance (Tamás *et al.*, 1999). Under these conditions glycerol can be regarded as a metabolic end or waste product and hence Fps1p serves as a waste product exporter.

When yeast cells are grown in high osmolarity medium and then shifted to low osmolarity, they dispose of 80% of their accumulated glycerol within 5 minutes (Luyten *et al.*, 1995). In contrast, cells lacking Fps1p require 60 minutes to achieve the same low glycerol level and survive a hypo-osmotic shock in a 100-fold lower proportion than wild type cells. Those cells that survive resume growth more slowly (Luyten *et al.*, 1995; Tamás *et al.*, 1999) and, moreover, if the *fps1Δ* mutation is combined with a mutation that weakens the cell wall, a hypo-osmotic shock is lethal (Tamás *et al.*, 1999). Since cells lacking Fps1p grow in low osmolarity medium as well as the wild type, the *fps1Δ* mutant is specifically sensitive to a hypo-osmotic shock and thus far is the only yeast mutant known to display such a phenotype (Tamás et al., 1999; Ferreira and Hohmann, unpublished observations).

The role of Fps1p in osmoregulation and the control of cellular glycerol content are supported by further observations. Mutants lacking Fps1p exhibit diminished signaling through the HOG pathway, apparently because they can accumulate glycerol after a hyperosmotic shock faster than wild type cells (Tao et al., 1999; Tamás, Rep and Hohmann unpublished observations). Strikingly, *fps1Δ* mutant cells show a defect in cell fusion during the mating process of haploid yeast cells; this defect is apparently also associated with altered osmoregulation because it can be suppressed by deletion of the *GPD1* gene and hence by reduction of glycerol production (Philips and Herskowitz, 1997). Cell fusion requires local cell wall degradation and it appears that yeast cells have to relieve osmotic pressure at this point in order to prevent cell bursting.

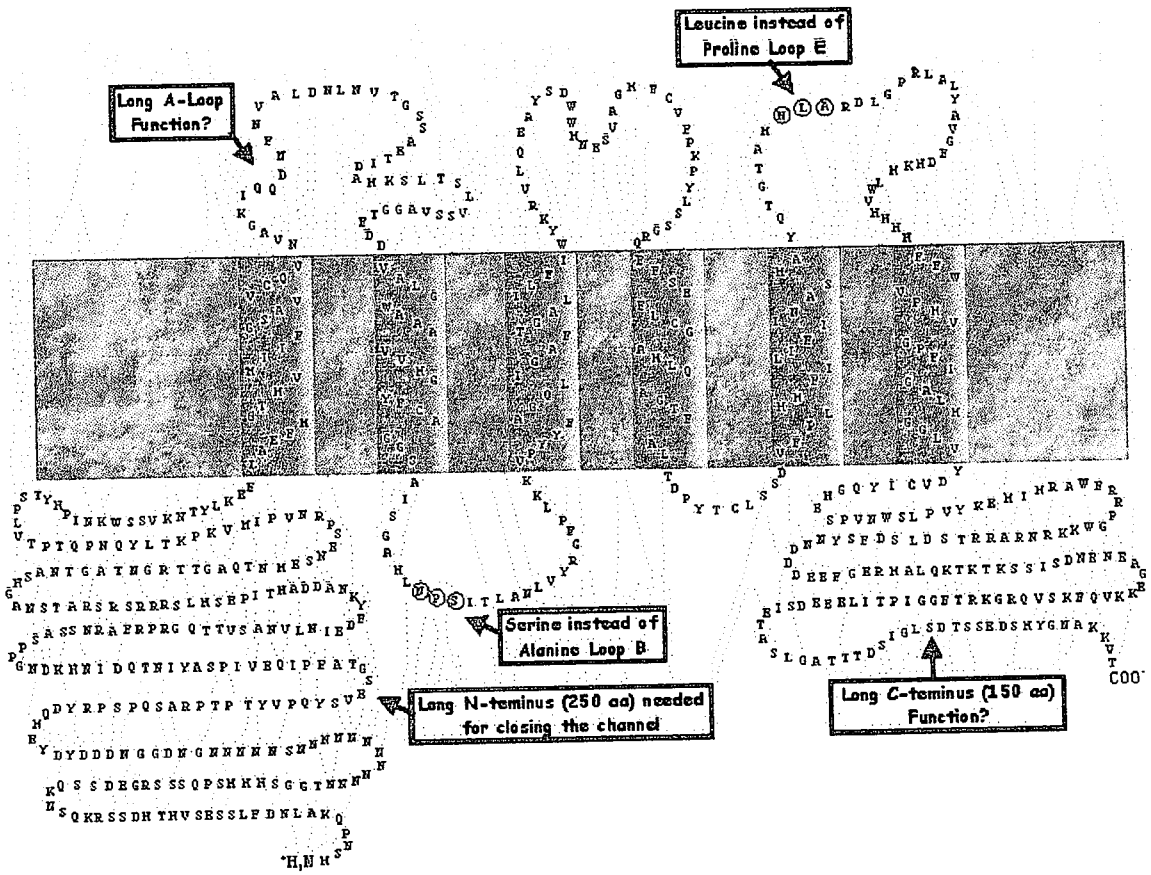

Figure 5. Yeast Fps1p topology

Fps1p differs from more typical members of the MIP family, such as GlpF, in a number of ways. For example the family's signature NPA motifs are replaced by NPS and NLA in Fps1p, as indicated. The letters A to E denote Fps1p's loops of which B and E are believed to be involved in the formation of the glycerol channel by dipping into the membrane to form an 'hourglass' (Jung *et al.*, 1994).

The observation that the presence of 1M sorbitol in the medium also corrects the mating defect of *fps1Δ* mutants supports the notion that this phenotype is due to a problem with osmoregulation and cell bursting (Philips and Herskowitz, 1997). This observation also illustrates that osmotic phenomena play a role in very different cellular processes not obviously related to osmoadaptation at first sight. Finally, although there is substantial biophysical evidence for the existence of osmolyte export systems in other organisms, especially in mammalian cells (Kwon and Handler, 1995), Fps1p is to date the only eukaryotic solute exporter characterized at the molecular level.

VII. Control of the function of microbial MIP channels

MIP channel function can be controlled at different levels, as also discussed in other chapters in this issue. For instance, in plant cells the expression of genes encoding aquaporins has been demonstrated to be controlled by stress (Bohnert *et al.*, 1995; Yamada *et al.*, 1995; Balk and de Boer, 1999) as well as by developmental cues (Gao *et al.*, 1999). AQP2 and 5 are paradigms of mammalian aquaporins whose localization to their target membrane is controlled by hormonal stimuli (Deen *et al.*, 1995; Kamsteeg *et al.*, 1999). In addition, gating mechanisms could potentially control the function of MIP channels within the membrane as has recently been suggested for the control of mammalian AQP6 (Yasui *et al.*, 1999) and plant PM28A (Johansson *et al.*, 1998; Kjellbom *et al.*, 1999). For microbial MIP channels, control of gene expression as well as gating has been demonstrated as a means of regulation.

Control of the expression of microbial MIP genes has already been discussed along with the analysis of their physiological roles. Indeed, their expression pattern often serves as a guide towards their physiological role, as illustrated by the co-regulation of bacterial glycerol facilitators with glycerol kinases in glycerol metabolism and the stimulated expression of *Escherchia coli aqpZ* under hypo-osmotic conditions (Calamita *et al.*, 1998). In addition we have mentioned the control of yeast *AQY2* by osmotic shock and that of yeast *AQY1* and *Dictyostelium wacA* by sporulation (Flick *et al.*, 1997; Chu *et al.*, 1998) although the involvement of these proteins in osmoadaptation and development, respectively, has not yet been demonstrated. In the following section we focus on the control of protein function, which we have thus far not specifically addressed.

A. Control of protein activity

Regulation of microbial MIP channels at the protein level has only been well studied in *Saccharomyces cerevisiae.* While the expression of the gene encoding the glycerol facilitator, *FPS1* does not change with growth conditions, the protein is regulated by osmotic shock: the channel apparently closes within seconds after a hyperosmotic shock and opens equally fast after a hypo-osmotic shock (Luyten *et al.*, 1995; Tamás *et al.*, 1999). This regulation ensures that glycerol can be accumulated under hyperosmotic conditions and be released after hypo-osmotic shock. However, the precise mechanism controlling Fps1p is not understood. Extensive analysis of the possible involvement of different signaling pathways in yeast, such as the HOG and PKC pathway, suggests that none of these systems is needed for gating of Fps1p (Luyten *et al.*, 1995; Tamás *et al.*, 1999). Moreover, a search for mutants that resemble the phenotype of mutants lacking Fps1p has revealed no gene other than *FPS1,* suggesting but not excluding the fact that Fps1p does not need any other protein for closing (Ferreira and Hohmann, unpublished results). Gustin and co-workers have reported the presence of a mechanosensitive ion channel in the plasma membrane of *Saccharomyces cerevisiae* that is activated by stretching of the membrane (Gustin *et al.*, 1988) but whether the channel activity of Fps1p could be regulated in a similar way is unknown.

A short domain within the amino-terminal extension of Fps1p apparently controls glycerol movement (Luyten *et al.*, 1995; Tamás *et al.*, 1999). Deletion of this sequence abolishes closing, thereby causing loss of glycerol from the cell during growth in high osmolarity medium, and sensitivity to hyperosmotic conditions. This regulatory sequence has been narrowed down by deletion analysis to fewer than 20 amino acids and certain amino acid replacements within this sequence have been found to abolish closing. It also appears that the spacing between this domain and the first transmembrane domain of Fps1p is critical for function (Tamás and Hohmann, unpublished data). However, the sequence does not reveal any hint as to the function of this domain and it is not understood how it controls gating. Detailed mutational analyses as well as novel genetic screens have been devised to address this question and the possible involvement of other parts of Fps1p in the control of the transport function. Interestingly, the glycerol facilitator GlpF from *Escherichia coli* can mediate glycerol transport into and out of yeast cells, as indicated above (Luyten *et al.*, 1995; Tamás *et al.*, 1999). However, its transport function is not regulated by osmotic shock in yeast and hence expression of GlpF also results in glycerol loss from the cell and in an osmosensitive phenotype, analogous to the expression of Fps1p lacking the regulatory domain

(Tamás *et al.*, 1999). Hence, GlpF provides a basis to study the parts of Fps1p that are required for it to be a gated channel.

VIII. Conclusions and future perspectives.

Microbial MIP channels are currently being identified at a rate of about one per week (Hohmann *et al.*, 2000). Although their sequences alone are a useful source of information for generating structure-function relationships (Heymann and Engel, 2000), the determination of their physiological roles is of fundamental importance. This field has been long neglected since MIP channels transport substrates that are able to – or thought to be able to - cross the lipid bilayer by simple diffusion. However, it has become clear that the permeability of microbial membranes for water and glycerol (and related substances) may be limiting, at least under certain conditions. Under such circumstances MIP channels may provide the means to control water and glycerol fluxes into and out of the cell. This principle is best illustrated for the glycerol channel, Fps1p, in yeast osmoregulation. The yeast plasma membrane appears to be relatively impermeable to glycerol and moreover there is evidence that yeast can actively control the permeability of glycerol through the lipid bilayer (Sutherland *et al.*, 1997; Tamás *et al.*, 1999). The presence of Fps1p, whose function is itself regulated by osmotic shock, allows yeast cells to control their intracellular glycerol content in response to external osmolarity.

The fact that glycerol and water can cross lipid bilayers has made analysis of the role of MIP channels more difficult. Phenotypes associated with the deletion of genes encoding MIP channels are not necessarily as clear as in the case of yeast Fps1p, where deletion of the gene causes a sensitivity to hypo-osmotic shock and a constitutively open channel results in sensitivity to high osmolarity. For instance, deletion of *Escherichia coli glpF* causes only a limited inability to catabolize glycerol (Truniger *et al.*, 1992; Voegele *et al.*, 1993) and deletion of the *aqpZ* gene in this organism causes a visible sensitivity to low osmolarity only after co-cultivation in competition with the wild type (Calamita *et al.*, 1998). Hence, to detect the phenotype of a MIP channel mutant may require very careful inspection as well as knowledge of the conditions the organism experiences in its natural environment. This is hardly surprising since in Nature, even subtle differences in viability under specific conditions may provide a major growth and survival advantage.

Moreover, analysis of deletion mutants should be combined with studies on the expression pattern of the relevant MIP, since this may provide further hints about the conditions under which a MIP channel may be functional.

The water channels Aqy1p from yeast and wacA from slime mold *D. discoideum* illustrate this latter aspect. Both proteins are developmentally regulated and hence a phenotype should be sought that is associated with the relevant developmental program: spore formation and germination in this case. It is likely that subtle phenotypes associated with mutation under conditions different from these, where the gene is not or poorly expressed, may be artifacts.

Other important principles are also illustrated by the role of yeast Fps1p. MIP channels can transport solutes and/or water in both directions and hence may serve a role in uptake and/or in efflux. In addition, MIP channels may also function in the export of metabolic end products along a concentration gradient, such as that observed for glycerol export through Fps1p under anaerobic conditions. It is at present not known whether this feature is shared by other MIP channels but is certainly worth considering.

Overall, microbial MIP channels provide an interesting field for the study of microbial physiology in processes such as osmoregulation and developmental programs which in turn can provide models for the study of higher organisms. In addition, microbial systems themselves provide a test bed for the analysis of MIP channels from higher organisms following their heterologous expression. So far, MIP channels have mainly been studied in *Xenopus* oocytes, but since it has been demonstrated that for instance the function of GlpF is affected by lipid composition, (Truniger and Boos, 1993) such heterologous systems may provide misleading results. Yeast expression and vesicles isolated from yeast are thus increasingly used to study aquaporin function. The yeast system has been demonstrated to be robust with respect to the routing of mutant MIPs, hence allowing their functional analysis. Undoubtedly, one power of functional expression in yeast, combined with proper test systems, is the direct investigation of transport and regulation by genetic analysis.

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CHAPTER 3

Growth, Conservation and Release of Osmolytes by Yeasts during Hypo-osmotic Stress

Abstract

In response to fluctuations in environmental osmolarity, yeast cells adjust their intracellular solute concentrations in order to maintain a constant turgor pressure and ensure continuation of cellular activity. In this study, the effect of hypo-osmotic stress on osmolyte content of osmotolerant yeasts was investigated. All yeasts investigated released glycerol upon a hypo-osmotic shock. However, the osmotolerant yeast *Z. rouxii* also released arabitol whereas *P. sorbitophila* released erythritol in addition to arabitol and glycerol. Osmolyte release was very rapid and specific and was neither affected by reduced temperatures nor inhibited by the channel blocker gadolinium or the protonophore carbonyl cynide m-chlrophenyl hydrazone (CCCP). Extracellular osmolyte levels increased drastically suggesting that osmolytes were not metabolised but mainly released upon exposure to hypotonic conditions. The export process is well controlled and the amount of osmolyte released is proportional to the shock intensity. Osmolyte release occurs without cell lysis and thus the survival as well as the subsequent growth of yeast cells is unaffected after hypo-osmotic shock. The kinetics and patterns of osmolyte export suggest the involvement of channel proteins but the molecular nature of this export pathway remains to be investigated.

INTRODUCTION

In their natural habitats, yeast cells are often exposed to severe changes in osmolarity. Similar changes can also occur during biotechnological processes where yeasts are used. To survive under such stressful environments, yeast cells evoke a series of molecular, physiological and morphological events commonly known as the osmotic stress response (Blomberg and Adler, 1992). The most pronounced response to osmotic stress appears to be the production and intracellular accumulation of osmoprotective solutes (osmolytes) such as glycerol, arabitol, mannitol, and erythritol (Brown and Simpson, 1972; Yancey, *et al.*, 1982; van Eck, *et al.*, 1993). The ability to produce, conserve or export these osmolytes is a well-controlled process and appears to be an intrinsic feature of all living organisms in dealing with osmotic stress. Osmotolerant yeasts such as *Pichia sorbitophila* and various species of *Zygosaccharomyces* have developed better controlled and more efficient responses than non-tolerant yeasts during osmotic stress (Attfield, 1998; Lages *et al.*, 1999).

To date, the adaptive processes of yeast cells to hyper-osmotic stress have been extensively studied but the aspect of hypo-osmotic stress has received less attention. Exposure of cells to hypo-osmotic stress results in a rapid inflow of water and cell swelling. Consequently, the turgor pressure increases and if allowed to continue for too long, the cell may rupture. To overcome this situation, the cell has to adjust its intracellular solute levels. This is mainly achieved by activation of membrane transporters that allow the efflux of solutes and ions thus enabling the cell to undergo a regulatory-volume-decrease (Chamberlin and Strange, 1989).

For instance bacterial cells rapidly release their osmolytes from the cytoplasm into the surrounding media (for review Poolman and Glaasker, 1998). Osmolyte export appears to be mediated mainly by mechanosensitive channels although some studies suggest that active (carrier) mechanisms might also be involved (Glaasker *et al.*, 1996). Mammalian cells also respond to hypo-osmotic stress by a rapid release of osmolytes. In fact, swelling-activated release of both organic and inorganic solutes has been reported from a wide range of mammalian cells and has been shown to play a significant role in the volume-regulatory response (for review, Kirk, 1997). However, the molecular nature of the proteins mediating osmolyte release in eukaryotic organisms remains to be elucidated.

In the budding yeast *Saccharomyces cerevisiae*, it has recently been shown that cells rapidly release their intracellular glycerol upon hypo-osmotic shock and this release is controlled by a MIP family membrane channel protein Fps1p (Tamás *et al.*, 1999). It appears that Fps1p

opens during hypo-osmotic conditions and closes during hyper-osmotic conditions thereby controlling the accumulation and release of glycerol during osmoregulation. *fps1Δ* mutants are sensitive to hypo-osmotic stress indicating that glycerol export is required for recovery (survival) following a sudden drop in external osmolarity (Tamás *et al.*, 1999). Whether other yeasts also employ a channel mediated glycerol export similar to that of *S. cerevisiae* has not yet been reported.

Physiological studies have indicated significant differences in the water relations of *S. cerevisiae* and those of osmotolerant yeasts such as *Zygosaccharomyces rouxii, Debaryomyces hansenii* and *P. sorbitophila*. For example, osmotolerant yeasts are capable of growing at a water activity (a_w) as low as 0.65 (sugar) or 0.86 (salt) whereas *S. cerevisiae* can only tolerate 0.89 a_w (sugar) or 0.93 a_w (salt) (Onishi, 1963; Norkrans, 1966; van Eck, *et al.*, 1993). Similarly, species of the fission yeast *Schizosaccharomyces* can only tolerate a_w in a range of 0.89 to 0.90 when glucose is the osmolyte, but the minimum a_w tolerance range is 0.94 to 0.985 when salts are the stressing agents (Ganthala, *et al.*, 1994). *Z. rouxii* and *S. cerevisiae* also show key differences in activities of several enzymes of central metabolism upon exposure to high salt (Brown and Edgley, 1980). In general, osmotolerant yeasts synthesize several osmolytes, retain high amounts of glycerol and have an osmotically inducible active glycerol transport system (Adler *et al.*, 1985; Van Zyl and Prior 1990; Van Zyl *et al.*, 1990; Lucas *et al.* 1990; Lages and Lucas, 1995; Lages *et al.*, 1999). The response of these yeasts to hypo-osmotic stress has not been thoroughly investigated although hypo-osmotic stress is one of the most common problems encountered by unicellular organisms in nature and in various biotechnological applications. The aim of the current study was to examine the growth, conservation and release of osmolytes from the osmotolerant yeasts during hypo-osmotic stress. Taken together, our results indicated that osmotolerant yeasts rapidly release their osmolytes during hypo-osmotic stress. The export process is well controlled and the amount of osmolyte released is proportional to the shock intensity. Osmolyte release occurs without cell lysis and thus the survival as well as the subsequent growth of yeast cells is largely unaffected after hypo-osmotic shock. The kinetics of osmolyte release suggests the involvement of channel proteins but the molecular nature of this export pathway remains to be investigated.

MATERIALS AND METHODS

Yeast strains and growth conditions

The osmotolerant yeasts *Pichia sorbitophila* CBS 7064 (CSIR Y170), *Zygosaccharomyces rouxii* NRRL Y2547 and *Debaryomyces hansenii* CBS 0767 (CSIR Y953) as well as the nontolerant strain *Schizosaccharomyces pombe* CBS 5682 (CSIR Y457) were obtained from the Industrial Biotechnology Microbial Resource Centre, Department of Microbiology, University of Orange Free State (South Africa). The *Saccharomyces cerevisiae* strain used was the haploid laboratory strain W303-1A (Thomas and Rothstein, 1989). All yeast strains were grown in the YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or defined medium glucose-YNB (2% glucose, 0.67% yeast nitrogen base supplemented with the required amino acids). For solid medium, 1.5% agar was added. Liquid cultures were grown at 30°C with continuous shaking of about 200 rpm. Yeast cells were kept on solid YEPD media at 4°C for a few weeks or at –80°C in 15% glycerol for long term storage.

Assay of viability after osmotic stress

An exponentially growing yeast culture was harvested (5000 rpm, 5 min), resuspended in YEPD media (pH 5.2) containing 0.86 M NaCl (5% w/v, 0.972 a_w) and incubated at 30°C for 3 h. At the end of that period, salt stressed cells (1.5 ml) were harvested, washed twice with the same media and finally resuspended in 0.1 ml of YEPD with salt (0.972 a_w). The resultant cell suspension was diluted serially in 1 ml YEPD with salt (iso-osmotic stress) or without salt (hypo-osmotic stress). Samples of 0.2 ml from a 10^{-6} dilution were plated in triplicates on media containing the same concentration of salt as the diluting medium. Agar plates were aerobically incubated at 30°C until colonies were visible. The mean number of colonies was determined and reported as colony-forming units.

Osmotic stress and efflux experiments

Yeast cells were grown in 250 ml glucose-YNB with amino acids until $OD_{600} = 1$. Cells were harvested (5 min, 5000 rpm), resuspended in 100 ml of glucose-YNB with 0.86 M NaCl (pH 5.2) and grown at 30°C for 3 h.

Cells were harvested (5000 rpm, 2 min) from samples (1.5 ml) and resuspended in 0.1 ml of the same medium. Hypo-osmotic shock was performed by diluting ten fold the cell suspension with glucose-YNB media lacking NaCl at either 30°C or 0°C. After a given time interval, cells were immediately sedimented and the pellet and the supernatant were saved for respective intra-and extracellular osmolyte determination. In some experiments, 0.86 M NaCl was replaced by isoosmolar glucose (1.67 M), PEG400 (0.75 M), sorbitol (1.63 M) or ethanol (1.09 M) as stressing agents. To test the effect of inhibitors on osmolyte release, cells were prepared as above and the compound was added 10 min before the hypo-osmotic shock. The channel blocker gadolinium (Gadolinium (III) chloride hexahydrate; Aldrich) and the protonophore carbonyl cynide m-chlrophenyl hydrazone (CCCP; Sigma) were used at final concentrations of 50 mM and 50 μM respectively (Lucas *et al.*, 1990).

Extraction and measurement of osmolytes

Intracellular osmolytes were extracted by boiling the washed cell suspension in Tris lysis buffer (van Eck *et al.*, 1989) for 10 min followed by centrifugation (13000 rpm, 5 min) to remove the debris. The supernatant was retained for analysis. Intra- and extracellular glycerol concentrations were determined spectrophotometrically at 340 nm with the aid of a commercial enzymatic kit (Kit No. 148270; Boehringer-Mannheim, Germany). Arabitol and erythritol concentrations were determined by high performance liquid chromatography (Dionex, MA1 column). Samples were diluted with double distilled milli-Q water and filtered through 0.22 μm filters (Millex^{R}) before injection.

Dry weight measurements

The log phase culture was diluted with sterile media and its turbidity measured using a spectrophotometer and a Klett-Summerson colorimeter at 640 nm (red filter) with sterile YEPD media as a blank. Triplicates of 10 ml from each dilution were centrifuged (5000 rpm, 4 min) and washed twice in distilled water. The pellets were then dried to a constant weight (105°C, 48 hrs) in dry and pre-weighed tubes. Samples were cooled in a desiccator containing silica gel before dry weight measurements. Standard curves of dry weight (g/l) versus turbidity were prepared for each organism for the determination of dry weight.

In most cases dry weight values were confirmed by the filter method. Cells were harvested (4 ml) by filtration (GF/C 25 mm Whatman) and washed with an equal volume of water. The filters containing cells were oven dried at 80°C overnight.

Data treatment and reproducibility of results

The osmolyte content at any particular point was expressed in mg/g dry weight and then calculated as a percentage of the initial concentration (5 min before the osmotic shock). All experiments were repeated at least twice until consistent results were obtained. Representative experiments are shown .

RESULTS AND DISCUSSION

Survival of yeast cells during hypo-osmotic stress

When yeast cells were subjected to a hypo-osmotic shock, more than 80% of the cells survived an osmotic shift from 0.972 a_w (0.86 M NaCl) to 0.998 a_w (0.0 M NaCl) (Table 1). No significant difference in the survival rate was observed between osmotolerant and less tolerant yeasts. Previous studies in *S. cerevisiae* showed that a significant loss of viability after hypo-osmotic shock occurs in cells that are defective in osmolyte export (Luyten *et al.*, 1995) as well as those with mutations in the PKC signalling pathway (Lee and Levin, 1992). Therefore, the observation that the osmotolerant yeasts survive a hypo-osmotic shock as a well as *S. cerevisiae* (Table 1) suggests that also these yeasts can sense and efficiently diminish their accumulated osmolytes upon exposure to hypo-osmotic shock.

Table 1. Survival of yeast cells after a hypo-osmotic shock from 0.972 a_w to 0.998 a_w.

Organism	Colony forming units per plate[a] (mean ± standard deviation of triplicate determinations)		Percentage[b] Survival %)
	Iso-osmotic condition	Hypo-osmotic condition	
S. cerevisiae	183 ± 7	159 ± 13	86.6 ± 4.5
Z. rouxii	198 ± 22	165 ± 14	83.3 ± 6.0
D. hansenii	228 ± 16	205 ± 6	89.7 ± 8.5
P. sorbitophila	216 ± 20	194 ± 4	90.7 ± 6.5

[a] Colony forming units per plate from a 10^{-6} dilution. [b] Survival percentage of hypo-osmotic condition relative to iso-osmotic condition.

Release of Osmolytes from Yeast exposed to Hypo-osmotic Shock

Yeast cells accumulate substantial amounts of polyols, such as glycerol and arabitol, as their major osmolytes (compatible solutes) during hyper-osmotic stress (Blomberg and Adler, 1992). Upon a decrease in external osmolarity, the different yeasts rapidly released their osmolytes and diminished the intracellular content in a matter of minutes (Fig. 1). For all strains, about 75 % of the intracellular osmolytes were lost in 5 min. This rapid decrease in osmolyte content was also observed at 0°C (Fig. 1). In the algae *Dunaliella tertiolecta* and in some protozoa, intracellular osmolytes are depleted by cellular metabolism in response to hypo-osmotic stress (Goyal, 1989; Gilles, 1987). This was not the case in yeasts since a decrease in the intracellular osmolytes corresponded to an increase in its external concentration. As shown in Fig. 2, extracellular glycerol levels increased drastically indicating that osmolytes were not metabolised but released after osmotic downshock. Interestingly, the amount of osmolyte released was proportional to the shock intensity (Fig. 3) suggesting that the release is a well controlled physiological process.

Osmolyte release results from effects of turgor stress (differences in osmotic pressure) but not from water stress per se.

We further investigated whether osmolyte efflux is a consequence of turgor stress or water stress. Cells were stressed with 0.86 M NaCl, harvested and resuspended in media containing isoosmolar concentrations of either glucose, PEG, or ethanol. No significant release of osmolytes was observed when cells were moved from NaCl to PEG or glucose and vice versa as long as the a_w of the stressing agent was equal to that of the solute on the external side (Fig. 4). The only exception was observed with ethanol where osmolyte release was apparent (continued unabated) when cells were moved from 0.86 M NaCl (0.972 a_w) to 1.09 M ethanol (0.972 a_w) (Fig. 4). Due to its small size and solubility, ethanol rapidly equilibrates across the yeast plasma membrane and affects water availability without affecting the turgor pressure (for review, Hallsworth, 1998). Therefore, the observation that the presence of ethanol in the external media did not affect efflux suggests that osmolyte release is a result of turgor stress but not water stress *per se*.

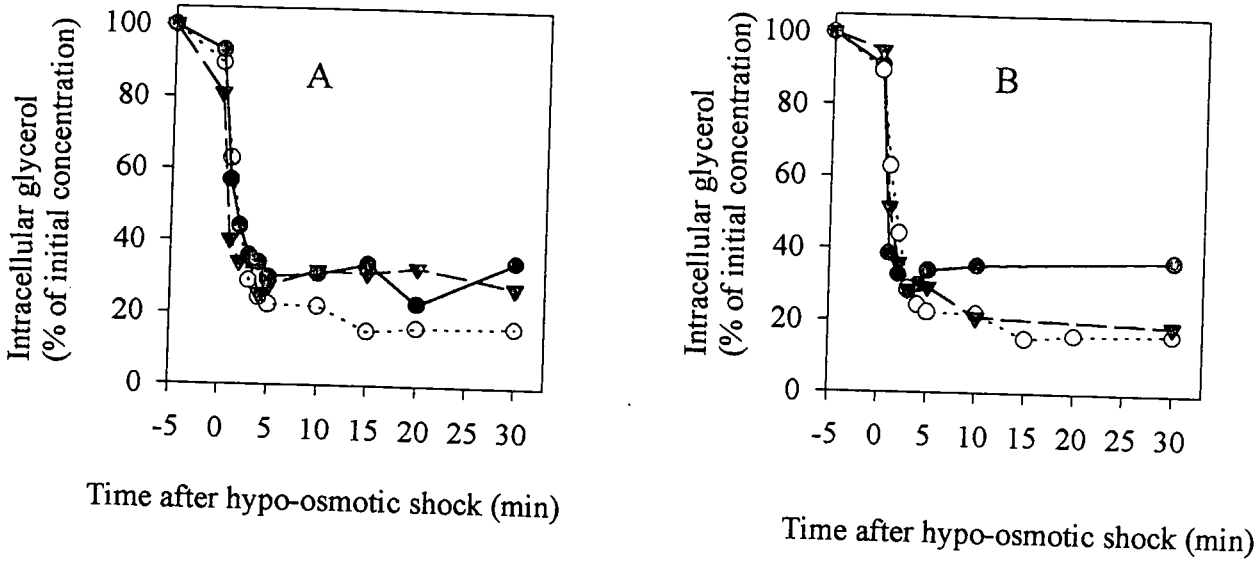

Figure 1a. Intracellular glycerol content of *S. cerevisiae* (○), *S. pombe* (◉) and *Z. rouxii* (▽) at 28°C (A) and 0°C (B) when subjected to hypo-osmotic shock (from 0.86 M NaCl to 0.09 M NaCl in glucose-YNB).

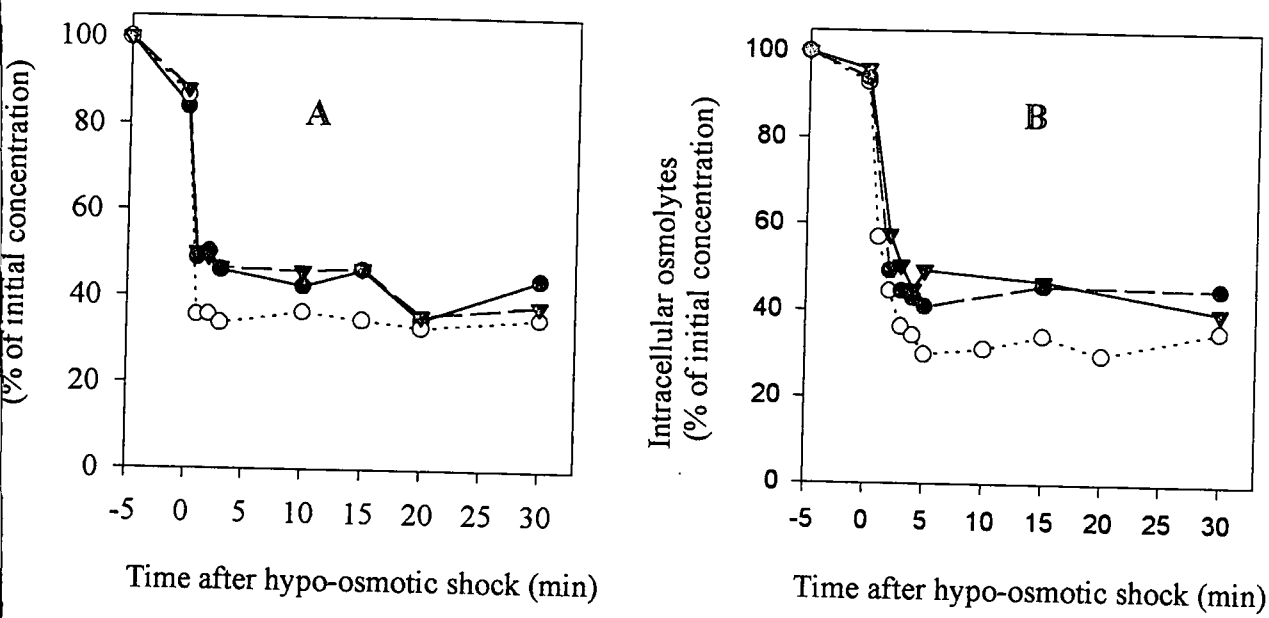

Figure 1b. Intracellular content of the osmolytes glycerol (○), arabitol (▼) and erythritol (●) by the *Pichia sorbitophila* at 28°C (A) and 0°C (B) after hypo-osmotic shock (from 0.86 M NaCl to 0.09 M NaCl in glucose-YNB).

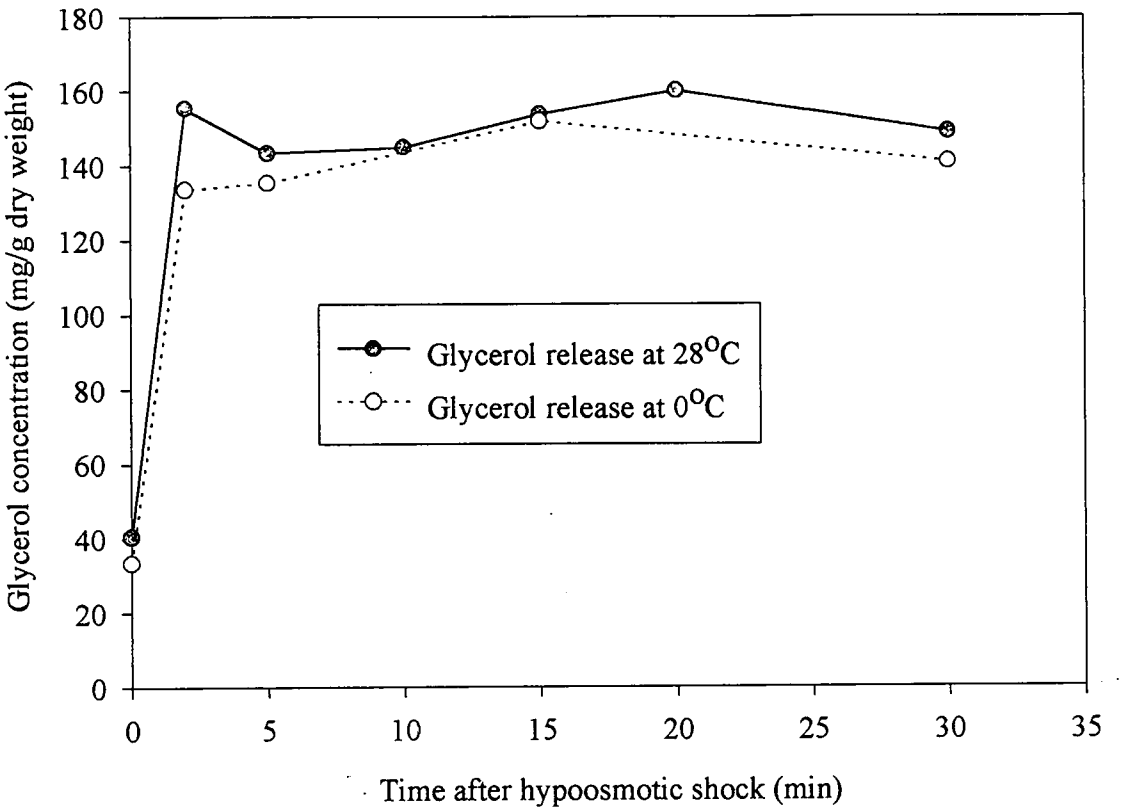

Figure 2. Extracellular glycerol from *Z. rouxii* after hypo-osmotic shock (0.86 M NaCl - 0.09 NaCl in YNB-glucose)

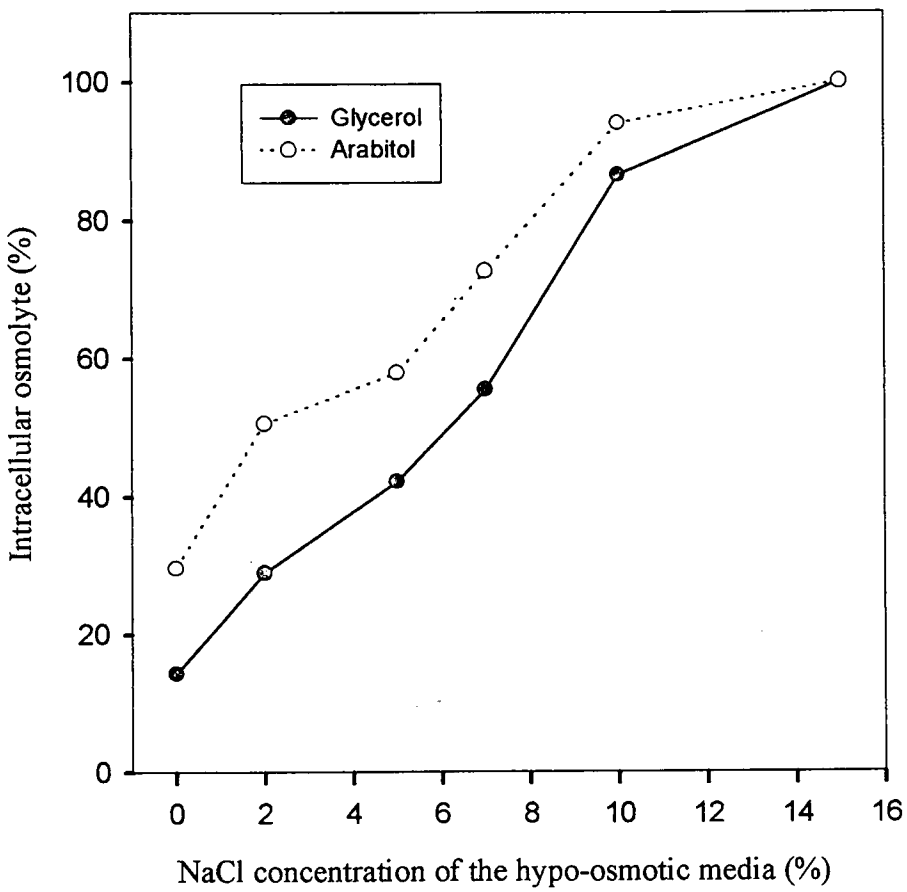

Figure 3a. Release of osmolytes (at 28^{O}C) as a function of shock intensity (15% to 0 % NaCl) in *Z. rouxii* NRRL 2547. Intacellular osmolytes were extacted 5 min after hypo-osmotic shock and expressed in percentages, taking the iso-osmotic values as 100 %

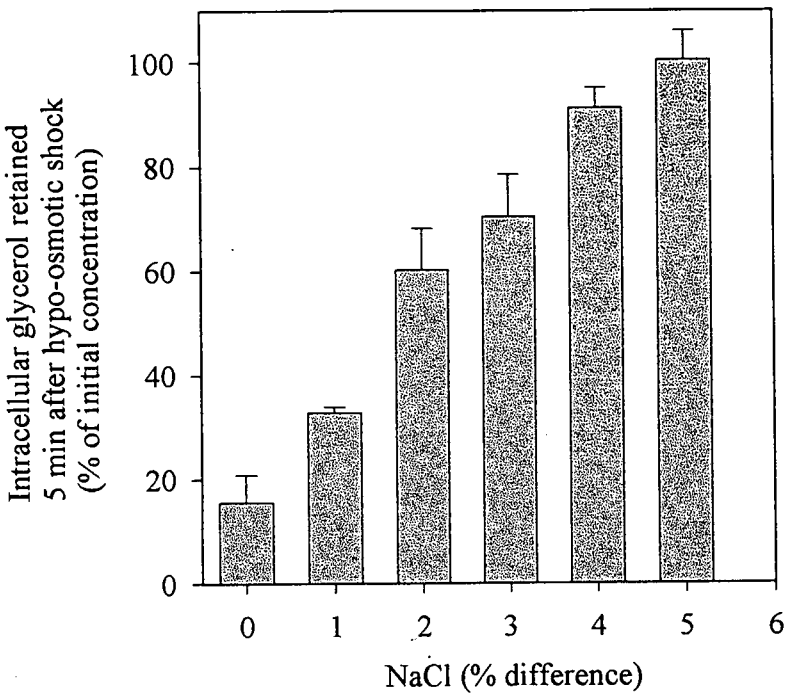

Figure 3b. Release of accumulated glycerol as a function of shock intensity in *S. cerevisiae*.

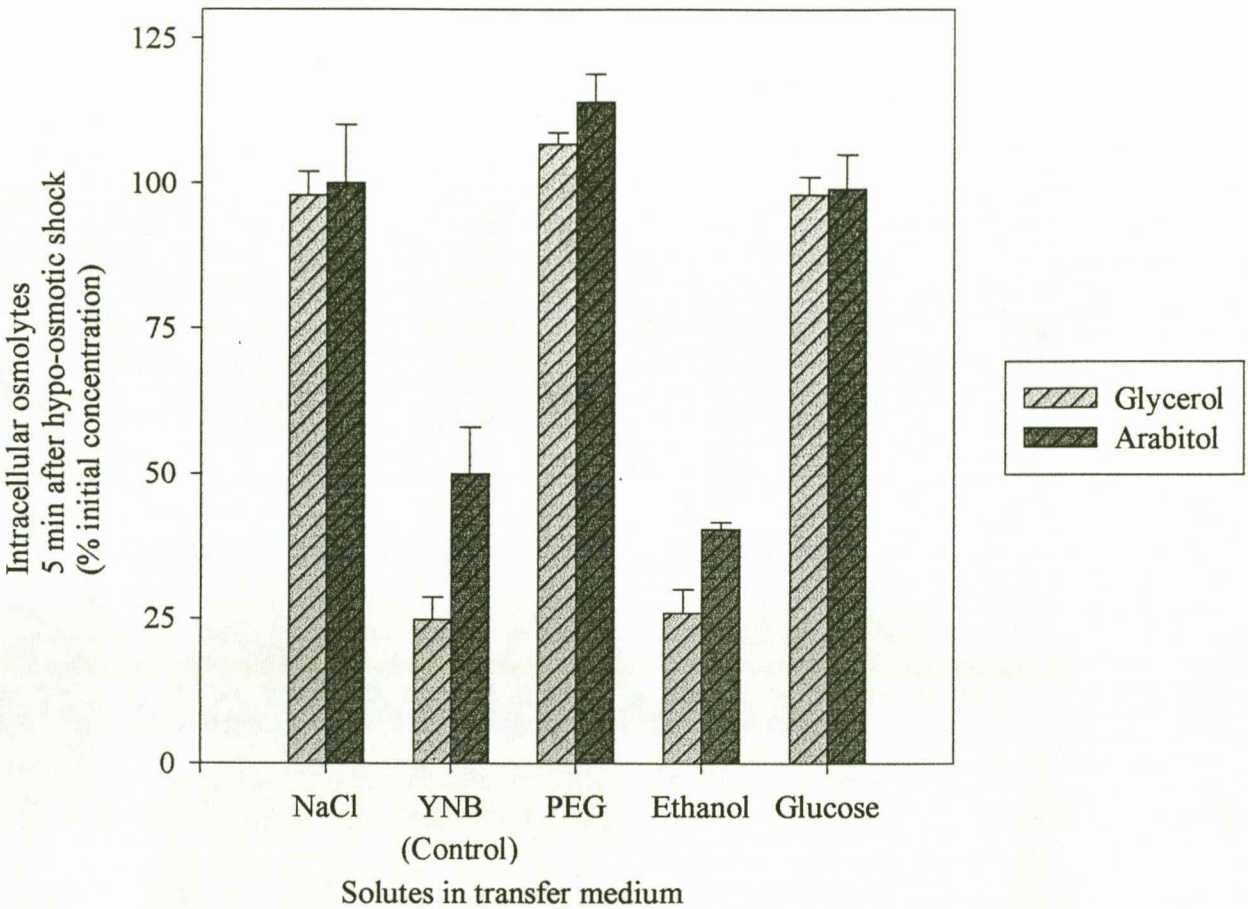

Figure 4. Retention of osmolytes (mean ± standard deviation of triplicate determinations) by *Z. rouxii* when cultivated at 0.972 a_w (NaCl) and transferred to iso-osmotic media or hypo-osmotic medium (control, lacking osmoticum).

Osmolyte release is unaffected by gadolinium and CCCP inhibitors

To examine the nature of the transport system mediating osmolyte release, various transport inhibitors were used. Mechanosenstive channels mediating solute efflux in certain bacteria may be inhibited by the presence of gadolinium ions (Yang and Sachs, 1989; Berrier, *et al.*, 1992; Schleyer *et al.*, 1993; Batiza *et al.*, 1996). Addition of gadolinium from concentrations of 5 mM to 50 mM either before or during hypo-osmotic shock had no effect on the release of osmolytes (glycerol, arabitol, erythritol) from either *Z. rouxii* (Fig. 5a) or *P. sorbitophila* (Fig. 5b). Gadolinium had no effect on Fps1-mediated glycerol efflux in *S. cerevisiae* (Tamás *et al.*, 1999) Carbonyl cynide m-chlrophenyl hydrazone (CCCP) is known to affect membrane potential, which is the driving force of active transport systems. Treatment of yeast cells with the protonophore CCCP (5-50 μM) did not lead to either inhibition or even a delay in solute efflux (Fig. 5). It is thus likely, that the putative efflux pathways in osmotolerant yeasts differ from the transport systems used for influx, since glycerol uptake for instance is prevented by CCCP (Van Zyl *et al.*, 1990; Lages and Lucas, 1995; 1997; Lages *et al.*, 1999).

Is osmolyte export a passive process, due to membrane leakage, carrier or channel mediated?

All osmolytes released by osmotolerant yeasts upon hypo-osmotic shock were low-molecular-mass polyols that can permeate the plasma membrane without the involvement of channel proteins or efflux carriers. However, the efflux process appeared to be very specific with a distinct preference for osmolytes, thus eliminating the possibility of membrane leakage. Furthermore, the fact that a rapid osmolyte efflux could still be observed at 0°C suggests the involvement of a specific efflux mechanism other than passive diffusion which is slower at 0°C. However, the rate of efflux is very high and presumably too fast to be catalysed by a carrier mechanism. If a carrier is involved, the presence of the efflux substrate on the external (*trans*-side) should theoretically cause an increase in efflux even at very low osmotic gradients (Ruffert *et al.*, 1997). The presence of glycerol (3g/l) in the diluting media instead abolished glycerol release from the cells. The same phenomena were observed for other osmolytes. It has long been observed that the carbon source present in the media determines transport system (Diaz, 1987) and influences polyol transport (Brown, 1974). No significant difference in glycerol release was observed in the absence or the presence of 2% glucose. Experiments performed with the uncoupler CCCP confirmed that indeed efflux carriers might not be involved whereas experiments with the channel blocker gadolinium indicated that mechanosensitive channels might also not be involved.

Similar observations have been made in *Corynebacterium glutamicum* (Ruffert *et al.*, 1997) where osmolyte efflux appears to be mediated by osmoregulated channels. Taken together, the pattern and kinetics of osmolyte release from osmotolerant yeasts upon an osmotic downshock suggest the presence of a channel-mediated transport system similar to that of Fps1p in *S. cerevisiae*. The Fps1-mediated glycerol transport in *S. cerevisiae* is very rapid, neither affected by reduced temperatures nor inhibited by gadolinium or CCCP. However preliminary molecular investigations using PCR and DNA probes did not indicate *FPS1* homologues in osmotolerant yeast (Kayingo *et al.*, 2000 and Chapter 5). It is thus likely that the genes encoding components involved in channel-mediated osmolyte export in osmotolerant yeasts are structurally different from that of *FPS1* in *S. cerevisiae* although the corresponding protein(s) seem to function in a similar way. Whether different osmolytes share the same transporter in a given organism or whether there are separate channels for arabitol and glycerol in *Z. rouxii* for instance remains to be established.

Regulation of osmolyte export: While the release of osmolytes is very important during hypo-osmotic stress, the cell must conserve these osmolytes during hyper-osmotic stress. Osmolytes such as glycerol are also involved in other physiological processes unrelated to osmoregulation. Therefore, osmolyte flux must be regulated to avoid interference with basic functions of the cell. In *S.cerevisiae,* the accumulation and release of glycerol is controlled by a glycerol channel Fps1p. During hyper-osmotic conditions, the *S. cerevisiae* glycerol channel apparently closes thereby conserving glycerol inside the cell whereas in hypo-osmotic conditions, Fps1p opens and intracellular glycerol release occurs rapidly (Fig. 1, Lutyten *et al.*, 1995). We have shown that the opening and closing varies according to the applied shock (Fig. 3b) and this phenomenon appears to be ubiquitous among yeasts. In bacteria, efflux channels open or become active only when the external osmolarity is lower than a certain value (Ajouz *et al.*, 1998). For osmotolerant yeasts, the threshold osmolarity change that triggers osmolyte release was however not determined. Various studies have indicated that osmolyte channels are not regulated at the level of expression but at the level of activity (Poolman and Glaasker, 1998; Ruffert *et al.*, 1997). Channel regulation via conformational rearrangements has been reported for the *Streptomyces lividans* K^+-channel KcsA (Perozo *et al.*, 1999). In *S. cerevisiae*, the transport function of Fps1p is rapidly regulated by changes in external osmolarity via its uniqiue N-terminal extension. However, the signalling pathway regulating the opening and closing of Fps1p are not known. Neither the HOG nor the PKC signalling pathway appears to be involved (Tamás *et al.*, 1999). Although very little is known about the molecular nature of osmolyte efflux systems in osmotolerant yeasts, it is likely that they are also regulated at the level of activity.

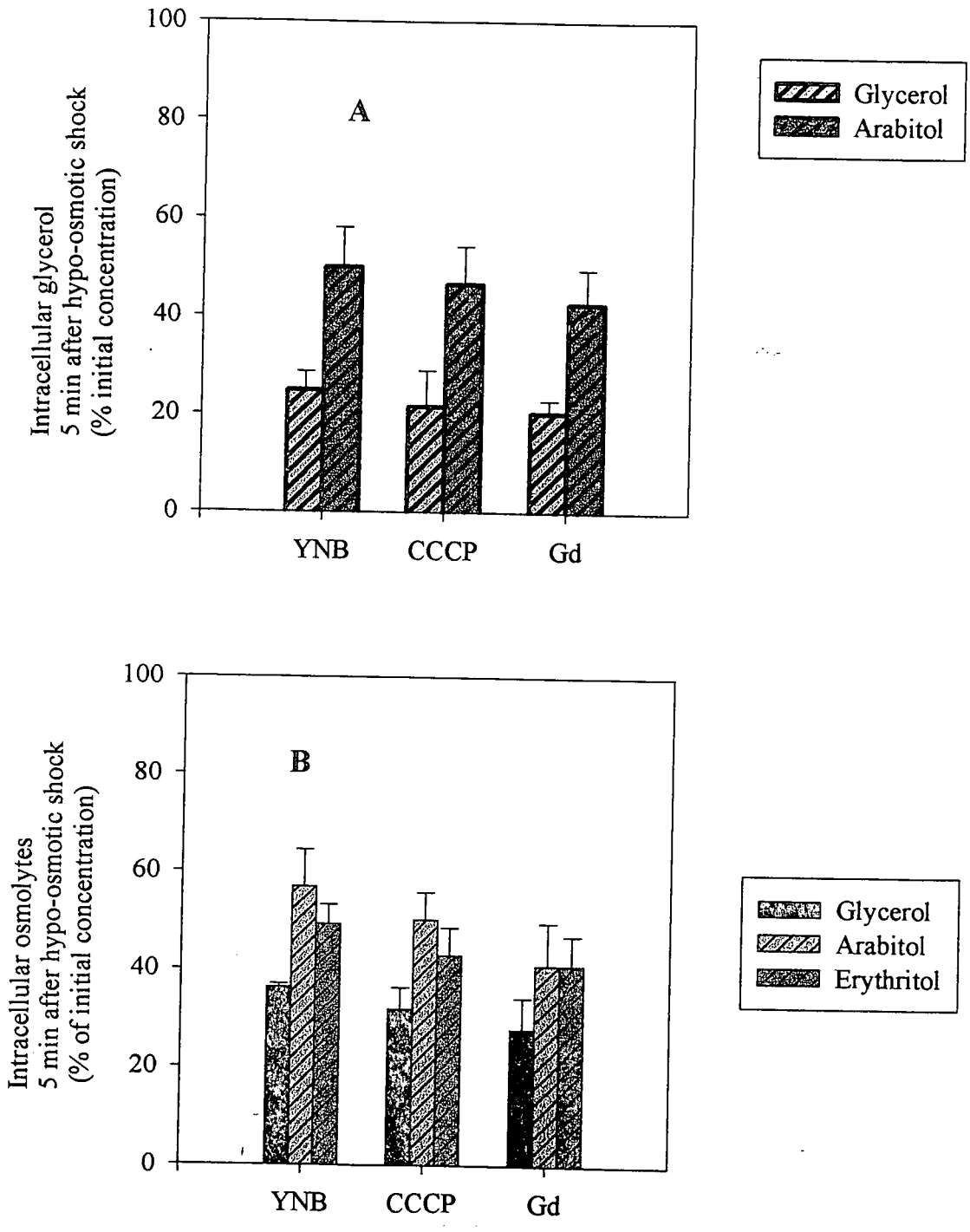

Figure 5. Effect of the protonophore CCCP (50 μM) and the channel blocker gadolinium (50 mM) on osmolyte release by *Z. rouxii* (A) or *P. sorbitophila* (B) during hypo-osmotic stress from 0.86 M NaCl (0.972 a_w) to 0.09 M NaCl (0.996 a_w).

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CHAPTER 4

Effect of Ergosterol on Survival and Glycerol Release from *Saccharomyces cerevisiae* cells after Osmotic Downshock

Abstract

Lipid composition influences the membrane permeability of solutes during adaptation of yeast cells to osmotic stress. In this study, we investigated the effect of ergosterol on survival and glycerol release from *Saccharomyces cerevisiae* cells after an osmotic downshock. The wild-type strain survived the osmotic downshock and grew to a similar degree in the presence or absence of ergosterol supplements. By contrast, *S. cerevisiae* cells lacking a glycerol facilitator (the *fps1Δ* strain), grew poorly upon an osmotic downshock, but apparently survived the shock better, and recovered more rapidly, if ergosterol was supplied. The *erg-1* disruption mutant, which is unable to synthesize ergosterol, survived and recovered from the osmotic shock more successfully at the higher ergosterol concentration. Transport studies showed a rapid efflux of glycerol from the wild-type cells upon osmotic downshock. The glycerol content of wild-type cells was reduced to about 20% of its initial value within 5 min regardless of addition of exogenous ergosterol. However, the glycerol content of the *fps1Δ* strain was only reduced to 80% of its initial value within 5 min, and remained at this level for at least 30 min. When exogenous ergosterol was supplied, the rate and amount of glycerol release was markedly enhanced in the *fps1Δ* mutant. The polyene antibiotic nystatin, which affects membrane permeability, caused *S. cerevisiae* cells to release a large amount of glycerol and equally inhibited the growth of wild-type and *fps1*deletion strains in medium containing 5% (w/v) NaCl. This study demonstrated the role of ergosterol in glycerol efflux and survival in *S. cerevisiae* after an osmotic downshock and provided additional evidence for the significance of membrane permeability and glycerol conservation in yeast osmoregulation.

INTRODUCTION

The role of glycerol and membrane lipid composition in yeast stress responses has been studied extensively (Swan and Watson, 1996; Prior and Hohmann, 1997; Attfield, 1998). It has been known for decades that most yeast cells accumulate substantial amounts of glycerol in their cytosol in response to hyper-osmotic stress. The accumulated glycerol serves as an osmolyte (compatible solute) and maintains turgor without interfering with cellular functions (Brown, 1978; Yancey et al. 1982). It is also well established that yeast cells regulate their membrane lipid composition in response to osmotic stress (Tunblad-Johansson and Adler, 1987; Watanabe and Takakuwa, 1987; Hosono, 1992; Yoshikawa *et al.*, 1995). However, little is known about the relationship between membrane lipid composition, glycerol transport and survival during hypo-osmotic stress. Studies with liposomes indicate that glycerol permeation is influenced by membrane composition (De Gier, 1993) but the key components in the yeast membrane that influence glycerol transport are not yet well defined.

In *Saccharomyces cerevisiae*, the movement of glycerol across the cell membrane occurs via active transport, by channel-mediated diffusion (Fps1 protein), and by passive diffusion across the plasma membrane (Sutherland *et al.*, 1997). The extent to which glycerol permeates the cell may then be influenced by the membrane lipid composition as observed in osmotolerant yeasts when grown under osmotic stress (Watanabe and Takakuwa, 1987). During hypo-osmotic stress, yeast cells rapidly release their intracellular glycerol and this release appears to be controlled by a membrane channel protein Fps1p (Luyten *et al.*, 1995; Tamás, *et al.*, 1999). Cells lacking the Fps1 glycerol channel grow poorly during hypo-osmotic stress, while those expressing an unregulated channel are sensitive to hyperosmotic stress (Tamás *et al.*, 1999). Sutherland *et al.* (1997) revealed that the deletion of *FPS1* results in diminished passive diffusion of glycerol and alters cellular lipid composition. Interestingly, the deletion of the glycerol facilitator (*glpF*) in *Escherichia coli*, a close homologue of *FPS1*, also reduces the permeability across the plasma membrane (Truniger and Boos, 1993). Recently, we have observed that *FPS1* deletion leads to an alteration in the ergosterol contents of the whole cell and the plasma membrane (Toh *et al.*, 2000). These observations raise the question whether a link between glycerol channel proteins, membrane permeability and cellular lipid synthesis existed. Therefore, the present study was carried out to investigate the relationship between ergosterol content, glycerol release and survival of yeast cells during hypo-osmotic stress. Ergosterol is the most abundant sterol in yeast membranes (Zinser *et al.*, 1993) and plays an important role in determining the integrity of biological membranes.

In turn, ergosterol apparently influences the membrane permeability to solutes (Rattray 1988; Van der Rest *et al.*, 1995). Here, we demonstrate the role of ergosterol in glycerol efflux and further highlight its significance in osmotolerance.

MATERIALS AND METHODS

Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains lacking a glycerol facilitator *fps1Δ::HIS3* (Tamás *et al.*, 1999) and *fps1Δ::LEU2* (van Aelst *et al.*, 1991) were derived from the haploid laboratory strain W303-1A (Thomas and Rothstein, 1989). The ergosterol (*erg1*-disruption) mutant and its corresponding wild-type strain (X2180-1B *MAT α, SUC2, mal, mel, gal2 cup1*^{R}) were kindly supplied by Drs. L.W. Parks and J.H. Crowley of North Carolina State University. All yeast strains were routinely grown on YEPD (10 g l^{-1} yeast extract, 20 g l^{-1} each of peptone and glucose, pH 6) or on defined medium (6.7 g l^{-1} yeast nitrogen base, 20 g l^{-1} glucose and amino acids, pH 6, glucose-YNB) with supplements as indicated in the text.

Osmotic downshock sensitivity experiments

Yeast cells were grown on medium (0.998 a_w) without ergosterol supplementation (control) and on medium with ergosterol supplementation at a final concentration of 10 μg ml^{-1} ergosterol. For the *erg1*-disruption mutant and the X2180-1B isogenic wild-type strains, ergosterol (Sigma) and fatty-acid supplements were supplied as described by Swan and Watson (1998). The preparation of osmotically stressed cells is similar to that described previously (Luyten *et al.*, 1995). At a standardised time point (OD_{600} 1.0), the cells were harvested and resuspended in the same media supplemented with 50 g l^{-1} NaCl (0.972 a_w) and incubated at 30°C for 3 h. At the end of that period, the cultures were harvested, quickly washed once in growth medium without salt (0.998 a_w) and finally resuspended in the same medium to a predetermined optical density. Spot tests were carried out by serial decimal dilutions of the cell suspension prior to plating (Luyten *et al.*, 1995). Five microlitres of the cell suspensions were spotted onto agar plates without NaCl addition and incubated at 30°C until the colonies were visible. Aliquots of the cell suspensions were also removed for biomass dry weight and intracellular glycerol determinations.

Nystatin sensitivity experiments

The polyene antibiotic nystatin (Sigma, USA) was dissolved in ethanol (20 mg ml^{-1}) and added to exponentially growing cells to evaluate its effect on the growth of *fps1Δ* mutant strain and its isogenic wild type. The final concentrations of the antibiotic in the culture media were, 0.02, 0.05, 0.1 or 0.2 μg ml^{-1} while that of ethanol was < 0.5 % (v/v). To investigate the effect of nystatin on intracellular glycerol levels, yeast cells were grown in glucose-YNB until early exponential phase, harvested and then resuspended in glucose-YNB containing 5% NaCl. After three hours in hyper-osmotic media, nystatin (0.2 μg ml^{-1}) was added and samples taken for glycerol determination.

Intracellular glycerol determination

Aliquots of cells exposed to hypo-osmolar media or to nystatin treatment were periodically taken in a 1.5 ml Eppendorf tubes. Cells were quickly harvested, resuspended in 1.0 ml Tris lysis buffer (van Eck *et al.*, 1989) and then boiled for 10 min in a water bath with regular vortex agitation. The suspensions were centrifuged at 16000 r.p.m. for 5 min to remove cell debris and the supernatant was retained for glycerol determination. Intra- and extracellular glycerol concentrations were determined spectrophotometrically at 340nm with the aid of a commercial enzymatic kit (Kit No. 148270; Boehringer-Mannheim, Germany).

RESULTS AND DISCUSSION

Effect of ergosterol on survival of yeast cells after osmotic downshock

Osmotic downshock sensitivity experiments were carried out to test the effect of cellular ergosterol levels on survival, the rate of glycerol release and subsequent recovery of the yeast cells after an osmotic downshock. The wild-type strain recovered from osmotic downshock, and grew to a similar degree regardless of ergosterol supplementation (Fig. 1B). By contrast, cells of the *fps1Δ* strain grew very poorly on glucose-YNB, but apparently survived the osmotic downshock better, and recovered more rapidly, if ergosterol was supplied (Fig. 1A). The difference between the growth of the *fps1Δ* strain in the presence or absence of exogenous ergosterol was apparent throughout the incubation period between 12 and 30 h.

The *erg-1* disruption mutant which is unable to synthesise ergosterol (Parks *et al.*, 1995) and X2180-1B wild-type strain were subjected to osmotic downshock, and their growth and survival were assessed. The wild-type strain survived and recovered from the osmotic downshock equally well irrespective of the concentration of ergosterol supplied (Fig. 1B). By contrast, the *erg-1* disruption strain survived and recovered from the osmotic downshock more successfully at the higher ergosterol concentration (Fig. 1B). This result was consistently observed regardless of the incubation time after the downshock (between 12 and 30 h).

Effect of ergosterol on the release of glycerol after osmotic downshock

It has been proposed that the *fps1Δ* mutant grows poorly upon an osmotic downshock due to its inability to rapidly dispose of the accumulated glycerol (Luyten *et al.*, 1995). To determine whether the improved survival upon a downshock observed above was associated with changes in solute transport, the effects of ergosterol supplementation on glycerol efflux was investigated by monitoring the intracellular glycerol concentrations prior to and immediately after an osmotic downshock. There was a rapid efflux of glycerol from cells of the wild-type strain upon osmotic downshock as reported previously (Luyten *et al.*, 1995). The glycerol content of W303-1A wild-type cells was reduced to about 20% of its initial value within 5 min, and remained at this level for at least 30 min, regardless the addition of exogenous ergosterol (Fig. 2A).

On the other hand, the glycerol content of *fps1Δ* cells was only reduced to 80% of its initial value within 5 min of downshock, and remained at this level for at least 30 min. However, when exogenous ergosterol was supplied, the rate and amount of glycerol release was markedly enhanced (Fig. 2A). In the presence of ergosterol, *fps1Δ* mutant reduced its intracellular glycerol concentration to around 60% of its initial value within 5 min. During iso-osmotic conditions, no glycerol release was observed in both the wild-type (Fig. 2B) and the *fps1*Δ strain (Fig. 2C) regardless the addition of exogenous ergosterol. Glycerol release occurs after an osmotic downsock to balance up the turgor pressure difference created as cells are moved from high to low osmolarity environments. Our observations that ergosterol may play a role in glycerol efflux is in agreement with previous reports in *Candida albicans* where mutants inhibited in ergosterol synthesis exhibited decreased permeability for glycerol (Pesti and Novak, 1984).

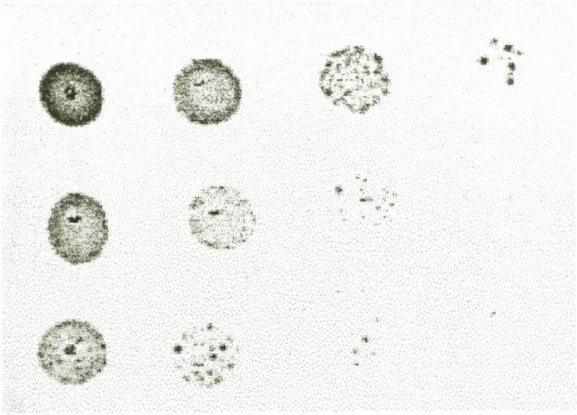
However, the mechanisms by which ergosterol improve glycerol release and survival of yeast cells are not yet clear. It could presumably be related to the membrane stablising effects of ergosterol (Hossack and Rose 1976) and the associated improvements in membrane fluidity (Arami *et al.*, 1997).

Effect of nystatin on the growth and release of glycerol from *fps1Δ* strains

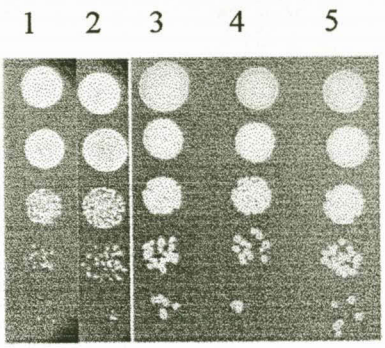
The susceptibility to nystatin is known to correlate with the ergosterol content in the plasma membrane of yeast cells (Hamilton-Miller 1973). For instance, mutants of *S. cerevisiae* that cannot synthesise ergosterol are more tolerant to nystatin (Bard, 1972). In this study, we investigated the effect of nystatin on the growth of *S. cerevisiae* wild-type cells and *fps1Δ* mutant which exhibits an alteration in the cellular ergosterol contents (Toh *et al.*, 2000). As shown in Figure 3, nystatin inhibited the growth of both strains in YEPD media with 5% NaCl whereas cells grown in media without NaCl were only slightly affected. The presence of 5% NaCl alone also caused a delay in the growth of yeast cells compared to the growth of cells without NaCl but addition of nystatin aggravated the effect. Both strains were equally sensitive to the various concentrations of nystatin tested. It has been suggested that nystatin interacts with ergosterol and forms an aqueous channel in the plasma membrane (Kobayashi and Medoff, 1977). These pores result in the leakage of intracellular osmolytes that are indispensable for growth of yeast cells in hyper-osmolar media (Hosono, 2000).

We further investigated the changes in intracellular glycerol levels after addition of 0.2 μg nystatin ml^{-1} to salt-stressed cells. As shown in Figure 4, nystatin caused salt-stressed cells to release large amount of glycerol. The intracellular glycerol decreased by more than 70% in 30 minutes of incubation with nystatin. Since the action of nystatin is generally thought to be due to an interaction with ergosterol in the membrane, it was anticipated that the *fps1* deletion strain which shows about 26% reduction in ergosterol content, could be less susceptible to nystatin compared to the wild-type. However, no significant difference was observed in susceptibility or glycerol release between the two strains. This result suggest that although a 26% difference in ergosterol content might be sufficient to alter membrane solute transport properties as we have shown in Figure 1 above, it might not be adequate to cause significant differences in susceptibility to the concentrations of nystatin used in this study. On the other hand, the ergosterol differences reported by Toh *et al.*, (2000) were observed during steady state conditions. The ergosterol content in the plasma membrane increases drastically when yeast cells are exposed to high salinity media. For instance, the ergosterol content in the plasma membrane of *Zygosaccharomyces rouxii* grown in YEPD media containing 15% NaCl increased 2. 9 fold higher than that of unstressed cells (Hosono, 1992). It is therefore possible that when S. *cerevisiae* cells were grown in media containing 5% NaCl, the ergosterol content of both the wild-type and the *fps1* deletion mutant increased to proportions that could not allow detectable differences in susceptibility to polyene antibiotics.

A



B



1 = *erg-1* disruption mutant + 5 µg ergosterol ml^{-1}

2 = *erg-1* disruption mutant + 10 µg ergosterol ml^{-1}

3 = Wild-type without ergosterol

4 = Wild-type + 5 µg ergosterol ml^{-1}

5 = Wild-type + 10 µg ergosterol ml^{-1}

Figure 1. Survival of *S. cerevisiae* W303-1A and *fps1Δ* strains (**A**) as well as the *erg-1* desruption mutant and the X2180-1B wild type strains (**B**) grown on glucose-YNB after an osmotic downshock with and without ergosterol supplementation.

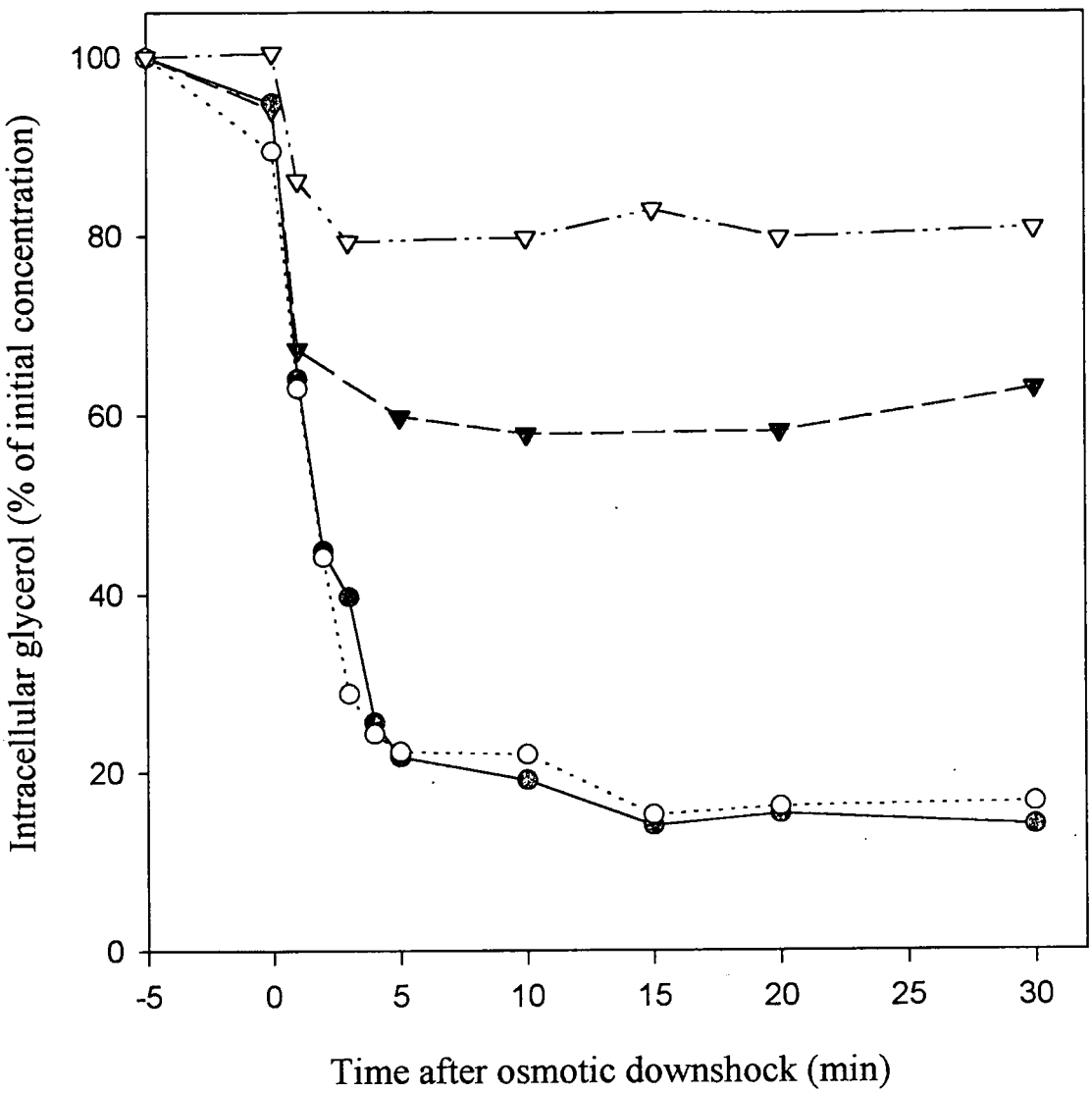


Figure 2A. Glycerol release from the *fps1*Δ (triangles) and W303-1A wild-type strain (circles) before and after osmotic downshock with (solid symbols) and without (open symbols) ergosterol supplementation.

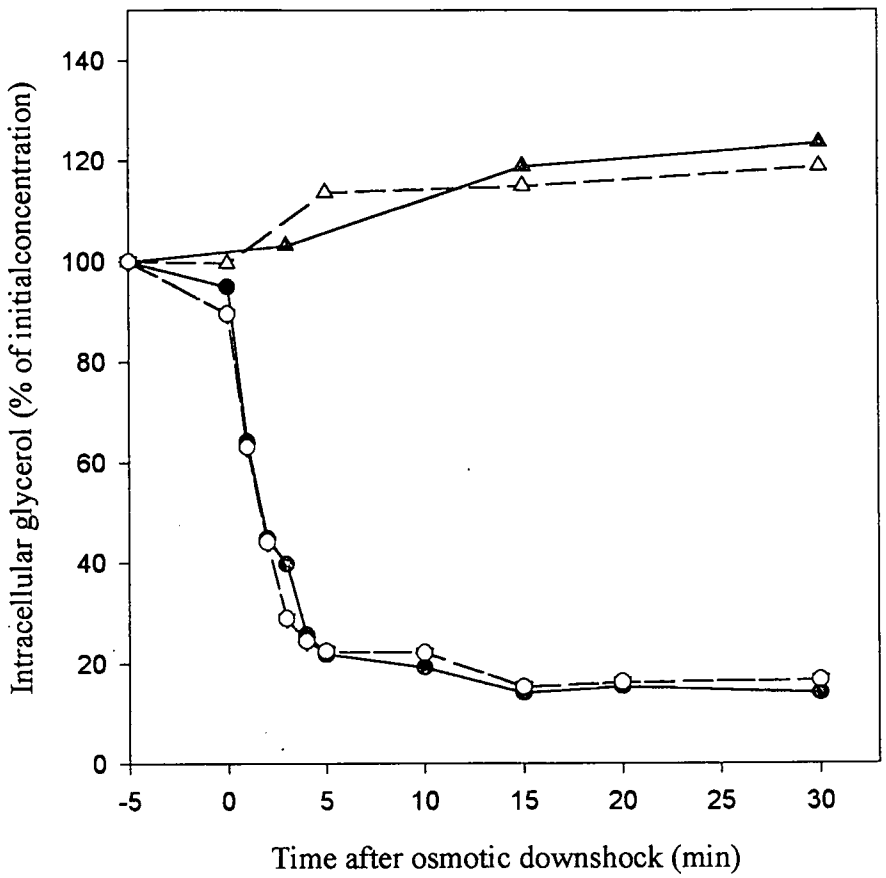


Figure 2B. Glycerol release from *S. cerevisiae* W303-1A wild-type strain during iso-osmotic conditions (triangles) or after osmotic downshock (circles) with (solid symbols) and without (open symbols) ergosterol supplementation.

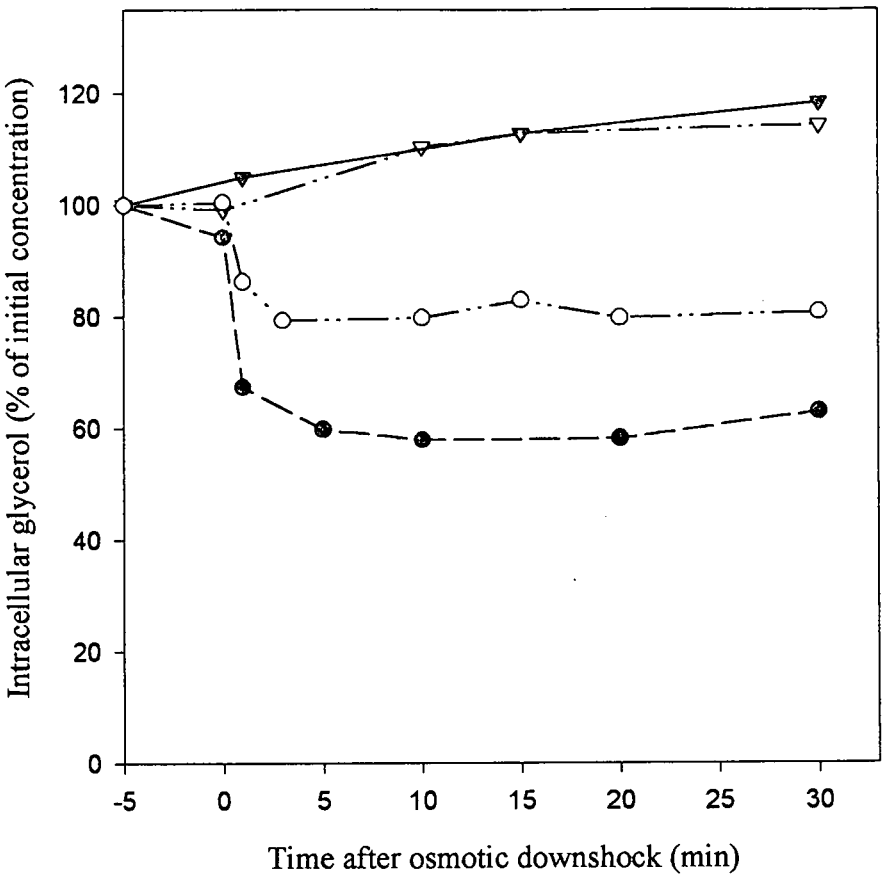
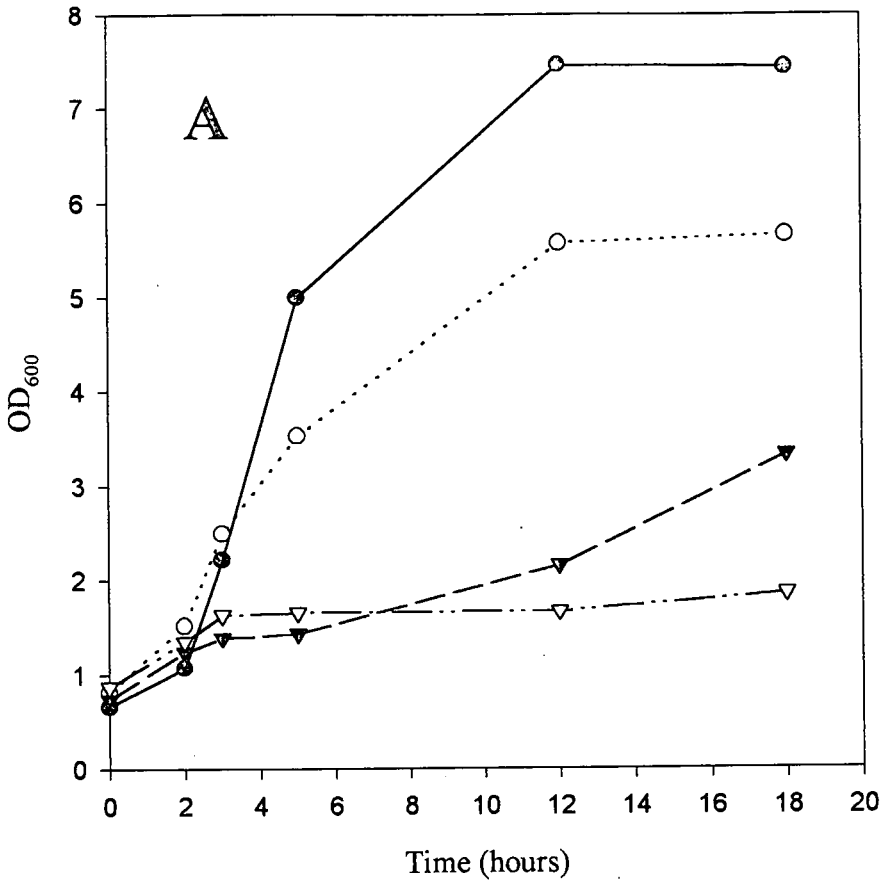


Figure 2C. Glycerol release from *fps1Δ* strain during iso-osmotic conditions (triangles) or after osmotic downshock (circles) with (solid symbols) and without (open symbols) ergosterol supplementation.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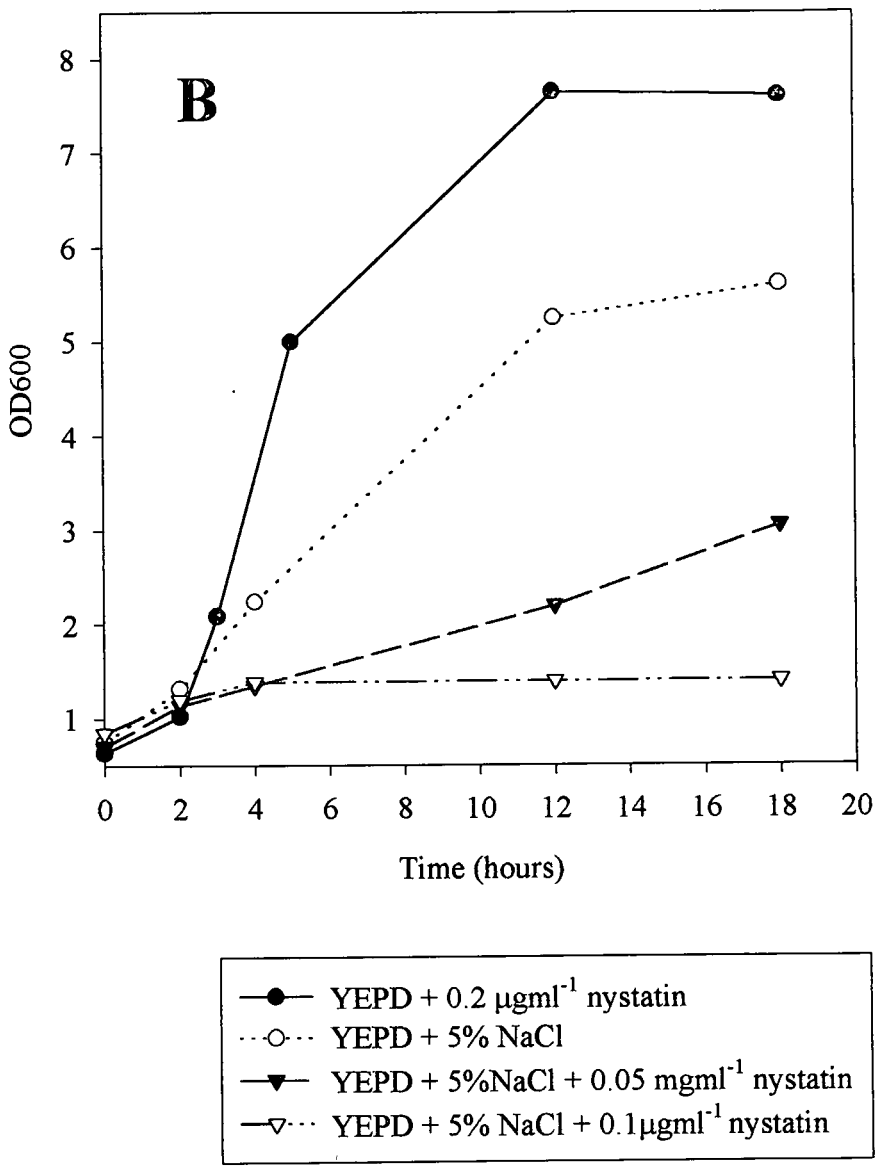

Figure 3. The effect of nystatin on the growth of *S. cerevisiae* W303-1A wild-type strain (**A**) and the *fps1Δ* mutant (**B**). The polyene antibiotic nystatin was dissolved in ethanol (20 mg ml^{-1}) and added to exponentially growing cells to the final concentrations, 0.05, 0.1 or 0.2 μg ml^{-1} while that of ethanol was < 0.5 % (v/v).

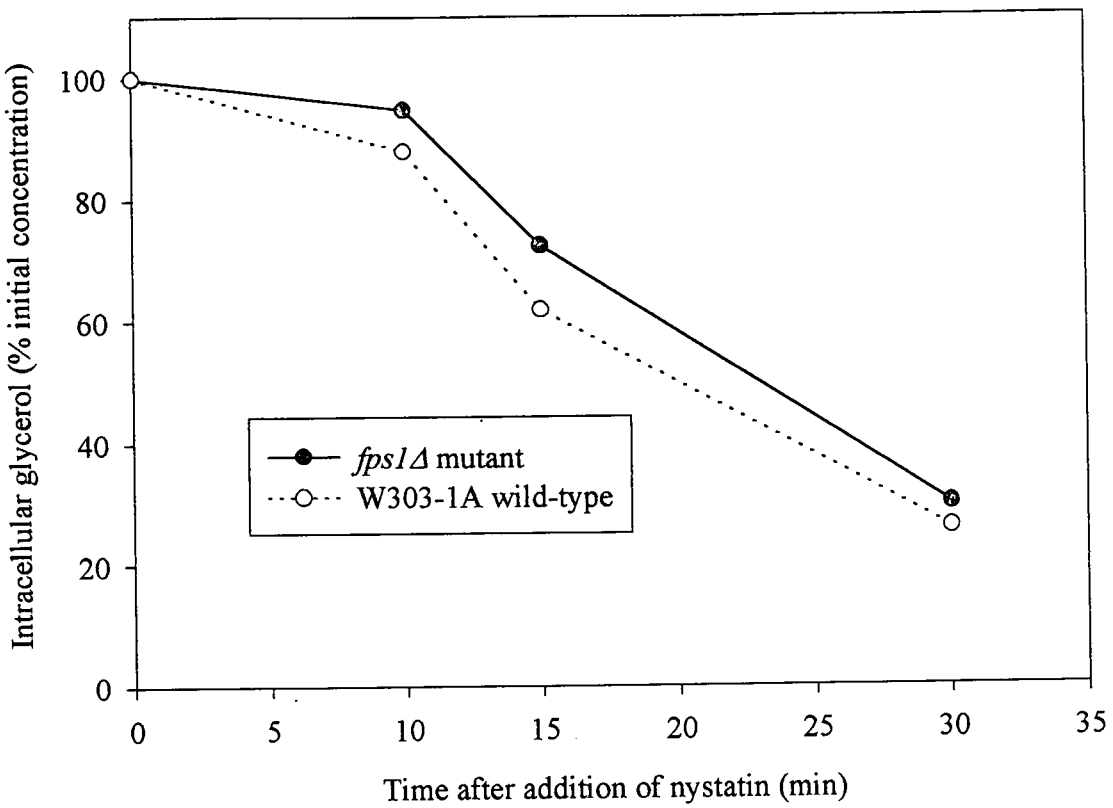

Figure 4. The effect of nystatin on the intracellular glycerol content of *S. cerevisiae* W303-1A wild-type and the *fps1Δ* mutant strains during osmotic stress (5% NaCl). Yeast cells were grown in glucose-YNB until early exponential phase, harvested and then resuspended in glucose-YNB containing 5% NaCl. After three hours in hyper-osmotic media, nystatin (0.2 μgml^{-1}) was added and samples taken for glycerol determination.

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CHAPTER 5

An investigation of the possible existence of homologues of *FPS1*, a glycerol facilitator of *Saccharomyces cerevisiae*, in the osmotolerant yeasts *Zygosaccharomyces rouxii* and *Pichia sorbitophila*

Abstract

Yeast species differ in their mechanisms of accumulating and transporting glycerol across the plasma membrane. In *S. cerevisiae*, glycerol accumulation is mainly controlled by a transmembrane channel protein Fps1p during osmoregulation. The yeast Fps1p is a member of the MIP family of water channels and glycerol facilitators. In this study, we investigated the presence of channel-mediated glycerol transport and *FPS1* homologues in the osmotolerant yeasts *Z. rouxii* and *P. sorbitophila*. Transport studies revealed a rapid glycerol efflux which was characteristic of glycerol facilitators. Analysis of *FPS1* homologues by PCR and DNA probes resulted in weak hybridisation signals suggesting that the putative glycerol channel-encoding gene might have low sequence similarity to *FPS1*. *S. cerevisiae* mutants, in which *FPS1* has been deleted, survive poorly an osmotic downshock due to an inability to rapidly dispose of the accumulated glycerol. This phenotype was used to screen a *Z. rouxii* library for genes that might complement or suppress this growth defect. Analysis of complementing clones revealed genes encoding homologues of the *S. cerevisiae CDC10* and *DOM34* These genes, none of which is a MIP family member, have been implicated in the yeast cytoskeleton, cell cycle, or cell integrity pathway. Functional analysis indicated that the *S. cerevisiae dom34* mutants are sensitive to osmotic stress and that the *Z. rouxii DOM34* complements this growth defect suggesting that the *DOM34* gene is involved in yeast osmotolerance. Physiological and genetic data point to the occurrence of glycerol facilitator protein(s) in the osmotolerant yeasts *Z. rouxii and P. sorbitophila*, previously assumed to permeate glycerol only via active transport and simple diffusion. However, these homologues might have low sequence similarity to *FPS1* as indicated by Southern blot hybridisation and PCR analysis.

INTRODUCTION

Glycerol plays a protective role during osmotic stress and its formation is essential for redox balancing in yeast (Brown, 1990; Ansell *et al.*, 1997). Yeast cells have consequently developed adaptive mechanisms to control glycerol flux within limits suitable for growth including; modulation of glycerol production and dissimilation, conservation and increased retention as well as regulating glycerol transport across the plasma membrane (for review see Prior and Hohmann, 1997; Attfield, 1998). However, the mechanisms of glycerol transport and its intracellular conservation differ considerably between yeasts and may also differ according to growth conditions (Sutherland *et al.*, 1997; Lages *et al.*, 1999).

In *S. cerevisiae*, glycerol conservation is mainly controlled by a transmembrane channel protein Fps1 (Luyten *et al.*, 1995; Sutherland *et al.*, 1997; Tamás *et al.*, 1999). Fps1p is a member of the MIP (Major Intrinsic Protein) family of transport proteins and is closely related to bacterial glycerol facilitators. However, Fps1p is unique among glycerol facilitators; the characteristic NPA motifs found in MIP proteins are not fully preserved. Secondly, Fps1p has long amino- and carboxy- terminal hydrophilic extensions, resulting in a protein of 669 amino acids, compared with the 250-300 amino acids for most MIP proteins (for recent review, Hohmann *et al.*, 2000).

The gene encoding Fps1p was originally identified as a multicopy suppressor of the growth defect of the *fdp1* mutant in controlling glycolysis (van Aelst *et al.*, 1991). Subsequent studies have shown that Fps1p is indeed a glycerol transport protein controlling both glycerol influx and efflux (Luyten *et al.*, 1995; Sutherland *et al.*, 1997). However, Fps1-mediated glycerol transport appears to be more important during efflux than in uptake (Tamás *et al.*, 1999).

S. cerevisiae mutants lacking *FPS1* cannot rapidly release glycerol and subsequently fail to cope with hypo-osmotic stress (Luyten *et al.*, 1995). In addition, *fps1Δ* mutants grow poorly under anaerobic conditions and exhibit slightly diminished osmotic induction of *GPD1* expression (Tamás *et al.*, 1999). Deletion of *FPS1* also causes cell fusion defects during mating (Philips and Herskowitz, 1997) and leads to prolonged phosphorylation of the Mpk1p kinase in the PKC pathway after a hypo-osmotic stress. All these effects are consistent with a role of Fps1p in controlling the intracellular glycerol content. It appears that Fps1p opens during hypo-osmotic conditions to release glycerol and closes during hyper-osmotic conditions thereby conserving glycerol inside the cell (Luyten *et al.*, 1995; Tamás *et al.*, 1999).

Whether a similar mechanism of glycerol conservation occurs in other yeasts is still unknown and no other yeast protein has been reported to be homologous to Fps1p both in structure, function and regulation. Glycerol transport in the osmotolerant yeast *Z. rouxii* is so far known to occur via simple diffusion and by an osmotically active transport system (Edgley and Brown, 1978; Van Zyl and Prior 1990; Van Zyl *et al.*, 1990). The purpose of this study was to explore the possible occurrence of a channel mediated glycerol transport and *FPS1* homologues in the osmotolerant yeasts *Z. rouxii* and *P. sorbitophila*, which in turn might provide additional insights into yeast stress tolerance.

MATERIALS AND METHODS

Strains, growth conditions, and transport experiments

The following yeast strains were used: *Pichia sorbitophila* CBS 7064 (CSIR Y170), *Zygosaccharomyces rouxii* NRRL Y2547, *Z. rouxii* NRRL Y998, *Z. rouxii* NRRL Y225, *Schizosaccharomyces pombe* CBS 5682 (CSIR Y457) and *Debaryomyces hansenii* CBS 0767 (CSIR Y953). These wild-type yeast strains were obtained from the Microbial Resource Centre (MIRCEN), Department of Microbiology and Biochemistry, University of the Free State, South Africa. The various laboratory strains used in this study are listed in Table 1. The *Saccharomyces cerevisiae* strains lacking a glycerol facilitator *fps1Δ::HIS3*, *fps1Δ::LEU2 mpk1Δ::TRP1* (Tamás *et al*, 1999) and *fps1Δ::LEU2 tps1Δ::TRP1* (Luyten *et al.*, 1995) were derived from the haploid laboratory strain W303-1A (Thomas and Rothstein, 1989). The *S. cerevisiae dom34* mutant was constructed and obtained from the laboratory of Dr J Engebrecht (State University of New York). A diploid strain MA121-3 of *Z. rouxii* (Ushio *et al.*, 1988; 1996) was derived from the strain NRRL 2547 (*MATa*, prototrophic [cir^0], i.e., harbouring no plasmid). All wild-type yeast strains were routinely grown on YEPD (10 g l^{-1} yeast extract, 20 g l^{-1} each of peptone and glucose, pH 6). Mutant strains were grown on defined medium (6.7 g l^{-1} yeast nitrogen base, 20 g l^{-1} glucose and amino acids, pH 6) with supplements as indicated in the text. For the growth of the *tps1Δ fps1Δ* double mutant, glucose was replaced by 2% galactose whereas the *fps1Δ mpk1Δ* double mutant was grown in presence of 1 M sorbitol. The *Escherichia coli* cells (TOP10F') were routinely grown at 37°C overnight in LB media (10 g l^{-1} NaCl, 10 g l^{-1} bacto-trypton, 5 g l^{-1} yeast extract). Osmotic stress and transport experiments were carried out as described previously in Chapter 3 and 4.

Molecular and genetic techniques

Yeast and bacterial transformations were carried out using the lithium acetate method (Gietz and Schiestl, 1995) and the calcium chloride method (Maniatis *et al.*, 1982) respectively. In general, nucleic acid manipulations were carried out according to Sambrook *et al.* (1989) unless otherwise mentioned. We routinely isolated plasmid DNA from bacteria using cetyltrimethylammonium bromide (CTAB) following the method of Del Sal *et al.* (1988). To isolate plasmids from yeast, an overnight culture was harvested, disrupted, and homogenised using acid washed glass beads. The homogenised cell suspension was centrifuged (13000 rpm, 5 min) and plasmid DNA was recovered from the supernatant. For sequencing experiments, plasmid DNA was purified using the Nucleobond AX100 cartridges of Macherey-Nagel, Germany (Cat. No. 740521). Yeast genomic DNA was prepared from mid-stationary cultures as described by Sherman *et al.* (1986) whereas total RNA was isolated from exponentially growing yeast cells cultivated in YEPD with or without 5% NaCl. Cells were harvested and lysed with Zymolase (0.2 mg/ml) and β-mercaptoethanol (8 µl/ml) in extraction buffer (1 M sorbitol, 100 mM Na_3-citrate, 60 mM EDTA, pH 7.0). After cell lysis (3 hours at 37°C) and homogenization, total RNA was isolated from the yeast spheroplasts using the Rneasy kit (Qiagen, Cat. No. 74103). Poly A^+ mRNA was purified from total RNA following the oligotex (Qiagen, Cat. No. 70022) purification protocols.

Southern Blot Hybridisation

Digested yeast genomic DNA (10 µg) was fractionated by gel electrophoresis and transferred to a positively charged nylon membrane (Magna Graph, MSI, Cat. No. NJ0HY00010) by capillary blotting (Sambrook *et al.*, 1989). The entire reading frame or a 426 bp fragment of *FPS1* corresponding to the most conserved region (Fig. 1), were generated by PCR from *S.cerevisiae* W303-1A genomic DNA, and used as probes. Hybridisation was carried out overnight at 68°C followed by stringent washes (2 X 5 min, 2 X SSC, 1%SDS at room temperature, then 2 X 15 min, 0.1X SSC, 0.1% SDS) under constant agitation. Less stringent hybridisation was performed at 55°C with a single wash (2 X 5 min, 2 X SSC, 1% SDS) at room temperature. Probe labelling and detection were performed using the DIG system (Boehringer Mannheim).

Polymerase chain reactions

Oligonucleotide primers: Two pairs of specific primer were designed from *S. cerevisiae* sequences. Pair A corresponds to the Fps1p amino acid sequences, ISGAHL (sense) and ARDLGP (antisense) just outside the NPS and NLA boxes respectively (Fig. 1A). Pair B and was designed from the N- and C-termini nucleotide sequences of the *FPS1*.

Pair A

G5 5' ATCTCAGGTGCTCATTTG 3'

G6 5' TGGGCCCAGATCACGAGC 3'

Pair B

FPSFO 5' GCTCTAAACGACTTTCTGTCCA 3'

FPSRE 5' CCATAATGCGAATCTTCTGATG 3'

Also, two pairs of degenerate primers were designed (Fig.1). Pair C was designed from the LNPSIT (sense) and NLARDL (antisense) motifs of the *FPS1* gene. Pair D was designed from the consensus motifs of the entire MIP family LNPAVT (sense) and NPARSF (antisense).

Pair C

sense G1 5' GGGATCCYTNAAYCCNTCNATHAC 3' *BamH*1

antisense G2 5' CGGAATTCRTCNCGNGCNARRTT 3'..... *EcoR*1

Pair D.

sense G3.5'CTTCTAGAYTNAAYCCNGCNGTNAC 3' Xba1

antisense G4.5'CGGAATTCAANSWNCKNGCNGGRTT3'.....EcoR1

IUB (international union of Biochemistry) codes are used to represent mixed positions (degeneracy); R = A/G M = A/C W = A/T S = G/C K = G/T Y = C/T V = A/G/C D = A/G/T H = A/C/T B = G/C/T N = A/G/C/T. With 0.5 μM specific primers, PCR amplifications were carried out (94°C, 1 min; 52°C, 50 sec; 72°C, 1 min; 30 cycles) by 2.5 units of *Taq* DNA polymerase in presence of 200 μM dNTPs with 1.5 mM $MgCl_2$ (Boehringer Mannheim). In all cases template DNA (50-100 ng) was denatured for 2 min at 95°C.

Degenerate Reverse Transcriptase Polymerase Chain reaction (RT-PCR)

To optimize the degenerate RT-PCR procedure for homology cloning (Fig. 1B), several parameters such as primers, $MgCl_2$ and template concentration were adjusted until the desired amplification was reached (Fig. 2). *S. cerevisiae* and *E. coli* from which the MIP homologues are already known were also included as positive controls.

The Titan^{TM} One Tube RT-PCR system (Boehringer Mannheim Cat. No. 1888382) was used in presence of 2.5 μM primers, 1 mM dNTPs and 3 mM $MgCl_2$ concentrations. The reaction also included Dithiothreitol (DTT) at a final concentration of 5 mM, 8 units of RNase inhibitor and the following cycling parameters.

50°C	30 min (reverse transcription)	94°C	2 min (initial denaturation)
94°C	1 min (cycle denaturation)	46°C	1 min (annealing)
68°C	3 min (elongation)	for	35 cycles
68°C	7 min (prolonged elongation).		

Samples were analysed on 1.2% agarose gel. Bands of about 400 bp (typical of the MIP family) were obtained and immediately purified (Boehringer Mannheim purification system Cat. No. 1732676). To obtain large quantities of DNA for subsequent manipulations, purified samples were reamplified. Aliquotes from purified PCR products were digested by respective enzymes, ligated into pUC18 and transformed into *E. coli.* A schematic scheme for the cloning strategy and the optimised conditions are shown in Figure 1B. Plasmid DNA was isolated from positive clones digested and analysed for the presence of the correct insert (Fig 2B). Candidate clones were picked and prepared for sequencing (Thermo sequenase dye terminator cycle sequencing system, Amersham Kit No. 204279). Sequence data analysis and homology searches were performed using the FASTA program and the BLAST network service (National Center for Biotechnology Information).

Complementation experiments

To investigate the occurrence of functional homologues or suppressors in *Z. rouxii*, *fps1*Δ mutants were transformed with a genomic library constructed in the autonomously replicating yeast plasmid pKU24 using partially digested DNA from *Z. rouxii* NRRL 2547 (Ushio *et al.*, 1996). Transformed cells were grown on selective media (YNB-glucose without leucine) for about 5 days until a colony size of about 1-2 mm. About 300,000 transformants were replica-plated on 5% NaCl YEPD plates. After 3 days, salt stressed cells were replica-plated on plates without NaCl (osmotic downshock) and monitored for survival following an osmotic downshock. Fast recovering transformants (compared to the control cells transformed with empty plasmid) were selected and rescreened using spot assay. For these assays, 5 µl of a 10-fold dilution of cell suspension (OD_{600} = 1) was spotted on defined media and monitored for 3 days. Each promising isolate was compared with the wild-type and the *fps1Δ* strain for growth during osmotic stress (hyper- and hypo-osmotic stress), and anaerobic conditions. Isolates were also analysed for the ability to release glycerol after a hypo-osmotic shock as described in detail in chapters 3 and 4. To confirm activity, plasmid loss experiments were carried out by growing the transformants in non selective media (YEPD) followed by several rounds of replica-plating on YEPD agar plates for single colonies. Yeast cells (colonies) that could not grow on selective media (i.e those that had lost the plasmid) were selected and monitored again for survival during osmotic downshock. Plasmids containing the putative complementing or suppressing activity were isolated from yeast cells and subcloned into the shuttle vector YEp*lac*195. The different subclones were transformed back into the *fps1Δ* strain to confirm and localise activity (Fig. 3). Promising subclones were isolated and the corresponding inserts were sequenced for analysis. The DNA fragments were also labelled and used as a probe in Southern blots to confirm that indeed they originate from *Z. rouxii*. In addition to the *fps1Δ* mutant, complementation experiments were also performed using the *tps1Δ fps1Δ* and the *gpd1Δ gpd2Δ* double mutants, which are unable to grow on glucose or on high osmolarity media (such as 3 M glycerol) respectively. The *tps1Δ fps1Δ* double deletion mutant was grown in YEPGalactose and transformed with the *Z. rouxii* library as mentioned above. Complementation in the transformed strains was analysed on YNB-glucose. For the complementation of the *gpd1Δ gpd2Δ* double mutant, cells were transformed with the *Z. rouxii* library and grown on selective media (YNB-glucose without leucine) until colonies were visible. About 20,000 transformants were replica-plated on the same selective plates containing either 2 or 3 M glycerol or 1 M xylitol and monitored for survival for about 7 days.

Heterologous expression of the *S. cerevisiae fps1-Δ1* ungated channel in *Z. rouxii*.

A diploid strain MA121-3 of *Z. rouxii*, was transformed with the truncated construct (*fps1-Δ1*) lacking the N-terminal 13-230 amino acids that are essential for the closing and opening of the Fps1p channel (Tamás *et al.*, 1999). The YEp*FPS1* construct containing the *FPS1* gene on a 3.8 kb *Sal*1/*Hind*111 fragment (Van Aelst *et al.*, 1991) and the construction of YEp*fps1-Δ1* have been described previously (Tamás *et al.*, 1999). The YEp*fps1-Δ1* construct was digested by *Sal*1 and *Hind*111 to release the truncated *FPS1*. The resulting fragment was then gel purified and ligated into the *E. coli* - *Z. rouxii* shuttle vector to create the construct pKU24*fps1-Δ1*. This construct was subsequently transformed into the *Z. rouxii* MA121-3 strain and its effects on osmotolerance monitored.

Table 1. Laboratory yeast strains used in this study

Strain	Genotype	Reference
S. cerevisiae W303-1A	*MAT a leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*	Thomas and Rothstein, 1989
S. cerevisiae YMT 2	W303-1A *fps1Δ::HIS3*	Tamás *et al.*, 1999
S. cerevisiae YSH 6.161.-2D	W303-1A *gpd1Δ::TRP1 gpd2Δ::URA3*	Ansell *et al.*, 1997
S. cerevisiae YSH 302	W303-1A *tps1Δ::TRP1 fps1Δ::LEU2*	Luyten *et al.*, 1995
S. cerevisiae YSH 7.86.-1A	W303-1A *mpk1Δ::TRP1 fps1Δ::LEU2*	Tamás *et al.*, 1999
S. cerevisiae Y743	Σ1278b *MATa / MATα ura3/ura3*	Davis and Engebrecht, 1998
S. cerevisiae Y739	Σ1278b *MATa / MATα ura3/ura3 dom34Δ::LEU2/ dom34Δ::LEU2*	Davis and Engebrecht, 1998
Z. rouxii MA121-3	*MATa / MATα leu2/leu2*	Ushio *et al.*, 1996

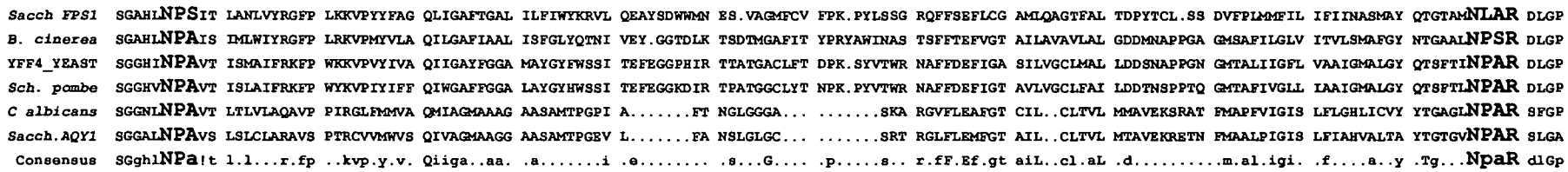

Figure 1A. Partial alignment of Fps1p and other fungal /yeast members of the MIP family

The two most conserved regions (consensus motifs NPA about 400 bp apart) used for designing primers are represented in bold.

Two pairs of degenerate primers were designed. Pair C was designed from the Fps1p amino acid sequences LNPSIT (sense) 5' and NLARDL (antisense). Pair D was designed from the consensus motifs of the entire MIP family (Park and Saier, 1996); LNPAVT (sense) and NPARSF (antisense). The aligned proteins and their accession numbers were, *S. cerevisiae* Fps1p (P23900), *Botrytis cinerea* putative glycerol facilitator (AL112633), YFF4_Yeast, the *S. cerevisiae* putative glycerol facilitator Yfl054p (P43549), *S. pombe* putative glycerol facilitator (SPAC977.17), *Candida albicans* putative aquaporin (contig4-2389), and *S. cerevisiae* aquaporin (AAC69713).

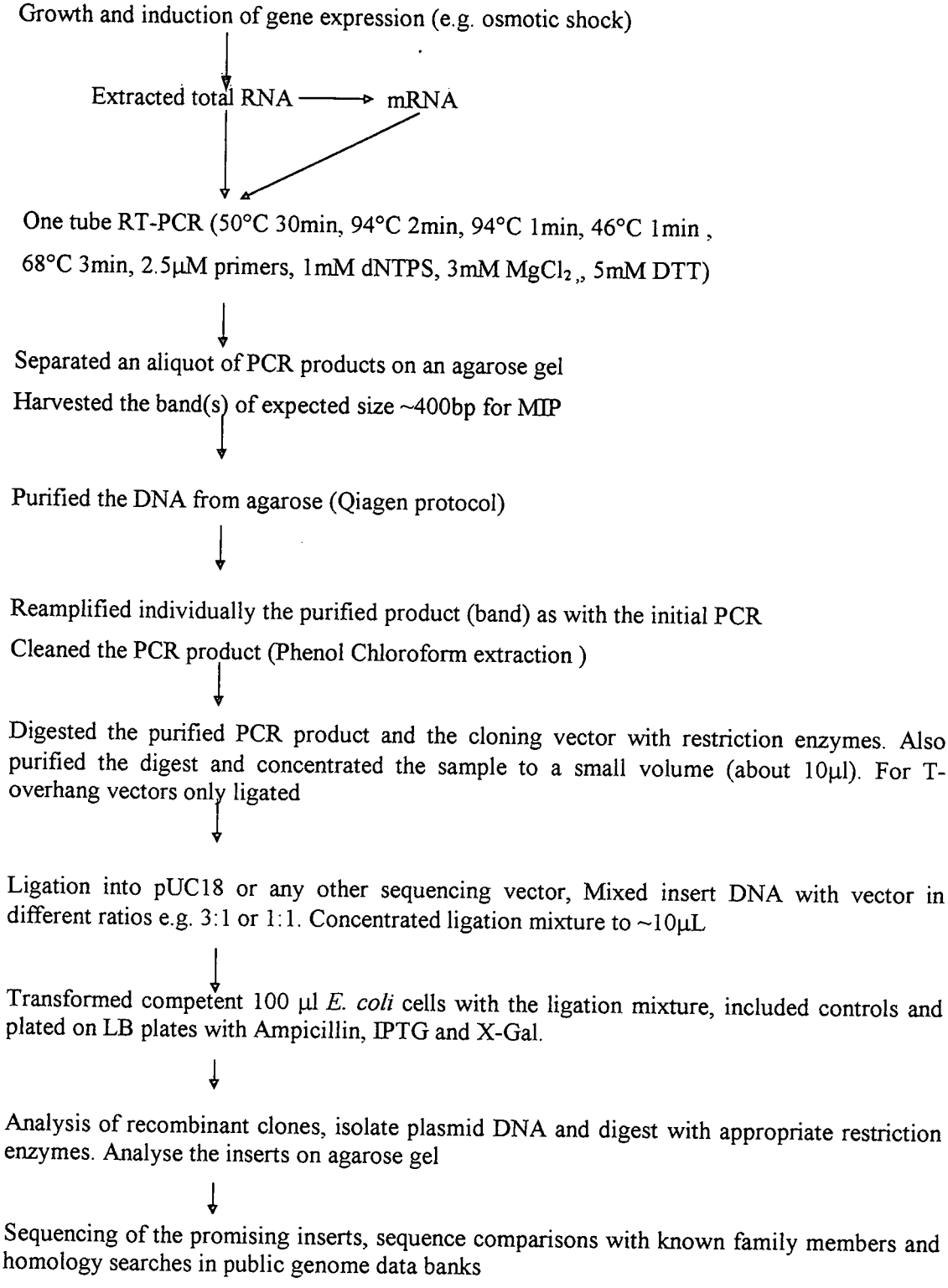

Figure 1B. Optimised procedure used for isolating MIP family genes following the degenerate Reverse Transcriptase Polymerase chain reaction (RT-PCR).

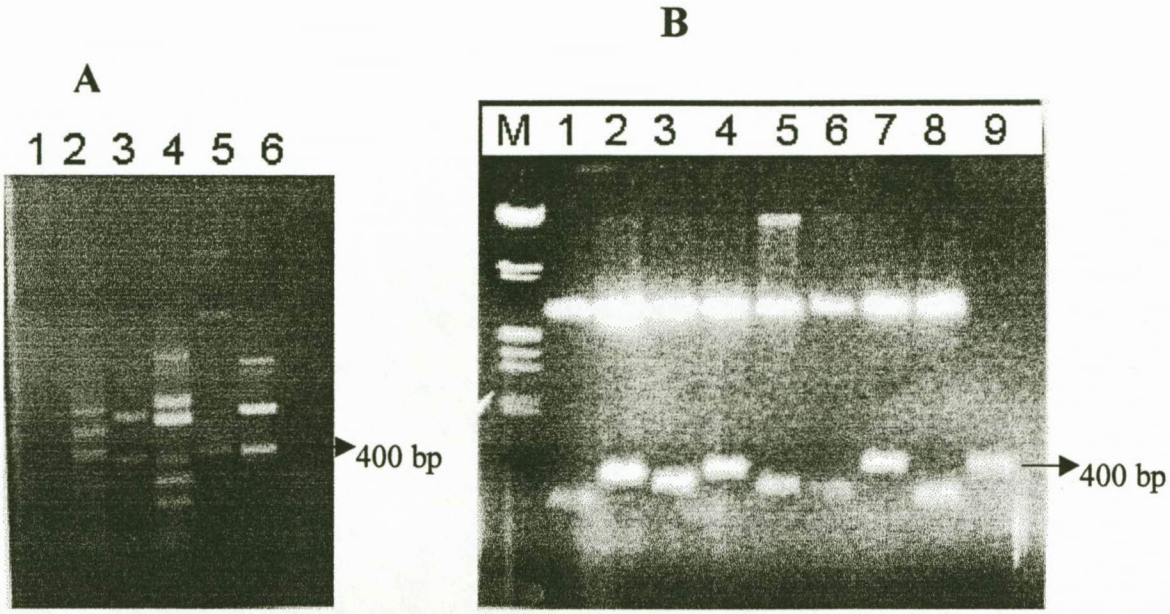

Figure 2. The RT- PCR and cloning of the amplified products.

Reactions were performed as described in materials and methods with different concentrations of primer pair D (panel A); Lanes: 1, 0.5 μM; 2, 1.5 μM; 3, 2.5 μM; 4, 5.0 μM with *Z. rouxii* RNA and lanes 5, 2.5 μM (*S. cerevisiae* RNA) 6, 2.5 μM (*E. coli* RNA). Cloned PCR products (350-450 bp) were analysed by restriction digestion and gel electrophoresis (Panel B; lanes 1-8). Insert sizes were compared with the *FPS1* control (lane 9) and *EcoR*1+*Hind*111 lambda markers (M).

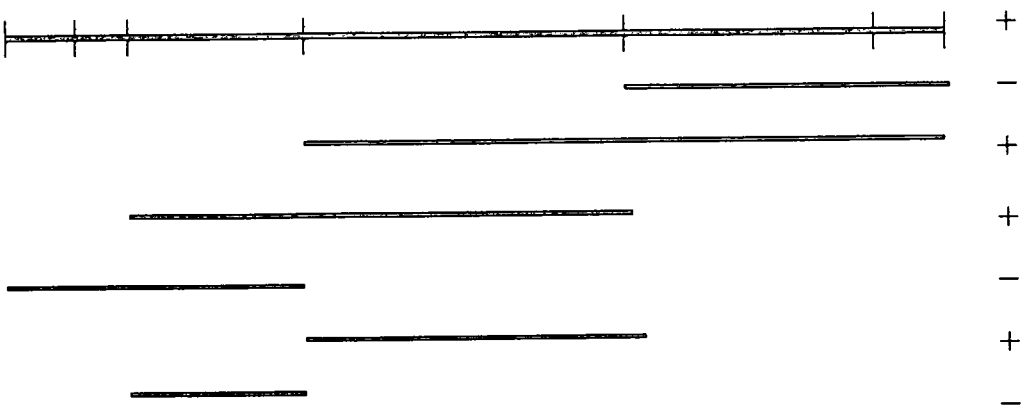

Figure 3. Restriction map, subcloning and functional analysis of a 4.6 kb insert in plasmid pZrcomp9. Plasmid DNA was digested with different restriction enzymes; *Hind*111 (H), *BamH*1 (B), *Xba*1 (X), *Sph*1 (S). The digested DNA fragments were purified, cloned into the yeast vector YEp*lac*195 and transformed into the *fps1Δ* strain. Subclones that could still complement the downshock senstivity are indicated with a plus sign (+) while those that lost activity are indicated with a minus sign (-). Not drawn according to scale.

RESULTS AND DISCUSSION

Analysis of *FPS1* homologues by PCR and DNA probes

Although glycerol accumulation in *Z. rouxii* occurs by an osmotically active transport system (Van Zyl *et al.*, 1990), the pattern and kinetics of glycerol release upon an osmotic downshock (Fig. 4) suggest the presence of a channel-mediated transport system similar to that of *S. cerevisiae* Fps1p (Chapter 3). However, when *Z. rouxii* was transformed with the construct pKU24*fps1-Δ1* encoding constitutively open glycerol channel from *S. cerevisiae*, the ability to grow on high salt concentration was only slightly affected unlike *S. cerevisiae* where the same construct severely affected growth on media containing 1 M NaCl (data not shown). We therefore explored the occurrence of *FPS1* homologues in osmotolerant yeasts using DNA and PCR probes. Digested genomic DNA from various yeasts was separated by agarose gel electrophoresis, blotted on nylon membranes and probed with either the entire *FPS1* reading frame or a 426 bp fragment corresponding to the most conserved regions in the MIP family (Fig. 1A). Under stringent conditions, hybridisation was only observed in *S. cerevisiae*. However, with less stringent conditions, hybridisation also occurred in the three *Z. rouxii* strains investigated (Fig. 5A). Two bands were observed in *BamHI/HindIII* digests of *Z. rouxii* strains, the strongest of which was approximately 6.1 kb. More bands could be observed under non-stringent conditions when the entire *FPS1* reading frame was used as a probe. Some degree of DNA to DNA cross-hybridisation has been observed among bacterial MIP family genes (Calamita *et al.*, 1995). Lack of strong hybridisation to the *FPS1* probes under stringent conditions might suggest a low nucleotide sequence similarity with genes from other yeasts. This is consistent with the observation that MIP family genes generally share low nucleotide sequence similarity and some members display less than 20% identity. The *S. cerevisiae* Fps1p appears to be the most divergent MIP family protein so far known (Hohmann *et al.*, 2000). For example, neither of the family's signature NPA boxes is fully preserved, being NPS and NLA, respectively (Fig. 1A). Fps1p also differs from other MIP family members by having long amino- and carboxy-terminal hydrophilic extensions. The protein has 669 amino acids, compared with 250-300 amino acids for most of the family members (Van Aelst *et al.*, 1991).

The occurrence of *FPS1* homologues in various yeasts was further investigated by PCR using specific primers (Pair B) directed against the end terminals and conserved portion of *FPS1* (Pair A). Apart from *S. cerevisiae*, no amplification was observed from other strains. Amplification was, however, achieved with degenerate primers corresponding to *FPS1* amino acid sequences LNPSIT (sense) and NLARDL (antisense). Since several fragments ranging from 300 to 600 bp were obtained, Southern blots were prepared and probed with the *FPS1* gene (426 bp fragment) to find out which of these PCR products was homologous to *FPS1*. Under stringent conditions, only a single band of about 400 bp from *S. cerevisiae* hybridised (not shown). However, with less stringent conditions, strong hybridisation occurred with *S. cerevisiae* and with *P. sorbitophila* (Fig. 5B) and all hybridising fragments were between 400-500 bp. Weak signals were also observed for *Z. rouxii* (data not shown). Physiological and genetic analysis pointed to a possible existence of a glycerol facilitator in *Z. rouxii* that might be homologous to MIP channel proteins. These observations promoted us to clone the corresponding gene(s) using RT-PCR (Fig. 1B) and degenerate primers from the conserved portions of the MIP family (Fig. 1A). Successful PCR amplification was observed in *S. cerevisiae* and *E. coli* as well as in *Z. rouxii* (Fig. 2). Products within the expected size range of 350-450 bp were cloned for further analysis. Sequence analysis of the cloned fragments from *S. cerevisiae* and *E. coli* yielded sequences corresponding to previously reported members of the MIP family (Park and Saier, 1996). However, analysis of 32 inserts from *Z. rouxii* revealed sequences that were neither related to *FPS1* nor to others members of the MIP family.

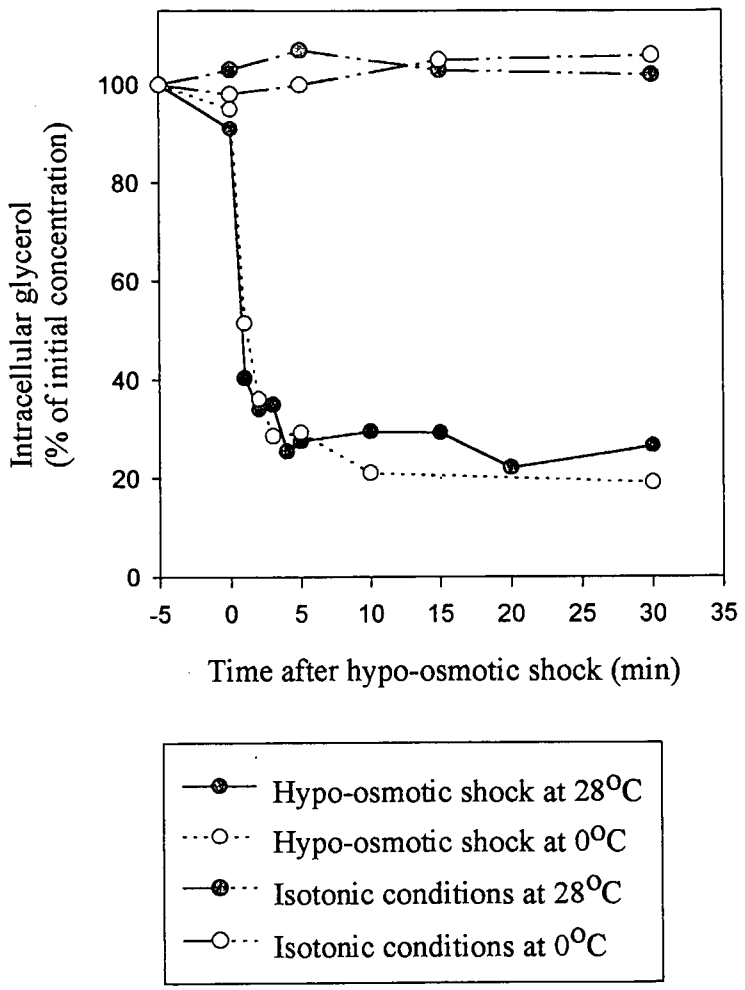

Figure 4. Intracellular glycerol content after a hypo-osmotic shock (0.86 M NaCl to 0.09 M NaCl) in *Z. rouxii* NRRL Y2547 grown in glucose-YNB.

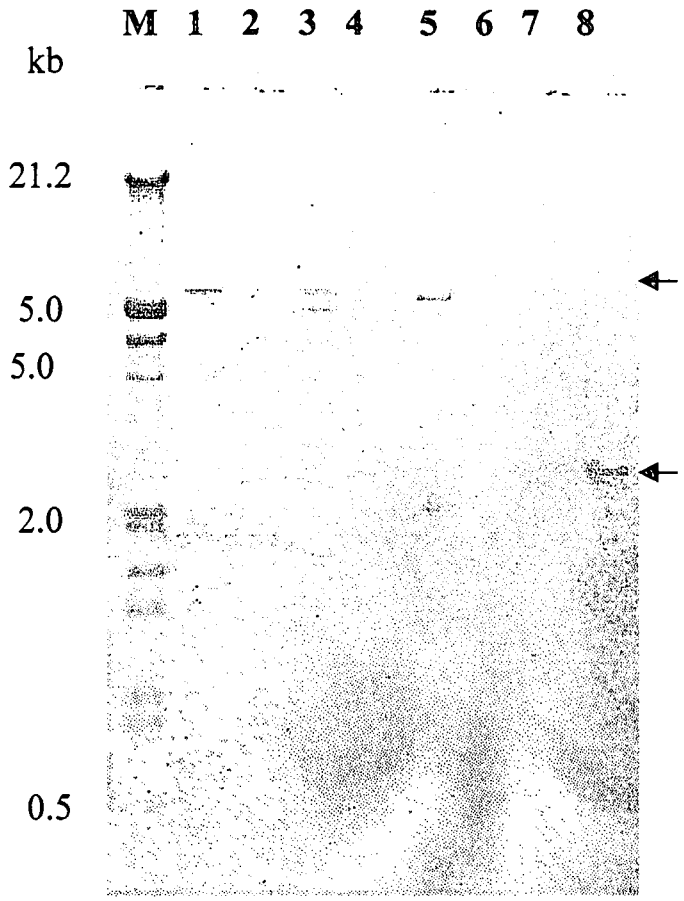

Figure 5A. Southern blot analysis: The *FPS1* (426 bp) probe hybridised to restriction digest (*Bam*H1 /*Hind*111) of genomic DNA from (1) *Z. rouxii* NRRL Y998, (2) *P. sorbitophila* CBS7064, (3) *Z. rouxii* NRRL Y2547, (4) *S. pombe* CBS 5682, (5) *Z. rouxii* NRRL Y225, (6) *D. hansenii* 0767, (8) *S. cerevisiae* W303-1A under medium stringent conditions: Hybridisation at 55°C overnight, followed by 2 X 10 min wash in 2 x SSC , 0.1% SDS at RT. The probes were generated by PCR using primers; oligoG5 5' ATCTCAGGTGCTCATTTG 3' and oligoG6 5' TGGGCCCAGATCACGAGC 3' which corresponds to the Fps1p amino acid sequences, ISGAHL (sense) and ARDLGP (antisense) respectively.

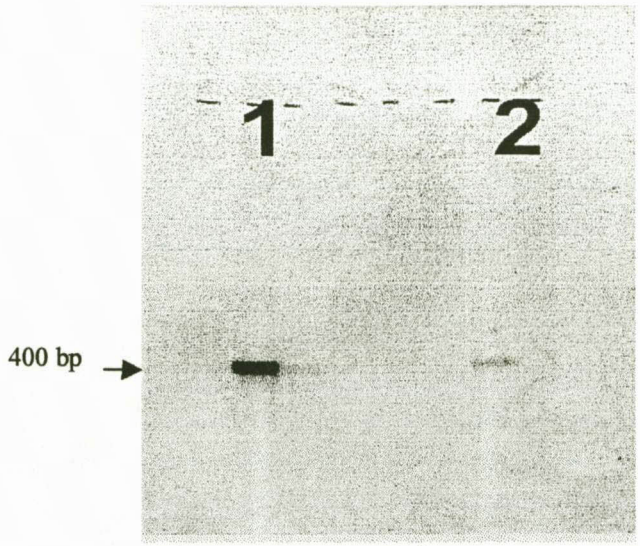

Figure 5B. Degenerate PCR products were probed with a probe from the most conserved fragment of *FPS1*. Strong hybridisation occurred on *S. cerevisiae* (1) as well as on *P. sorbitophila* (2).

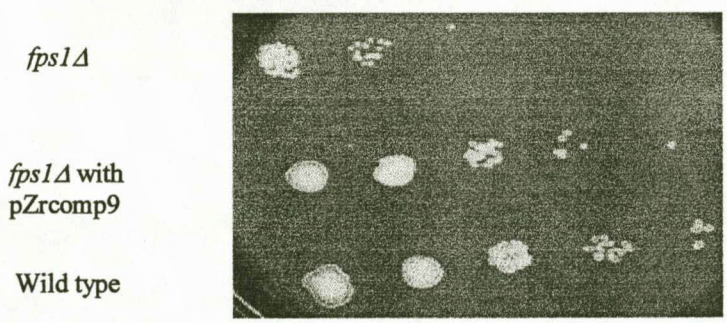

Figure 6. Complementation analysis: *fps1Δ* mutants were transformed with plasmids containing *Z. rouxii* genes (genomic library) and monitored for survival following a hypo-osmotic shock. Promising colonies were further re-screened using a spot assay.

Complementation analysis

Since no *FPS1* homologues were found using thePCR method, an alternative approach. Was tried. *S. cerevisiae* mutants lacking Fps1p do not show a growth defect on glycerol as a sole carbon source but show defects related to glycerol export and glycolysis that could be utilised in investigating the occurrence of functional homologues or suppressors by complementation analysis. Transforming the *tps1Δ fps1Δ* double mutant with a multcopy plasmid carrying the *FPS1* gene or the *E. coli* glycerol facilitator *glpF*, has been shown to restore growth on fermentable carbon sources (Luyten *et al.*, 1995). Unfortunately, when this double mutant was transformed with the *Z. rouxii* library, nearly 25% of the transformed cells grew on glucose including a control without insert. The use of the *mpk1Δ fps1Δ* mutant, which can not grow in media without osmotic stabilisers (Tamás *et al.*, 1999), was also unsuccessful. The growth of the osmosenstive *gpd1Δ gpd2Δ* double mutant is known to be improved by glycerol transporters during hyper-osmotic stress (Karlgren *et al.*, 2000) and was thus used for complementation analysis. Out of the 20,000 transformants screened, 14 colonies survived at 2 M glycerol but they grew much slower compared to the control experiments even after 10 days (data not shown) and were therefore not considered for further analysis.

The most clear-cut *fps1Δ* phenotype so far observed is that mutants survive poorly an osmotic downshock (hypo-osmotic shock) due to inability to dispose of accumulated glycerol. This defect is partially suppressed by over-expression of plant and bacterial glycerol facilitators (Luyten *et al.*, 1995; Weig *et al.*, 2000). From about 300,000 *fps1Δ* colonies transformed with a *Z. rouxii* library, 9 transformants grew almost like the wild-type in 24 hours of hypo-osmotic stress; the rest of the transformants grew much slower. Promising transformants were re-screened using a spot assay (Fig. 6). Complementing plasmids were isolated and the inserts analysed by restriction digestion mapping (Fig. 3) and sequencing.

Sequence analysis of the most strongly complementing fragments revealed genes that play a role in yeast cytoskeleton, cell cycle or the cell integrity pathway, that are unrelated to the MIP family. Of the most promising plasmids, pZrcomp9 contained an insert encoding a gene homologous to the *S. cerevisiae CDC10* gene encoding a septin (Fig. 7A). Septins belong to a family of conserved proteins that has been implicated in a variety of cellular functions and in changes in the cell shape. The biochemistry and localisation of septins suggest that they form a novel cytoskeletal system or that they function as scaffolds for the assembly of signalling complexes (for review Flescher *et al.*, 1993; Longtine *et al.*, 1996; Field and Kellogg, 1999).

Septin mutations affect the yeast cytoskeleton, budding, morphogenesis and consequently cell integrity (Cid *et al.*, 1998). The mechanism by which the *Z. rouxii CDC10* improved growth of the *fps1*Δ mutant during hypo-osmotic stress is still unclear as it does not appear to improve glycerol release (Fig. 8). However, there appears to be a link between cell cycle, osmoregulation, cell integrity and the external environment (Chowdhury *et al.*, 1992; Brewster and Gustin, 1994; Shiozaki and Russell, 1995; Degols, *et al.*, 1996; Schoch *et al.*, 1997; Fillinger, *et al.*, 2000).

The yeast *DOM34* is required for osmotolerance

While screening for genes from the *Z. rouxii* library that could alleviate the growth defects of the *S. cerevisiae fps1Δ* mutant in hypo-osmotic media, we isolated plasmid pZrcomp9A that contained an insert with a full ORF homologous to *DOM34* (Fig. 7B). The *S. cerevisiae DOM34* gene (Duplication Of Multilocus region) is similar to genes found in diverse eukaryotes and archaebacteria. Analysis of *dom34* mutants showed that they are defective in multiple development pathways such as the failure to undergo sporulation, exhibit a G1 delay and fail to correctly execute pseudohyphal development (Davis and Engebrecht, 1998).

We investigated whether the yeast *DOM34* gene could also be relevant in osmotolerance. Preliminary experiments indicated that the *S. cerevisiae dom34* mutants are sensitive to osmotic stress and that the *Z. rouxii DOM34* complements this growth defect (Fig.9).

CONCLUSION

In *S. cerevisiae*, glycerol efflux is regulated and mediated by the MIP family glycerol channel Fps1p. Osmotolerant yeasts such as *Z. rouxii* appear to have a similar protein-mediated glycerol release mechanism under hypo-osmotic stress. Whether the protein(s) mediating the rapid glycerol efflux in *Z. rouxii* is homologous to Fps1p in structure and function required further investigation. Recent studies have indicated that most of the genes involved in glycerol metabolism are very well conserved among yeasts. For instance, the *Z. rouxii GPD1* (AJ251481), *DAK1* (AJ294719) and *HOG1* (AB012146) are highly homologous to those from *S. cerevisiae* and *S. pombe*. Furthermore, DNA to DNA cross-hybridisation among glycerol channel encoding genes has been observed in bacteria so we theoretically anticipated cross

hybridisation between *FPS1* and its homologues from other yeasts especially *Z. rouxii* whose G+C content is closely related to that of *S. cerevisiae* (Kreger- van Rij, 1984).

However, lack of strong hybridisation signals with *FPS1* probes suggests that the putative glycerol channel-encoding gene may be divergent from *S. cerevisiae FPS1*. It is also possible that *Z. rouxii* releases osmolytes via other mechanisms as observed in bacteria where osmolyte export after hypo-osmotic shock is mediated by mechanosensitive channels. Thus, cloning and identification of the putative glycerol facilitating proteins was attempted to obtain additional information on the molecular mechanisms of glycerol transport in yeast.

A versatile homology –based cloning procedure was developed but efforts to isolate glycerol facilitators from *Z. rouxii* and *P. sorbitophila* were not successful most likely due to structural diversity amongst fungal glycerol channel encoding genes. Possibly, the use of short oligonucleotide probes or antibodies, although laborious, might provide alternative strategies in identifying glycerol facilitators in other yeasts.

A large number of eukaryotic secondary transport systems have been cloned by complementation of yeast mutants defective in the corresponding transporter (Kranz and Holm, 1990; Anderson *et al.*, 1992; Sentenac *et al.*, 1992; Fromer *et al.*, 1993). The use of complementation strategies to isolate functional homologues yielded genes that are unrelated to *FPS1* and the mechanism by which these genes (homologous to cell division control genes) improved growth of the *fps1Δ* strain during hypo-osmotic stress is still unclear. Overall, the study signifies the complexity of yeast osmoadapation and suggests that *FPS1* might be involved in other physiological process in addition to glycerol export.

A

```
S. cerevisiae  CDC10    ALKRLTEIANVIPVIGKSDTLTLDERTEFRELIQNEFEKYNFKIYPYDSEELTDEELELN
Z. rouxii Put  CDC10    ALKKLTEIANVIPVIAKADTLTLEERAQFREIIQQEFKKHKFRIYPYDTDELTEEELELN
C. albicans    CDC10    ALKKLSEIANVVPIIAKSDSLTLDERSEFKKLLQSEFMKYNFNIYPYDSEDLYEEERQLN
S. pombe       SPN2     VLKRLTEVVNVVPVIAKSDSLTLEERAAFKQQIREEFVKHDINLYPYDSDDADEEEINLN
                        .**:*:*:.**:*:*.*:*:***:**: *:: ::.** *:.:.:****::: :** :**

                        RSVRSIIPFAVVGSENEIEINGETFRGRKTRWSAINVEDIN-QCDFVYLREFLIRTHLQD
                        ESIRSIVPFAVVGSEREIEVNGETFRGRKTRWGAVNVEDIN-QCEFVYLREFLIRTHLED
                        EDIKSLIPFAIAGSETEIEINGEMVRGRKTKWGAINIEDVS-QCEFVFLRDFLTRTHLQD
                        AAVRNLIPFAVVGSEKAIIVDGRPIRGRQNRWGVVNVDDEKPLRVCFFFVTFLMRTHLQD
                          ::.::***:.***  * ::*. .***:.:*..:*::* .     .::  ** ****:*

                        LIETTSYIHYEGFRARQLIALKENANS--RSS-----AHMSSN-AIQR------------
                        LIETTSYIHYEGFRARQLIALKENASS--RSS-----AGPANGGAYQPLIRLEWIGTLAL
                        LIETTALTHYETFRSKQLIALKENASNPNRQSQLQKDQGQTSQQSNQDLKNASGVPNAPM
                        LIETTSYYHYEKFRFKQLSSLKEQSSLATRMG-----SPAPVYPSEPHLHTATAQ-----
                        *****:  *** ** :** :***::.   * .        .   :

                        -----------
                        VLF---------
                        FQSTTGTAAAR
                        -----------
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B

```
S.cerevisiae Dom34  AWYGEKEVVKAAEYGAISYLLLTDKVLHSDNIAQREEYLKLMDSVESNGGKALVLSTLHS
Z.rouxii putDom34   AWYGEAEVMKAVDLGAVNTLLITDTLMRSDDIDQRKRFLELAQQVERLGGKVAVF-----
Drosophila Pelota   AFYGKKHVLQAAESQAIETLLISDNLFRCQDVSLRKEYVNLVESIRDAGGEVKIFSSMHI
                    *:**: .*::*.:  *:. **::*.:::.:::  *:.:::* :.:.  **:. ::
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Figure. 7. Partial sequence alignment of the putative *Z. rouxii CDC10* with its closest fungal homologues; *S. cerevisiae CDC10* (P25342), *Candida albicans CDC10* (P39827), *S. pombe* septin homologue *SPN2* (Q09116) (Panel A). Partial alignment of the putative *Z. rouxii* DOM34 with the *S. cerevisiae DOM34* (P33309) and the *Drosophila melanogaster* PELOTA protein (P48612) (Panel B).

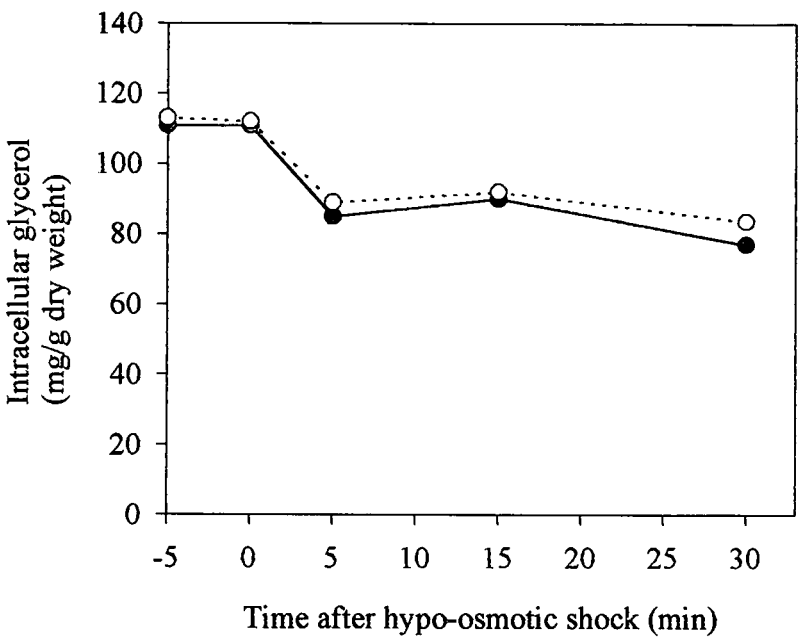

Figure 8. Glycerol release from the *S. cerevisiae fps1Δ* mutant transformed with pKU24 only (open cycles) or with pZrcomp9 (filled cycles) when the yeast cells were subjected to hypo-osmotic stress from 0.86 M NaCl (0.972 a_w) to 0.09 M NaCl (0.996 a_w).

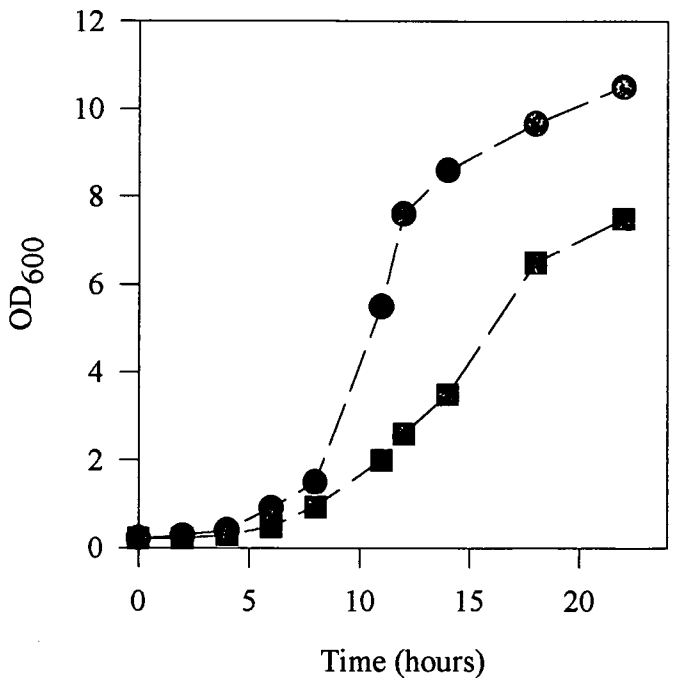

Figure 9A. Growth of the *S. cerevisiae* wild-type at 30°C in YEPD media without osmoticum (circles) or with 1 M sorbitol (squares).

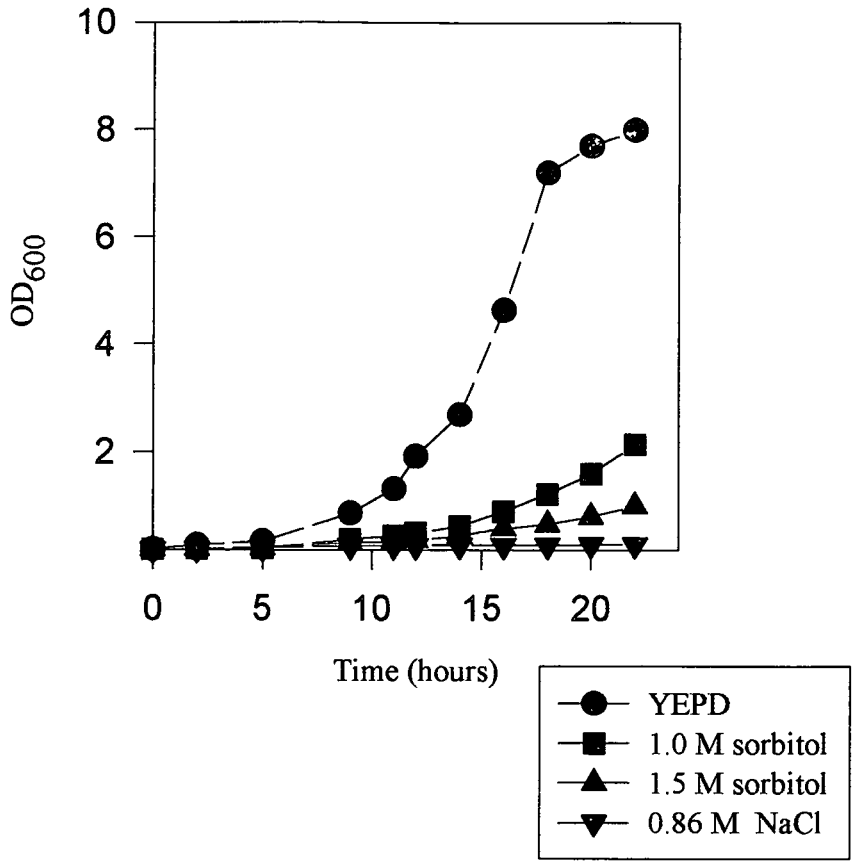

Figure 9B. Growth of the *S. cerevisiae dom34* mutant at 30 ^{o}C in YEPD media with different osmotica.
Control without osmoticum (circles), 1 M sorbitol (squares), 1.5 M sorbitol (triangles) and 0.86 M NaCl (inverted triangles).

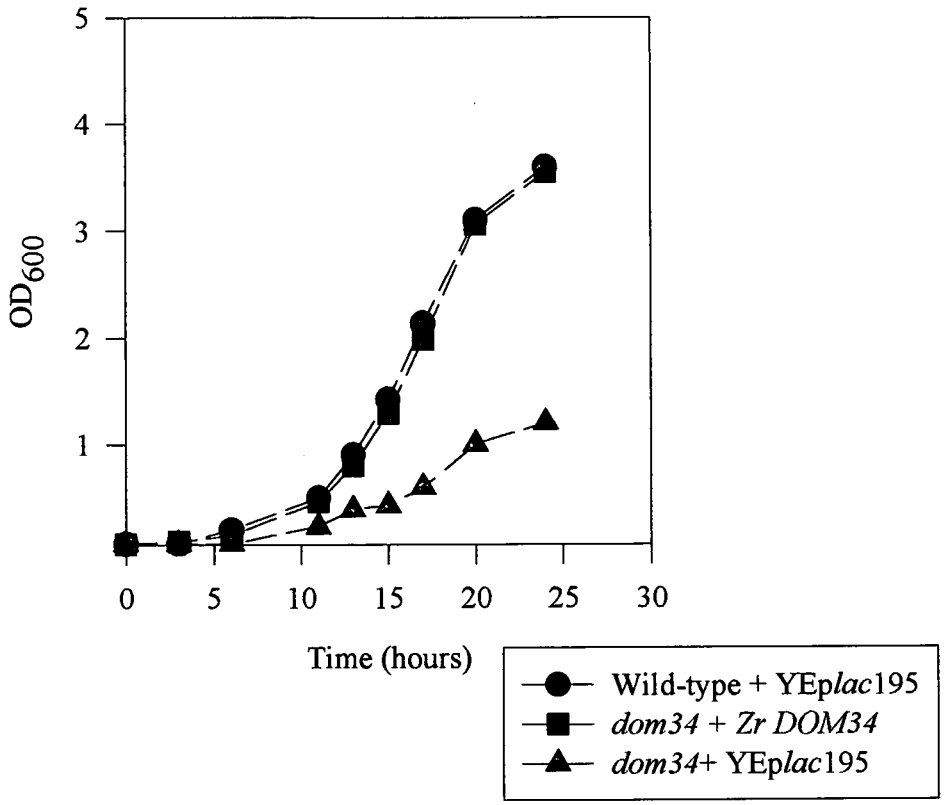

Figure 9C. *Z. rouxii DOM34* complements the osmosenstive phenotype of the *S. cerevisiae dom34* mutant.
The *S. cerevisiae* wild type and the *dom34* mutant strains were transformed with either the YEp*lac*195 containing *Zr DOM34* or with the empty vector. Growth of the transformed strains was then monitored in YNB-glucose containing 1 M sorbitol.

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CHAPTER 6

Isolation and Characterisation of the TIM10 Homologue from the Yeast *Pichia sorbitophila*: A putative component of the mitochondrial protein import system.

Abstract

The *Saccharomyces cerevisiae TIM10* gene encodes one of the few essential mitochondrial proteins that are required for the import of nuclear–encoded precursor proteins from the cytosol and their subsequent sorting into the different mitochondrial compartments. While searching for *FPS1* homologues, we isolated and characterised a putative homologue of *TIM10* from the halotolerant yeast *Pichia sorbitophila.* The *Pichia TIM10* gene encodes a protein of 90 amino acids with 66% identity to *S. cerevisiae* Tim10p. It was capable of suppressing the temperature sensitivity of *tim10-1* mutant in *S. cerevisiae* suggesting that *Pichia TIM10* is both a functional and structural homologue of *S. cerevisiae TIM10*. The putative *Pichia TIM10* gene product contains all the four conserved cysteine residues and the two CX_3C motifs typical of the Tim family proteins in the mitochondrial intermembrane space. Using anti-Tim10p serum, Western blots detected a protein of about 10-kDa and suggested that the *Pichia* Tim10p is a mitochondrial protein. The results suggest that mitochondrial import and sorting systems might be also strongly conserved in other fungi. The coding sequence of the *P. sorbitophila TIM10* has been deposited in the EMBL Nucleotide Sequence Database under Accession Number **AJ243940**

INTRODUCTION

The uptake of nuclear-encoded precursor proteins into the mitochondrion and their subsequent sorting into the different mitochondrial compartments are mediated by translocases; many of which have recently been identified. These include translocases in the outer membrane-TOM system and corresponding proteins in the inner membrane-the TIM system (for review, Rassow *et al.*, 1999). Translocase components in the intermembrane space include Tim9p, Tim10p and Tim12p which are essential for mitochondrial biogenesis and viability of yeast cells (Jarosch *et al.*, 1996, 1997; Sirrenberg *et al.*, 1998; Koehler *et al.*, 1998; Adam *et al.*, 1999).

Precursor proteins carrying a cleavable amino-terminal targeting sequence are imported into the mitochondria by a general import pathway composed of cytosolic chaperons, outer membrane receptors and a general import channel (Hachiya *et al.*, 1995; Hill *et al.*, 1998). After crossing the outer membrane, these proteins are translocated into the matrix by the TIM17-23 complex in co-operation with Tim44p and the mitochondrial Hsp70p (Rassow *et al.*, 1999). However, many integral inner membrane proteins such as the metabolite carriers, are synthesised without the amino- terminal targeting signal and therefore translocated by a separate import machinery. This import machinery has recently been identified and shown to consist of the intermembrane space proteins Tim9p, Tim10p Tim12p, and the inner membrane proteins Tim22p and Tim54p (Koehler *et al.*, 1998). Upon entering the intermembrane space, multispanning carrier proteins first bind to Tim9/10 complex, are then handed over to Tim12p, and finally enter the inner membrane via Tim22p (Rassow *et al.*, 1999).

Despite their high sequence similarity, Tim9p, Tim10p and Tim12p are not functionally equivalent and neither can one substitute for the other suggesting a co-operative mode of action in mitochondrial preprotrein import. Tim10p and Tim12p were first discovered as multicopy suppressors of mitochondrial RNA splicing defects and were initially termed Mrs11p and Mrs5p (Waldherr *et al.*, 1993; Jarosch *et al.*, 1996, 1997). Tim9p has only been recently identified from a screen of other import components that interact with Tim10p (Adam *et al.*, 1999). Here, we report on the molecular cloning and functional characterisation of a gene from the halotolerant yeast *Pichia sorbitophila* that shares high sequence similarity to the Tim9-Tim10-Tim12 family of mitochondrial preprotein transporters. The cloned gene exhibits 66% amino acid identity to Tim10p and was capable of restoring growth of the *TIM10* temperature-sensitive mutants of *S. cerevisiae* at 37°C. The putative *Pichia TIM10*

gene product contains all the four typical cysteine residues and also shows similarity to zinc-finger proteins.

MATERIALS AND METHODS

Strains and growth conditions

The following yeast strains were used: *Pichia sorbitophila* CBS 7064 (CSIR Y170), *Zygosaccharomyces rouxii* NRRL Y2547, *Schizosaccharomyces pombe* CBS 5682 (CSIR Y457) and *Debaryomyces hansenii* CBS 0767 (CSIR Y953) were obtained from the Industrial Biotechnology Microbial Resource Centre, Department of Microbiology, University of Orange Free State (South Africa). The temperature-sensitive (*tim10-1*) mutant of *S. cerevisiae* (Koehler *et al.*, 1998) and its isogenic wild type strain GA74-6A, (*MATα leu2, ura3, his3, trp1, ade8*) were kindly provided by Dr C.M. Koehler. All yeast strains were grown in the YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or defined medium (2% glucose, 0.67% yeast nitrogen base) supplemented with the required amino acids. *Escherichia coli* cells (TOP10F' InVitrogen) were routinely grown at 37° C overnight in LB media (1% NaCl, 1% bacto-tryptone, 0.5% yeast extract).

Nucleic acid manipulation and analysis

Standard procedures were carried out according to Sambrook *et al.* (1989) unless otherwise mentioned. Yeast genomic DNA was isolated as described by Sherman *et al.* (1986). The polymerase chain reaction was used to screen *P. sorbitophila* for the occurrence of membrane channel proteins homologous to the *S. cerevisiae* Fps1p. The following primers were consequently designed from *S. cerevisiae FPS1* sequences.

sense 5' ATCTCAGGTGCTCATTTG 3'

antisense 5' TGGGCCCAGATCACGAGC 3'

PCR amplifications were carried out (92°C, 2 min.; 52°C, 50 sec; 72°C, 1 min; 30 cycles) with 0.5μM specific primers, 2.5 units of Taq DNA polymerase, 200 μM dNTPs, 1.5 mM $MgCl_2$ (Boehringer Mannheim) with *P. sorbitophila* genomic DNA as a template. A PCR product of about 400 base pairs was purified (High pure™ PCR purification kit; Boehringer Mannheim) and then used to probe the *P. sorbitophila* (CBS 7064) genomic library in

YEplac352 (Hill *et al.*, 1986). The insert DNA of one hybridising clone was digested with several restriction endonucleases. Relevant restriction fragments were purified using the Qiagen kits and then subcloned into the sequencing vector pUC18. Both strands of each subclone were sequenced with an automated sequencer using the thermo sequenase dye teminator cycle sequencing kit (Amersham). Sequence data analysis and homology searches were performed using the FASTA program and the BLAST network service (National Center for Biotechnology Information). The 1.5 kb *Eco*RI/*Sal*I fragment containing the entire putative *TIM10* homologue was considered for further investigation. Southern blot hybridisation was carried out overnight at 68°C followed by stringent washes; 2 x 15min, 0.1 x SSC, 0.1% w/v SDS at 68°C (room temperature for medium stringent washes) under constant agitation. Probe labelling and detection were performed using the digoxigenin (DIG) system (Boehringer Mannheim).

Isolation of mitochondria and western blotting

For preparation of mitochondria, *P. sorbitophila* and *S. cerevisiae* were grown to stationary phase (OD_{600} = 3) in the medium containing per litre: 25 ml of 80% lactic acid, 3.0 g yeast extract, 0.5 g glucose, 0.5 g $CaCl_2$. $2H_2O$, 0.6 g $MgCl_2.2H_2O$, 1.0 g KH_2PO_4, 1.0 g NH_4Cl, and 8.0 g NaOH. The final pH was adjusted to 5.5 with KOH (Daum *et al.*, 1982).

Homogeneous preparations of yeast mitochondria were isolated from spheroplasts by differential centrifugation as described (Glick and Pon, 1995). Protein concentrations were determined using the Bradford assay (Read and Northcote, 1981). Mitochondrial proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) consisting of a 4% stacking gel and a 15% separating gel (Laemmli, 1970). Separated proteins were either stained by Coomassie Blue or transferred to the polyvinylidine difluoride membrane by semidry electroblotting. Membranes were probed with a polyclonal antibody raised against *S. cerevisiae* Tim10p (kindly provided by Dr C. M. Koehler). Other procedures for immunoblotting and immunodetection were performed with the chemiluminescence western blotting kit (Boehringer Mannheim).

Functional complementation analysis

The *tim10-1* temperature sensitive mutant of *S. cerevisiae* was transformed with both single and multicopy plasmids harbouring the putative *TIM10* homologue from *P. sorbitophila.* As a negative control, temperature-sensitive mutants and the wild-type strain GA74-6A were transformed with the empty vectors YEplac195, Yeplac181 and YCplac111 (Gietz and

Sugino, 1988). Yeast transformations were carried out using lithium acetate (Gietz and Schiestl, 1995). Functional complementation was analysed by comparing growth of transformants at 25, 30 and 37°C. For plate assays, 5 µl of a 10-fold dilution of cell suspension (OD_{600} = 1) was spotted on defined media and monitored for 3 days. To compare growth rates in liquid media, transformants were cultivated in 50ml cultures and monitored spectrophotometrically for 3 days. All experiments were done in triplicate and the results of a representative experiment are shown

RESULTS AND DISCUSSION

Isolation and characterisation of the *Pichia TIM10 gene*

During the analysis of membrane channel proteins homologous to the yeast glycerol facilitator Fps1p, a 4.6 kb DNA fragment was isolated from *P. sorbitophila* genomic library and digested into smaller fragments for subcloning and sequencing. The sequence of the 1.5 kb *Eco*RI/*Sal*I fragment revealed an open reading-frame with homology to the Tim9-Tim10-Tim12 family of mitochondrial intermembrane space proteins. As shown in Figure 1, there is an uninterrupted open reading frame that codes for a protein of 90 amino acids with a predicted molecular weight of 9,935 kDa and a pI of 5.54. The gene has a GC content of 44% and a codon adaptation index (CAI) of 0.186. Similarly, the homologous genes *TIM10, TIM12* and *TIM9* from *S. cerevisiae* exhibit CAI of 0.24, 0.07 and 0.22 respectively which implies that codon usage is little biased and that these genes are poorly expressed (Bennetzen and Hall, 1981, Sharp *et al.*, 1986). Southern blotting confirmed that, indeed, the cloned *TIM10* gene is from *P. sorbitophila* by revealing strong signals of about 4 kb and 5.1 kb in a *Eco*RV and *Hin*dIII digestions respectively (Figure 2). Cross hybridisation was also observed in other yeasts (*S. cerevisiae, Z. rouxii, Sz. pombe* and *D. hansenii*) under medium stringency hybridisation conditions (data not shown). Since phylogenetic analysis points to the *Pichia* homologue being most closely related to Tim10p (Figure 3) based on sequence identity and size, the gene has been tentatively designated as *Pichia TIM10.*

Similarity to other proteins in the public databases; the Tim family of proteins in the mitochondrial intermembrane space

Sequence comparisons and similarity searches revealed other proteins homologous to the Tim10/Tim12/Tim9 proteins from various eukaryotic organisms including a putative homologue (85% identity) from the preliminary sequence data of *Candida albicans* (Stanford 5476/ con4-3104). Although other mitochondrial proteins share sequence similarity with bacterial proteins as seen between Tim17p/ Tim22p/ Tim23p and the prokaryotic amino acid permease LivH, (Rossow *et al.*, 1999), no bacterial homologue of Tim10p was detected.

Figure 3A shows the amino-acid sequence alignment of *Pichia* Tim10p with other Tim proteins detected from the public databases. The putative *Pichia TIM10* gene product is closely related to Tim10 proteins from *S. cerevisiae* (66% identity), *Emericella nidulans* (53%) and *Ciona intestinalis* (44%). It was closer to Tim12 than to Tim8, Tim9 or Tim13 proteins. Over all, Tim proteins are more divergent in their C and N terminals and may be as small as 80 to 120 amino acid residues in length.

Alignment of amino acids indicated that all highly conserved regions in Tim proteins including the twin CX_3C motif (Koehler *et al.*, 1999) are present in the *Pichia* Tim10p (Figure 3A). Sirrenberg *et al.* (1998) reported that zinc binding is essential for the function of Tim10p and Tim12p in protein import. The four cysteine amino acid residues that are known to constitute a zinc-binding site in Tim10p, Tim12p and Tim9p (Adam *et al.*, 1999) are also present in the *Pichia* homologue. This suggests that the putative *Pichia* Tim10p may also be a zinc finger protein. Besides the highly conserved cysteine residues, other residues consistently conserved were recognized (Figure 3A).

Whether these residues might have a specialised physiological role remains to be established. Interestingly, these residues distinguish Tim10 proteins from other Tim family proteins. For instance, a consensus signature sequence $EX_{10}NX_5CX_2KC_{x9}LX_3EX_2CLDRCVXK$ was observed in Tim10 proteins but was not conserved in Tim9 proteins. Figure 3B shows a phylogenetic tree indicating the relationship between *Pichia* Tim10p and other selected homologues. It is apparent that *Pichia* Tim10p is more closely related to other fungal/yeast homologues than to Tim proteins from other taxa. Four subgroups were evident and the division presented in the tree concurs with the taxonomic relationships generally accepted.

Pichia Tim10p cross-reacts with *S. cerevisiae* Tim10p antiserum

To investigate whether *Pichia* Tim10p might be a mitochondrial protein, Western blots were performed on mitochondrial extracts and probed with antiserum raised against the *S. cerevisiae* Tim10p. A peptide of approximately 10 kDa was detected from both *S. cerevisiae* and *P. sorbitophila* (Figure 4). This was consistent with the predicted molecular mass of Tim10p (9935 Da) and suggested that the *Pichia* Tim10p is located in the mitochondrion. Additional localisation studies are necessary to confirm this suggestion. In addition, the antibody also reacted with proteins of much higher molecular weight (70 kDa) albeit with a weaker signal in both organisms.

Pichia TIM10 is a functional homologue of *S. cerevisiae TIM10*

Temperature-sensitive mutants (*tim10-1*) of *S. cerevisiae* cannot grow on any carbon source at 37°C (Koehler *et al.*, 1998). To investigate whether the gene product of *P. sorbitophila TIM10* was a functional homologue of *S. cerevisiae* Tim10p, the *tim10-1* mutant strain was transformed with plasmids harbouring the *Pichia TIM10* gene. As shown in Figure 5, the temperature-sensitive mutant transformed with YEp/*PichiaTim10* grew like the wild type at the restrictive temperature (37°C) while those transformed with the empty vector could not grow. However, at the permissive temperature (25°C) both mutants with or without the *Pichia* gene grew at wild-type rates. These results demonstrated that the *Pichia TIM10* functionally complements the *S. cerevisiae tim10-1* mutant enabling it to grow at 37°C.

The ability of *Pichia Tim10* to restore growth at the restrictive temperature suggests a role in the import and sorting of mitochondrial carrier proteins which are defective in *tim10-1* strains. Despite their ability to synthesise some proteins, mitochondria have to import most of their protein precursor from the cytosol. The intermembrane space proteins Tim9, Tim10 and Tim12 are some of the few essential proteins mediating this import through interaction with other Tim and Tom proteins. Defects in the import machinery may affect the viability of yeast cells and cause many mitochondria-related diseases in humans (Baker and Schatz, 1991; Jarosch *et al.*, 1997; Koehler *et al.*, 1999, Larsson and Clayton, 1995). For instance, depletion of Tim10p results in accumulation of Hsp60 precursors, loss of cytochromes and changes in mitochondrial morphology as growth stops. On the other hand, overproduction of Tim10p restores respiration ability in yeast with mitochondrial RNA splicing defects (Jarosch *et al.*, 1997).

The role of *Pichia* Tim10p in suppressing mitochondrial RNA splicing defects was not tested. However, its structural and functional homology to *S. cerevisiae* Tim10p tempts one to speculate that it might also suppress mitochondrial RNA splicing defects.

To conclude, we have shown that the yeast *P. sorbitophila* possesses a structural homologue of the Tim10p that complements the *tim10-1* defective gene in *S. cerevisiae* enabling the strain to grow at 37°C. Immunoblotting with the anti-Tim10p serum shows that the homologous protein is located in the mitochondria. Sequence analysis from public databases and Southern blotting further revealed the occurrence of Tim10p homologues in other organisms. Taken together, the results suggest that mitochondrial preprotein import and sorting systems might be strongly conserved throughout eukaryotic organisms. It is anticipated that a clear understanding of yeast homologues might pave the way in elucidating the mechanisms for mitochondrial preprotein import in higher eukaryotes.

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1          TTATGCGCACAACTACGACGCCAAGAAAGAGGATTTCGGTGGTCCAGGCGATGAGGCCAA
61         TGAAGGCTCTCCCATTTTCGAAGCGGAGCTTCGTGCCTGCCACCACACAGCATTGTACTA
121        CAAGAGCGAGGAGCGCAACTACCTCTTTTTCATGGGTGGATTCTCAAACAGCTACCTCAG
181        GCTATTTGACCCCGAGCCCTACGTGTCAGACAAGCTTGACGTGTCGAAGTACGCCAAGTT
241        CAACCTCGCCCGCAGCAACAACCAGTCCAGGGTGTTGGCACTCAACCTCCGCACGCAGAA
301        GTGGTCGTTTTTCAGATACTTCCATGACTGCAGCGAAGCCGTATCAAAGAGCTTCACTCG
361        CAGGTTGCCGATCGACCGTCCGCTAGAGGACGTCGAATTTTGCAACTACGGCGGTGCCAT
421        ATCGCTCGACGGGAAGTGCATTAACATGTGCCACGGCCTCGCCTGCCCTGTTCCGGTTAA
481        TGCAGAGGAGTACGAAGCCCTCAAAACAGAGACTTCGGAATTGAGTTTCCTATGGGGCAT
541        TGTGACCCATTTCACCTTCCCTGGCCTATAATAGCCTATAATAGCCTACGATGGCCTGTG
601        ATGCTCGTGGTGGCCCACAACGGCCCATAAATTGCTGCATTTCGACACATTCTACCAGTT
661        CGCTCCTACTATACATATTACTTCTTAGATAGACTGGACAAACTGTTTCATAATTTTACG
721        TTACTTTTAATGGTACATACTACTATATAGACTTATCCAACTATCCCTCCGCTAGTGCTC
781        GCTTTCCAACACCTTTTTTTTCAGATTTCGACTTTGTGTTGCTTTGCTAAAATCGACAAC
841        TTCAAGAAAAAAATTGGCGAATCTTTCAAACTACAGTTACAAGAAGTAGAAGTATTTATA
901        AGATTGAAAATGTTTGGATTAGGAGGAGCACCACAGATATCGTCAGAGCAGAAATTGCAA
                     M  F  G  L  G  G  A  P  Q  I  S  S  E  Q  K  L  Q

961        GCCGCTGAGGCGGAGTTGGATATGGTTACCGGAATGTTCAACCAGTTGGTTGATCAGTGC
            A  A  E  A  E  L  D  M  V  T  G  M  F  N  Q  L  V  D  Q  C

1021       CACTCTAAGTGTATTAACAAGAGCTACGGTGATTCCGACATTACCAAGCAAGAAGCACTC
            H  S  K  C  I  N  K  S  Y  G  D  S  D  I  T  K  Q  E  A  L

1081       TGTTTGGACAGATGTGTTGCCAAGTATTTCGATACCAATGTTCAAGTTGGAGAACACATG
            C  L  D  R  C  V  A  K  Y  F  D  T  N  V  Q  V  G  E  H  M

1141       CAAAAGTTGGGACAATCTGGTCAGTTTATGGGGAGAAAATAAGCGTACATAGAATCTTCA
            Q  K  L  G  Q  S  G  Q  F  M  G  R  K
1201       GAGTAGCCGGAGACAGAAATCATATTTAAGGATGTATGATATGTAATCTATATTTCGAAA

1261       ATGCACACTTAGCGTGATAGAATACATGTATTTACAAGAGGATGGGCCAGGTGGCGTCTG
1321       TCGATGGGAGGCCGGGGGCAGGCATCAAGCAAGACCGACGCTGCGCATCAAGCGGAGCTT
1381       CACGCCCTCCCAGGAGTTGAGCTTCGGTCTTGCGTCTATGAAAAAGCTGGCGCCCTATCC
1441       GTTGCCCGTGTAGAAGTCGATGACGTACTCGACCTGTGTGCCGCAGCGGTCGAT
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Figure 1. Nucleotide sequence of the 1.5 kb *Eco*R1/*Sal*1 subclone and the deduced amino acid sequences of the *P. sorbitophila TIM10* in single-letter code.

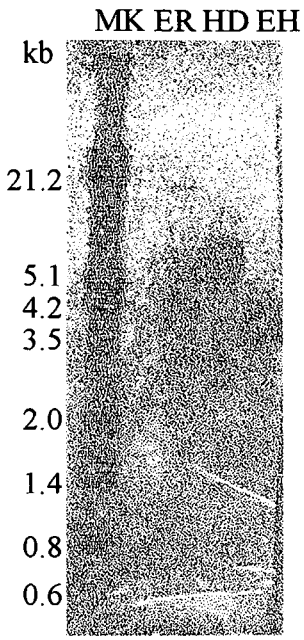

Figure 2. Southern blot analysis. Genomic DNA extracted from *P. sorbitophila* was digested by *Eco*RV (ER), *Hin*dIII (HD) and *Eco*RV/*Hin*dIII (EH) separated on a 0.7 % agaraose gel electrophoresis and probed by the entire coding region of the *Pichia TIM10* gene under stringent conditions.

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P. sorbitophila Tim10p  ........MF GLGGA.PQIS SEQKLQAAEA ELDMVTGMFN QLVDQCHSKCINKS.YGDSD ITKQEALCLD RCVAKYFDTN VQVGEHMQKL .GQSGQFMGR K.........  90
C. albicans     Tim10p  ........MF GLGGTTPQIS SQQKLQAAEA ELDMVTGMFN ALVSQCHTKCINKS.YNEAD ISKQESLCLD RCVAKYFETN VQVGENMQKL .GQSGQFMGR R.........  91
S. cerevisiae   Tim10p  ......MSFL GFGGGQPQLS SQQKIQAAEA ELDLVTDMFN KLVNNCYKKCINTS.YSEGE LNKNESSCLD RCVAKYFETN VQVGENMQKM .GQSFNAAGK F.........  93
E. nidulans     Tim10p  ........MS FLFGGAPKMS SEQKIAAAET EVEMITDMFN RLSESCSKKCIPND.YREGD LNKGESVCLD RCVGKFFEVN IKVSEKMQGV AGQQQGGAGL SL........  93
C. intestinalis Tim10p ..........  .......MDP QEAQKLAAEL EVEMMADMYN RMTSSCHKKCISTR.YDTGD LEKGEAVCID RCVAKYLDIH EQIGKKLTEM SQTDEEAMSK MSQKPGYSCK  92
R. norvegicus   Tim10p  ..........  .......MDP LRAQQLAAEL EVEMMADMYN RMTSACHRKCVPPH.YKEAE LSKGESVCLD RCVSKYLDIH ERMGKKLTEL SMQDEELMKR VQQSSGPA..  90
H. sapiens      Tim10p  ..........  .......MDP LRAQQLAAEL EVEMMADMYN RMTSACHRKCVPPH.YKEAE LSKGESVCLD RCVSKYLDIH ERMGKKLTEL SMQDEELMKR VQQSSGPA..  90
D. melanogaster Tim10p  .......... MALPQISTAD QAKLQLMQEM EIEMMSDLYN RMTNACHKKCIPPR.YSESE LGKGEMVCID RCVAKYLDIH EKIGKKLTAM FMQDEELMKK MSS.......  92
C. elegans      Tim10p  ..........  .......MAT DAQMAQVAEL EVEMMSDMYR RMTNSCQAKCIATA.FRESE LTKGEAVCLD RCVAKYLDVH EKLGKRLTSM SQGDEAALQK IAQQ......  86
S. cerevisiae   Tim12p  ......MSFF LNSLRGNQEV SQEKLDVAGV QFDAMCSTFN NILSTCLEKCIPHEGFGEPD LTKGEQCCID RCVAKMHYSN RLIGGFVQTR GFGPENQLRH YSRFVAKEIA 104
L. esculentum   Tim10p  ..........  .MAGVPSNLE REQIFSMAEK EMEYRVEMFN KLTHTCFKKCVENK.YKDSE LNMGENSCID RCVSKYWQVT NLVGTLLGNT RPM.......  ..........  81
A. thaliana     Tim10p  ..........  MASPIPVGVT KEQAFSMAQT EMEYRVELFN KLAQTCFNKCVDKR.YKEAE LNMGENSCID RCVSKYWQVN GMVGQLLSAG KPPV......  ..........  83
S. cerevisiae    Tim9p  ..........  .MDALNSKEQ QEFQKVVEQK QMKDFMRLYS NLVERCFTDCVND..FTTSK LTNKEQTCIM KCSEKFLKHS ERVGQRFQEQ NAAL...GQG LGR.......  87
E. nidulans      Tim9p  ..........  .MDGLNAAEQ RELANRMERK QMKEFMTMYS KLVQRCFDDCVND..FTTKS LISREEGCVM RCVDKFMKGS QRLNERFQEQ NAAMMQSGQL PGR.......  90
C. elegans      Tim9Ap  ..........  ..........  ..MTSEQNIQ TFRDFLTQYN LVAEQCFNSCVNE..FGSRT VSGKEESCAN NCLDKFLKMT QRVSQRFQEH QLLNAQANGA AIKVENGGKI  86
H. sapiens      Tim9Ap  ..........  ........MA AQIPESDQIK QFKEFLGTYN KLTETCFLDCVKD..FTTRE VKPEETTCSE HCLQKYLKMT QRISMRFQEY HIQQNEALAA KAGLLGQPR.  89
C. elegans      Tim9Bp  ..........  ..........  ..MNTIQNIQ QLREFLTVYN TLSERCFNACARD..YTTST LTKDEGSCVS QCIDKQMLVN RRFMLVFAEQ APKALFKQGE QSPTEAIKSA  86
D. melanogaster  Tim9p  ..........  ..........  ....MDSNLR NLKDFFTLYN KVTELCFSRCVDN..LSQRD LGGHEDLCVD RCVTKFARFN QNMMKVYVDV QTTINAKRME EMEENARKAE  84
H. sapiens      Tim9Bp  ..........  ........ME RQQQQQQQLR NLRDFLLVYN RMTELCFQRCVPS..LHHRA LDAEEEACLH SCAGKLIHSN HRLMAAYVQL MPALVQRRIA DYEA...ASA  87
N. crassa        Tim8p  .MDIPQADLD LLNEKDKNE. .LRGFISNET QRQRVQGQTH ALTDSCWKKCVTSPIKT.NQ LDKTEAVCMA DCVERFLDVN LTIMAHVQKI TRGGSK....  ..........  92
S. pombe         Tim8p  MADATKNPIA DLSESEQLE. .LSKFIESEQ QKVKLQQAIH QFTSTCWPKCIGNI..G.NK LDKSEEQCLQ NCVERFLDCN FHIIKRYA.. LEKFGFLFCW LGFSC.....  98
H. sapiens      Tim8Bp  ........MA ELGEADEAE. .LQRLVAAEQ QKAQFTAQVH HFMELCWDKCVEKP..G.NR LDSRTENCLS SCVDRFIDTT LAITSRFAQI VQKGGQ....  ..........  83
D. melanogaster  Tim8p  ......MSDF ENLSGNDKE. .LQEFLLIEK QKAQVNAQIH EFNEICWEKCIGKP..S.TK LDHATETCLS NCVDRFIDTS LLITQRFAQM LQKRGGGDL.  ..........  88
A. thaliana      Tim8p  .........M DPSMANNPE. .LLQFLAQEK ERAMVNEMVS KMTSVCWDKCITSA.PG.SK FSSSESSCLT HCAQRYMDMS MIIMKRFNSQ  ..........  ..........  77
S. pombe        Tim13p  .MGIFGGNSG NAPSSEDKKS IFMKQIRQEL AVAQAGELIS KINENCFDKCIPEP..G.ST FDPNEKSCVS KCMERYMDAW NIVSRTYISR MQREQKNLN.  ..........  95
H. sapiens      Tim13p  MEGGFGSDFG GSGSGKLDPG LIMEQVKVQI AVANAQELLQ RMTDKCFRKCIGKP..G.GS LDNSEQKCIA MCMDRYMDAW NTVSRAYNSR LQRERANM..  ..........  95
C. elegans      Tim13p  MDQLLDVETL KKLSPEQQEQ VI.SGVKQQA ALANAQNLVT DISEKCTNKCITAP..G.SS LASGEKQCLQ RCMDRFMESW NLVSQTLQKR LQEEMASSGG MGGGFGQGPS 106
A. thaliana      Tim13p  .MDSYSSPPM GGSGSSVSPE VMMESVKTQL AQAYXEELIE TLRTKCFDKCVTXP..G.SS LGGSESSCIS RCVERYMEAT AIISRSLFTQ L.........  ..........  87
                 Consensus  ..........  ..........  .........E.  .........N  .....C..KC!.........  L...E..C..  RCV.K.....  ..........  ..........  ..........
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Figure 3. Members of the Tim family in the mitochondrial intermembrane space.

(A) Clustal amino acid sequence alignment of *Pichia* Tim10p with other homologues detected from the public databases using BLAST and FASTA searches. The four-conserved cysteine residues that constitute the twin CX_3C motif are indicated in bold. Protein sequences analysed included: *Saccharomyces cerevisiae* Tim10 (Z80875), Tim12* (Z35960), Tim9 (AF093244) (Z72966), *Emericella nidulans* Tim10 (AAD40003), Tim9 (AAD40016), *Ciona intestinalis* Tim10* (AAD40001), *Rattus norvegicus* Tim10 (AAD3997), *Homo sapiens* Tim10 (AAD39995), Tim9A (AAD4006), Tim9B* (AAD4006), Tim8B (AAD39994), Tim13 (AAD39951), *Drosophila melanogaster* Tim10 (AAD39998), Tim9* (AAD40010), Tim8 (AAD39162), *Caenorhabditis elegans* Tim10 (AAD40000), Tim9A* (AAD40014), Tim9B* (AAD40015), Tim13* (AAD39955), *Lycopersicon esculentum* Tim10 (AAD40002), *Arabidopsis thaliana* Tim10 (AAD39999), Tim8 (AAD39990), *Neurospora crassa* Tim8 (AAD39161), *Schizosaccharomyces pombe* Tim8 (AAD40476), Tim13 (AAD40477).

* partial sequences shown in the alignment.

(B) Phylogenetic tree showing the relation ship between Tim10 proteins from various eukaryotic organisms. Amino acid sequences were retrieved from public databases and used for generating the tree by the neighbour-joining method.

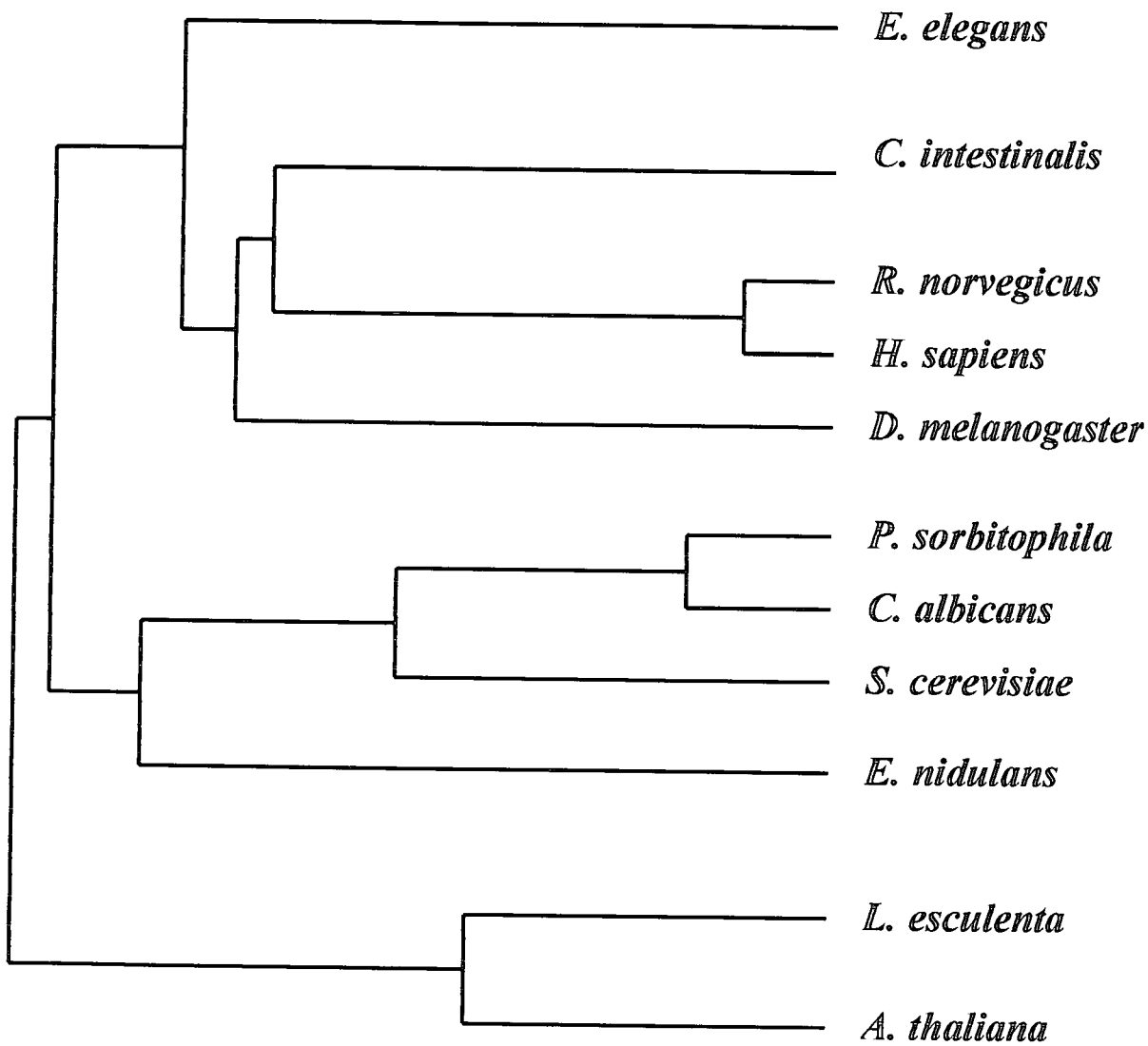


Figure 3B. Phylogenetic tree for Tim10 proteins
The tree was constructed using the Neighbor-Joining method after a clusterW aligned amino acid sequences.

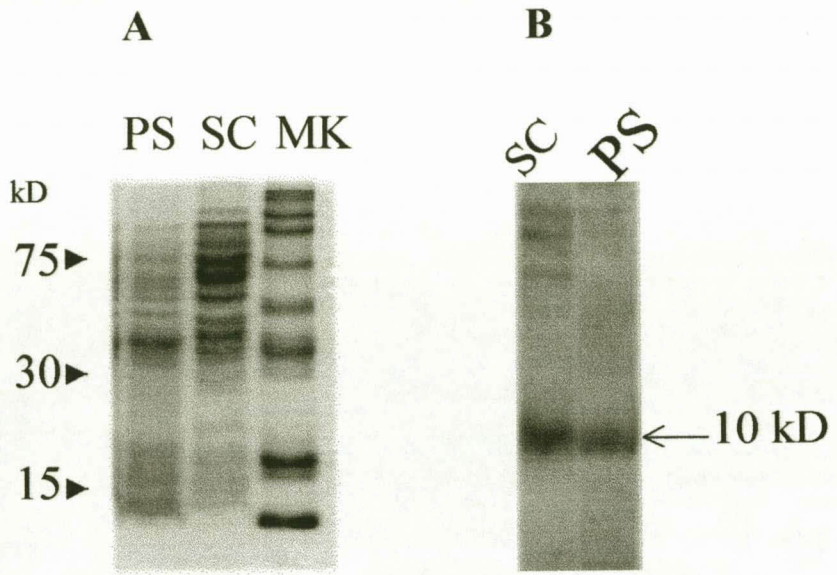
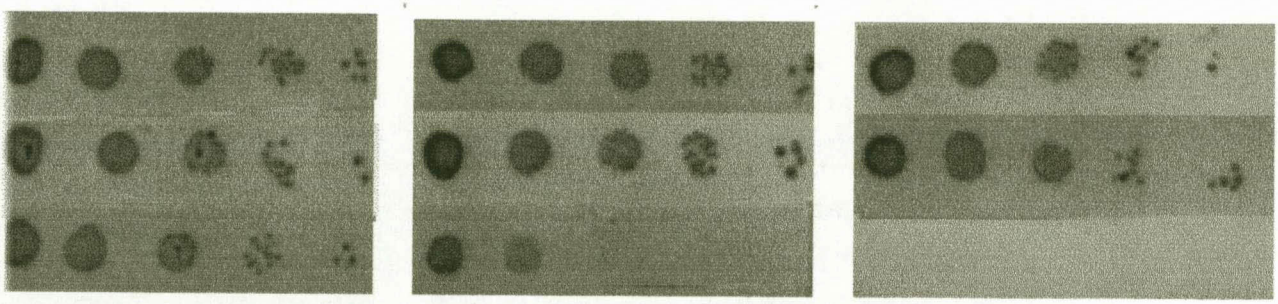


Figure 4. Western blot analysis. Mitochondrial proteins were isolated from spheroplasts of *S. cerevisiae* (SC) and *P. sorbitophila* (PS). Extracts were analysed on SDS-PAGE (A) and immunoblotting (B) with a rabbit anti Tim10p. Immunodetection was performed with a chemiluminescence kit (Boehringer Mannheim).



25°C 30°C 37°C

Figure 5. Functional complementation. Growth of *S. cerevisiae* wild type strain GA7-6A (1), *S. cerevisiae* temperature sensitive mutant *tim10-1* transformed with the *Pichia TIM10* (2) or with the empty plasmid (3) was monitored at permissive and restrictive temperatures.

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CHAPTER 7

Characterisation of a Putative Glycerol Facilitator in the Fission Yeast *Schizosaccharomyces pombe*

Abstract

The fission yeast *Schizosaccharomyces pombe* can utilise glycerol as a sole carbon source or as an osmolyte during osmoregulation. Transport studies indicated that *S. pombe* might control glycerol flux using facilitated diffusion or a channel-mediated mechanism but the proteins involved in this transport are not known. Comparative Blast searches of the *Schizosaccharomyces pombe* databases revealed three putative glycerol transport proteins two of which show considerable structural similarities to known MIP family glycerol facilitators. One complete open reading frame designated as *S. pombe mip1* encodes a putative transmembrane protein of 598 amino acids with 52% identity to the *Saccharomyces cerevisiae* putative glycerol facilitator Yfl054p including the unique N- and C - terminal extensions. The gene was subsequently isolated by PCR from the *S. pombe* CBS 5682 (CSIR Y457) genomic DNA, verified and cloned into yeast expression vectors for functional analysis. Expression of the *S. pombe mip1* into the *Saccharomyces cerevisiae* wild-type or the *fps1Δ* and *gpd1Δ gpd2Δ* strains, had no effect on osmotic sensitivity or glycerol release during hypo-osmotic stress. However, cells expressing *S. pombe mip1* had a lower intracellular /extracellular glycerol ratios as compared to control cells during osmotic stress. The osmosensitive phenotype of *fps1Δ* and *gpd1Δ gpd2Δ* mutants that is suppressed by the overexpression of a glycerol facilitator in the plasma membrane, were not suppressed by the overespression of *S. pombe mip1*. Northern blotting experiments revealed that *S. pombe mip1* expression is induced during osmotic stress suggesting a role in osmoregulation. However, deletion of the gene did not have any observable effect on the growth of *S. pombe* under osmotic stress or on glycerol as a sole carbon source. Therefore, the physiological role of this protein as well as the actual transporter (s) controlling glycerol flux in *S. pombe* remains to be elucidated.

INTRODUCTION

Most yeasts can utilise glycerol both as a sole carbon source or as an osmolyte in order to maintain osmotic homeostasis. Consequently, yeasts have developed elaborate mechanisms to regulate its flux across cell membranes. In the fission yeast *Schizosaccharomyces pombe*, glycerol appears to be the main osmolyte with which cells maintain osmotic homeostasis (Ohmiya *et al.*, 1995). Glycerol is synthesised from the glycolytic intermediate dihydroxyacetone phosphate in two steps that are catalysed by an NADH-dependent glycerol-3-phosphate dehydrogenase and a phosphatase (Gancedo *et al.*, 1968). Under osmotic stress conditions, the expression of genes involved in glycerol synthesis such as *gpd1* are upregulated by the Sty1- signal transduction pathway leading to the intracellular accumulation of glycerol (Aiba *et al.*, 1995). In absence of osmotic stress, the synthesised glycerol is either utilised or released rapidly out of the cell.

Glycerol was originally thought to permeate across yeast membranes only by simple diffusion. However, recent investigations have presented evidence that suggests the involvement of active transport systems and glycerol facilitating proteins (for review, Kayingo *et al.*, 2001). Indeed, transport systems that allow yeasts to maintain a steep glycerol concentration gradient across the membrane, have been already reported (Lages *et al.*, 1999). Little is known about the transport proteins and the genes encoding these proteins except in the yeast *S. cerevisiae*. Recently the gene *GUP1*, encoding a putative active glycerol uptake protein in *S. cerevisiae* has been identified and partially characterised (Holst *et al.*, 2000).

Furthermore, a gene *FPS1*, which encodes a MIP channel protein with similarity to bacterial glycerol facilitators, was described in *S. cerevisiae* (Van Aelst *et al.*, 1991). The Fps1p has been shown to control the movement of glycerol across the membrane and to play an important role in yeast osmoregulation (Luyten *et al.*, 1995; Sutherland *et al.*, 1997; Tamás, *et al.*, 1999).

Most glycerol facilitators so far identified are related to aquaporins such as AQP0, the bovine major intrinsic protein (Gorin *et al.*, 1984) from which the name MIP family was derived (Baker and Saier, 1990). These proteins exhibit a typical structure with six transmembrane spanning domains, an internal sequence repeat and highly conserved motifs (for recent review see Kayingo *et al.*, 2001). Functional analysis using heterologous systems and deletion mutants suggest that MIP channels play a critical role in osmoregulation by regulating water and solute transport across cell membranes (for reviews, Agre *et al.*, 1998; Hohmann *et al.*,

2000). MIP channel proteins may play additional roles in a wide range of physiological processes especially where a regulated water and solute transport is critical (Ludevid *et al.*, 1992; Kaldenhoff *et al.*, 1995).

To date, a significant number of MIP channels have been characterized in higher organisms and their role in mediating water and solute flux established. As far as microorganisms are concerned, MIP channels have only been well studied in a few organisms. In *Escherichia coli*, there are two MIP channel proteins, namely the water channel AqpZ (Calamita *et al.*, 1995) and the glycerol facilitator GlpF (Sweet *et al.*, 1990).

The *S. cerevisiae* genome revealed four genes encoding MIP channels (André, 1995) of which *FPS1* has been shown to be a glycerol facilitator whereas *AQY1* and *AQY2* are water channels (Bonhivers *et al.*, 1998; Laizé *et al.*, 1999). The function of the fourth MIP gene, *YFL054*, is not yet known. Although its expression is significantly induced after a diauxic shift from fermentation to respiration (DeRisi *et al.*, 1997) and during osmotic stress (Tamás, 1999), there is no evidence yet for its involvement in osmoregulation and/or cellular metabolism.

Recently, and mainly from genome sequencing projects, several MIP channels have been identified in many other unicellular organisms but little is known about their physiological roles (Kayingo *et al.*, 2001). The genome projects focusing on the fission yeast *S. pombe* has so far revealed two fragments encoding MIP channel proteins (http://www.sanger.ac.uk). The fragment SPAC977 on chromosome 1, appears to encode a complete open reading frame that we have called *S. pombe mip1*. To our knowledge, the functions of these genes have not been examined. Therefore, this study aimed at characterising the *S. pombe mip1* with respect to its structure-functional properties, patterns of gene expression as well as its phylogenetic relationship with other known MIP channels.

MATERIALS AND METHODS

Strains and growth conditions

The strains used in this study are shown in Table 1.

S. cerevisiae and *S. pombe* mutants were derived from the haploid laboratory strains W303-1A and L972 h^- respectively. All *S. cerevisiae* wild type strains were routinely grown on a rotary shaker at 30 °C in YEPD broth (10 g l^{-1} yeast extract, 20 g l^{-1} each of peptone and glucose) or on defined medium (6.7 g l^{-1} yeast nitrogen base, 20 g l^{-1} glucose and amino acids, pH 6) as discussed below. In some experiments, the glucose concentration was increased to 100 g l^{-1} or replaced by glycerol (3% v/v) as a sole carbon source. For transformations, *S. pombe* was grown in Edinburgh minimal medium (Moreno *et al.*, 1991) whereas YEPD was used for normal cultivation and routine maintenance. Anaerobic growth experiments were carried out in the anaerobic incubator (Forma Scientific USA) at 30 °C for 2-3 days.

Molecular and genetic methods

In general, nucleic acid manipulations were carried out according to Sambrook *et al.* (1989) unless otherwise mentioned. Large amounts of pure plasmid DNA was isolated from *E. coli* using the Nucleobond AX100 cartridges of Macherey-Nagel Germany (Cat. No 740521). For isolation of small quantities of plasmid DNA, cetyltrimethylammonium bromide (CTAB) was used following the method of Del Sal *et al.* (1988). Total RNA was isolated from exponentially growing yeast cells cultivated in YEPD with or without 5% NaCl. Cells were harvested and lysed with 0.2 mg/ml Zymolase and 8 µl/ml β-mercaptoethanol in extraction buffer (1 M sorbitol, 100 mM Na_3-citrate, 60 mM EDTA, pH 7.0). After cell lysis (3 hours at 37°C) and homogenization, total RNA was isolated from the yeast spheroplasts using the Rneasy kit (Qiagen, Cat. No. 74103) for small quantities or using the TRI™ reagent (Sigma, Cat. No. T9424) when large quantities of RNA were desired. Total genomic DNA was isolated from *S. pombe* and *S. cerevisiae* as described by Moreno *et al.* (1991) and Sherman *et al.* (1986) respectively. For blotting experiments, nucleic acids were fractionated by gel electrophoresis and transferred to a positively charged nylon membrane (Magna Graph, MSI, Cat. No. NJ0HY00010) by capillary blotting (Sambrook *et al.*, 1989).

Hybridization, probe labelling and detection were performed using the digoxigenin (DIG) system (Roche Diagnostics).

Isolation and cloning of *S. pombe mip1*

The polymerase chain reaction was used to amplify the entire reading frame encoding the *S. pombe* MIP channel. The following primers (Integrated DNA Technologies)

were used: Pombe up 5' gggatcc taactaaatgagcgtcc 3' *BamH*1

Pombe do 5' ggtcgac gagttcaattattctc 3' *Sal*1

PCR conditions: 94°C, 1min, 94°C, 30sec; 52°C, 30sec; 72°C, 1min; 25 cycles. Amplification was carried out with 2.5 units of *Taq* DNA polymerase (Roche Diagnostics) in presence of 200 μM dNTPS, 1.5 mM $MgCl_2$ and 0.5 μM primers.

The PCR product (1.8 kb) was purified (High Pure™ PCR purification kit; Roche Diagnostics, Cat. No. 1732676), cloned into pGMT-easy vector (Promega) and verified by restriction digestion analysis and sequencing. The insert was subsequently subcloned into yeast vectors YEp*lac*195 and pKU24 using the *Sal*1 and *BamH*1 linkers introduced into the primers. For heterologous expression of the *S. pombe mip1*, a yeast expression vector with a strong constitutive promoter and terminator sequences of the *S. cerevisiae PGK1* gene encoding phosphoglycerate kinase, was used. The 1.8 kb *Hind*111 fragment containing the *PGK* promoter and terminator sequences were subcloned into the YEp*lac*181 and the entire *S. pombe mip1* reading frame was then inserted into the *Bgl*11 and *Xho*1 isoschizomers respectively to create the construct pPGK-*Spmip1*. The pPGK-*Spmip1* plasmid (Fig. 1) was transformed into the *S. cerevisiae* W303-1A and *fps1*Δ strains and its effect on growth under hyper- and hypo-osmotic conditions as well its effects on the glycerol conservation were analyzed. Yeast transformations were performed using the lithium acetate method of Gietz and Schiestl (1995).

Disruption of the *S. pombe mip1*

The *S. pombe* MIP gene was disrupted in the one step procedure of Rothstein (1983) in the strain *ura4-D18* (Grimm and Kohli, 1988). The pGMT-*Spmip1* plasmid was digested with *Hind*111 which cuts inside *S. pombe mip1* to release a fragment of about 500bp. The 1.8kb *Hind*111 fragment encoding the *S. pombe ura4* gene was cut from the pCG1 plasmid (Grimm *et al.*, 1988) and then inserted into *S. pombe mip1* to create a disruption (Fig. 2). The linear fragment carrying the disrupted *S. pombe mip1* was gel purified (Qiagen) and transformed into the *S. pombe ura4-D18* strain. Transformants were selected on minimal media without uracil and then replica-plated several rounds on YEPD plates and back to selective plates. Surviving colonies were examined for Southern blot and PCR analyses.

Glycerol transport assays and osmotic stress experiments

Yeast cells were grown in 250 ml defined media (glucose-YNB) with amino acids (pH 6) until $OD_{600} = 1$. Cells were harvested (5000 rpm, 5 min) resuspended in 100 ml of glucose-YNB with 5% NaCl and grown at 30°C for 3 hours. Samples of 1.5 ml were taken from which cells were harvested (5000 rpm, 2 min) and resuspended in 0.1ml of similar media. Hypo-osmotic shock was performed by diluting (10X) that cell suspension with media without NaCl. After a given time interval, cells were immediately sedimented, washed and re-suspended in 1ml of water. Intracellular glycerol was extracted by boiling the cell suspension for 10 min followed by centrifugation (13000 rpm, 5 min) to remove the debris. Glycerol content was analyzed using chromatographic (Dionex , MA1 column) and enzymatic methods (Boehringer Manheimn Cat. No. 148270). For osmotic sensitivity experiments, cells were exposed to hyper-osmotic stress by increasing the NaCl concentration from 0 to 5% or hypo-osmotic stress by decreasing the NaCl concentration from 5 to 0 % as described previously (Tamás *et al.*, 1999). Spot tests were also carried out by serial dilutions of the cell suspension that were subjected to osmotic stress prior to plating. Five microlitres of the cell suspensions were spotted onto agar plates with or without NaCl addition and incubated at 30°C until the colonies were visible.

Table 1. Yeast strains used in this study

rain	Genotype	Reference
cerevisiae W303-1A	*MAT a leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*	Thomas and Rothstein, 1989
cerevisiae	W303-1A *yfl054Δ::TRP13*	Tamás *et al.*, 1999
cerevisiae	W303-1A *fps1Δ::HIS3*	Tamás *et al.*, 1999
cerevisiae	W303-1A *gpd1Δ::TRP1 gpd2Δ::URA3*	Ansell *et al.*, 1997
. pombe CBS 5682	Wild type	Chapter 3
. pombe 972h$^-$	*leu1-32 ura4-D18*	Grimm *et al.*, 1988
YGK2000	972h$^-$ *mip1::ura4*$^+$	This study

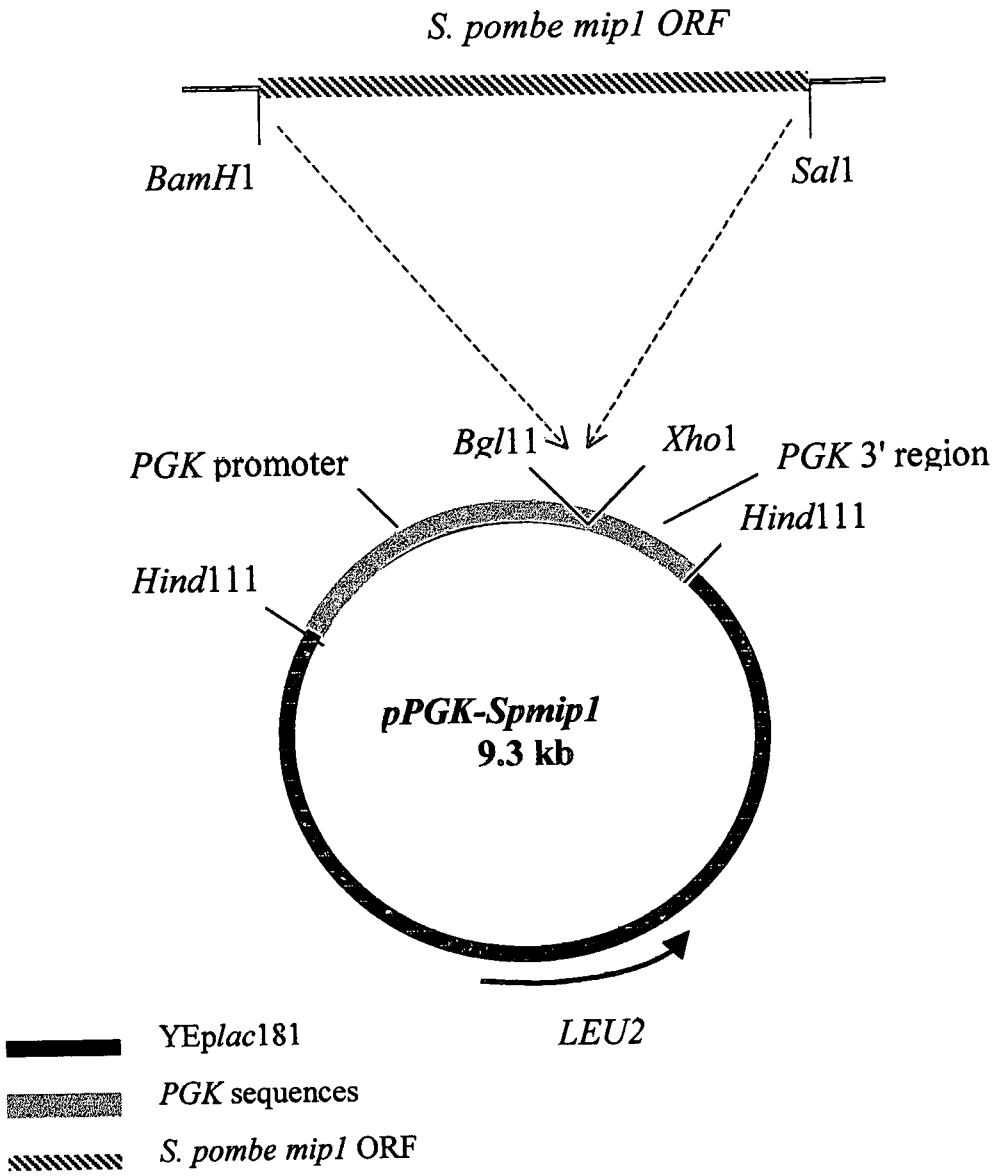


Figure 1. Construction of the yeast expression vector and insertion of the *S. pombe mip1* gene. The 1.8 kb *Hind*111 fragment containing the *S. cerevisiae PGK* promoter and terminator sequences were subcloned into the multicopy plasmid YEp*lac*181. A 1.8 kb *BamH*1/*Sal*1 fragment encoding the entire *S. pombe mip1* open reading frame was inserted into the *Bgl*11 and *Xho*1 isoschizomers respectvely to create the construct *pPGK-Spmip1*.

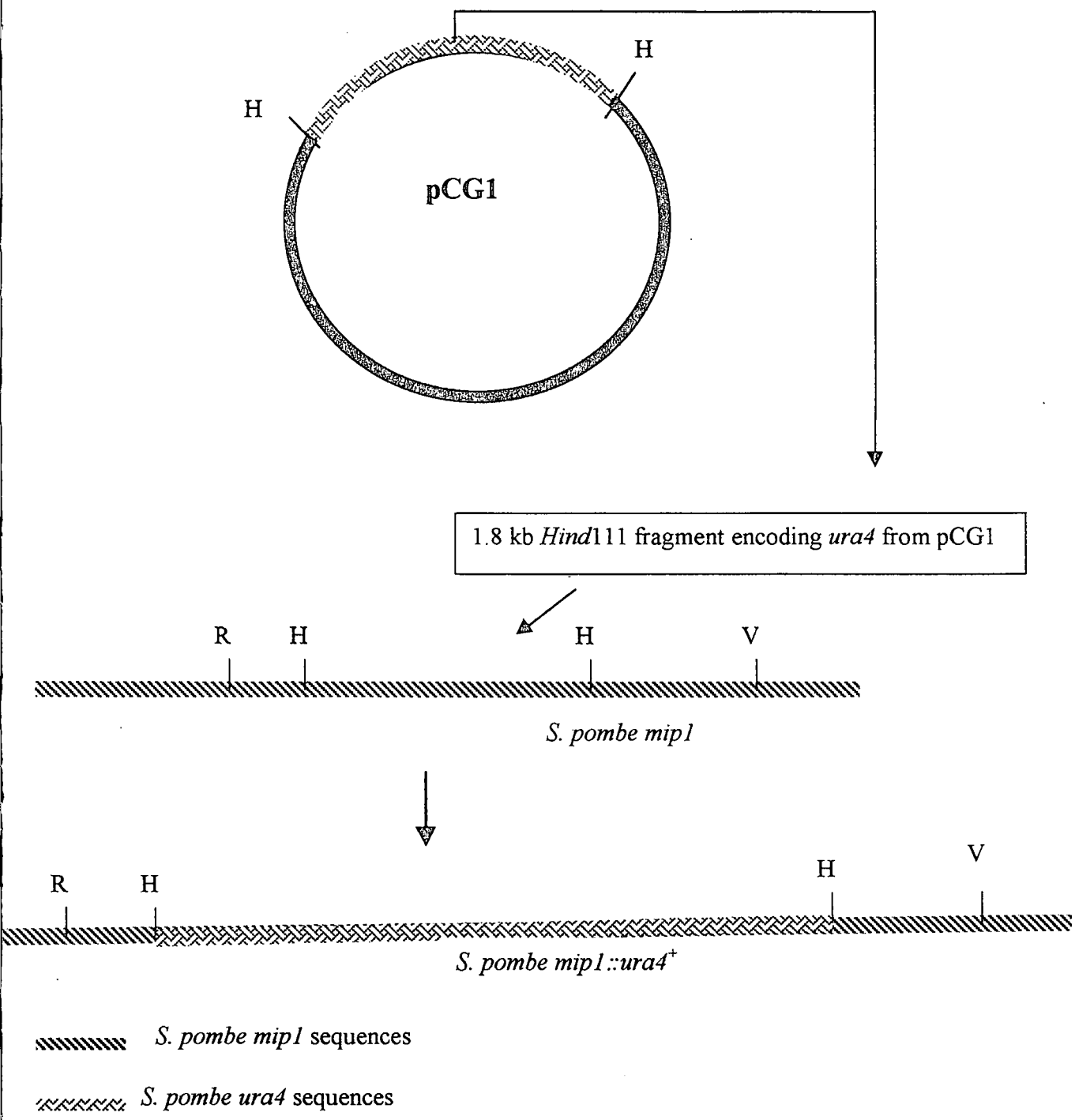


Figure 2. Partial restriction map and strategy for disruption of *S. pombe mip1*.

Partial restriction map indicating the relevant sites of the *S. pombe mip1::ura4* disruption construct. H, *Hind*111; R, *Eco*R1; V, *Eco*RV. Not drawn on scale.

RESULTS AND DISCUSSION

Glycerol transport in *S. pombe*

The patterns and kinetics of glycerol export from *S. pombe* upon a hypo-osmotic shock (osmotic downshock) are shown in Figure 3. There was a rapid glycerol efflux upon an osmotic downshock and cells lost more than 75 % of their intracellular glycerol in 5 minutes regardless of the downshock incubation temperature. The pattern of glycerol release suggests that a specific protein mediated glycerol transport system may exist in *S. pombe* with which cells might regulate glycerol flux across the membrane. These observations prompted a search for transporters that might be involved. Analysis of the *S. pombe* genome database (http://www.sanger.ac.uk) for sequences homologous to known glycerol transporters revealed three putative glycerol transport proteins. The one encoded by *SPAC24H6.01c* (ORF of 231 amino acid residues) is homologous (49% identity) to the N-terminal part of Gup1, a membrane protein involved in active glycerol uptake in *S. cerevisiae* (Holst *et al.*, 2000). The other two putative glycerol transporters encoded by *SPAC977.17* (598 amino acids) and by *SPAC186* (incomplete) are homologous to glycerol facilitators of the MIP family (Hohmann *et al.*, 2000; Kayingo *et al.*, 2001). In particular, the former is most closely related (52% identical) to the to the yeast putative glycerol facilitator YFL054p (Fig. 4A), and more diverse to MIP channels from other fungi (Fig. 4B). This gene designated as *S. pombe mip1* codes for a putative protein of 598 amino acids, with a predicted molecular weight of 65815.73 and a pI of 6.12.

Isolation and sequence analysis of *S. pombe mip1*

The *S. pombe* MIP homologue was isolated by PCR using primers designed from the sequence SPAC977.17 (WWW.sanger.ac.k/projects/s_pombe). A PCR product of about 1.8-kb was obtained from both *S. pombe* 972h$^-$ and *S. pombe* CBS 5682 (Fig. 5A). The PCR products were subsequently cloned into pGMT-easy vector and verified by restriction digestion analysis and sequencing. Southern and Northern blots probed with the labeled PCR product, confirmed that indeed the cloned gene originated from *S. pombe* (Fig. 5). No hybridization of this probe to other yeasts such as *Saccharomyces cerevisiae, Zygosaccharomyces rouxii, Debaryomyces hansenii, Pichia sorbitophila* and *Candida tropicalis* was observed when the Southern blotting was conducted even under medium stringent conditions (not shown).

Sequence analysis of the *S. pombe mip1* showed all typical features of the MIP family (Park and Saier, 1996) such as the canonical, tandemly arranged NPA motifs and other conserved residues (Fig. 4A and 6). A hydrophobicity plot (Kyte and Doolittle, 1982) indicated that the putative protein has six transmembrane domains like other MIP proteins (Fig. 4A, 6 and 7). The *S. pombe mip1* has an extended N-terminal extension of approximately 300 amino acids leading to a protein of about 598 amino acids unlike most MIP proteins which consist of about 250-300 amino acids. The *S. pombe mip1* exhibits the amino acid residues typical for glycerol facilitators (Fig. 6) suggesting that it might be involved in the transport of glycerol and /or other related solutes (Froger *et al.*, 1998; Kayingo *et al.*, 2001). However, unlike most glycerol facilitators where positions 4 and 5 (Fig. 6) are usually a proline followed by a nonaromatic residue, the *S. pombe* mip1 and its closest homologue Yfl054p possess an alanine in P4 and a tryptophan in P5.

Heterologous expression of *S. pombe mip1* in *S. cerevisiae* and functional analysis

PCR analysis and Northern blotting confirmed that *S. pombe mip1* was properly expressed in *S. cerevisiae* strains. A transcript was detected in cells transformed with pPGK-*Spmip1* as opposed to the control cells (Fig. 8). In this study, the effects of *S. pombe mip1* on growth and glycerol conservation during osmotic stress conditions were analyzed both in the *fps1*Δ strain and its isogenic wild-type *S. cerevisiae* W303-1A.

The growth of *S. cerevisiae* strains expressing the *S. pombe mip1* gene commenced later and grew slightly slower than the strains containing only the vector in glucose-YNB with or without 5% NaCl (Fig. 9) suggesting that expression of the gene affects growth properties. The intracellular glycerol content of cells transformed with the *S. pombe mip1* was significantlly lower than that of cells without the gene (Table 2) suggesting that the expression of *S. pombe mip1* in *S. cerevisiae* might interfere with the retention of glycerol in both wild-type and in a strain lacking the glycerol facilitator Fps1p. However, this gene does not appear to alleviate the inability of the *fps1*Δ strain to release glycerol upon hypo-osmotic shock (downshock). The pattern of glycerol release was identical in the *fps1*Δ strain expressing *S. pombe mip1* to the *fps1*Δ strain without *S. pombe mip1* (Fig. 10A). Furthermore, the expression of *S. pombe mip1* in the *fps1*Δ strain was unable to restore the hypo-osmotic stress sensitivity of the *fps1*Δ strain to the wild-type (Fig. 10B). These experiments suggest that the *S. pombe mip1* affects glycerol retention in *S. cerevisiae* but does not appear to act as a glycerol export channel similar to Fps1p found in *S. cerevisiae* (Luyten, *et al.*, 1995; Tamás *et al.*, 1999).

Previous studies have indicated that expression of bacterial or eukaryotic glycerol facilitators alleviate the *fps1*Δ growth and transport defects (Luyten *et al.*, 1995, Sutherland, *et. al.*, 1997; Weig and Jakob, 2000; Prudent *et al.*, 2000). Whether the *S. pombe* mip1 transports glycerol in ungated way albeit with a low turnover insufficient to alleviate the downshock sensitivity of *fps1*Δ strains, remains to be established. We tested further for growth phenotypes under conditions where glycerol export is required. Ansell *et al.* (1997) showed that during anaerobic growth, glycerol production is essential for redox balancing. Under these conditions, the *fps1*Δ mutants accumulate large amounts of glycerol inside the cell and grow much slower than the wild type (Tamás *et al.*, 1999). Expression of *S. pombe mip1* did not improve growth of the *fps1Δ* mutants during anaerobic conditions (Fig. 11).

To investigate whether the *S. pombe mip1* is involved in the uptake of glycerol and or other polyols such as xylitol, it was expressed in the *S. cerevisiae gpd1Δ gpd2Δ* strain. This strain is unable to produce glycerol (Ansell *et al.*, 1997) and is thus sensitive to hyper-osmotic stress caused by 2 M glycerol, 1 M erythritol or 1 M xylitol. However, its growth can be improved when transformed with a solute transporter that is capable of equilibrating the solute in and out of the cell (Karlgren *et al.*, 2000). Expression of *S. pombe mip1* did not improve growth of the *gpd1Δ gpd2Δ* mutants either on 2 M glycerol or 1 M xylitol (data not shown).

These results suggest that under the conditions tested, the *S. pombe mip1* was neither involved in the uptake of glycerol nor xylitol across the plasma membrane of *S. cerevisiae*. Whether the *S. pombe mip1* may require to be regulated by components not present in *S. cerevisiae* or whether it is involved in other functions unrelated to solute transport, is not yet clear.

The *S. pombe mip1*⁻ mutant phenotypes

To further investigate the function of *S. pombe mip1*, a disruption mutant was constructed (Fig. 2). The disruption was confirmed by PCR analysis and Southern blot using *ura4* as a probe (Fig. 12). The *S. pombe mip1::ura4*$^+$ mutant phenotypes were then tested under different physiological conditions where MIP channels have been shown to play a role. Lack of a glycerol facilitator has been reported to affect growth of some microorganisms on medium containing glycerol as a sole carbon source (Voegele *et al.*, 1993; Schweizer *et al.*, 1997). Disruption of the *S. pombe mip1* did not cause any observable growth defect on glucose or glycerol as a sole carbon source (Fig. 13). It is therefore likely that glycerol uptake for metabolic utilization does not occur via this channel. In addition, *S. pombe* does not present activity of mediated high or low affinity glycerol uptake (Lages *et al.* 1999) unlike *S. cerevisiae* where an active glycerol transport protein Gup1 (a non MIP protein), mediates glycerol uptake for the metabolic needs of the organism (Holst *et al.*, 2000). Therefore other transport systems may be involved. In addition to glycerol uptake, microbial MIP channels have been shown to play a role in osmoregulation (for reviews, Booth and Louis, 1999; Hohmann *et al.*, 2000). Therefore, the growth of *S. pombe mip1::ura4*$^+$ mutant under osmotic conditions was monitored. Surprisingly, the *S. pombe mip1::ura4*$^+$ mutant survived both the hyper- and hypo-osmotic shocks in a similar way as the wild-type cells (Fig. 14) suggesting that the *S. pombe mip1* might not be involved in short-term adaptation to osmotic stress. However, as pointed out previously (Chapter 2; Kayingo *et al.*, 2001), phenotypes associated with deletions of genes encoding MIP channels are not always clear and are difficult to interpret. They can be strain, concentration or growth phase dependent. For instance, deletion of *E. coli glpF* causes a growth defect only at low glycerol concentrations (Truniger *et al.*, 1992, Voegele *et al.*, 1993). Furthermore, deletion of the *aqpZ* gene causes a visible sensitivity to low osmolarity only after co-cultivation in competition with wild type (Calamita *et al.*, 1998). Hence, to detect the phenotype of a MIP channel may require very careful inspection as well as knowledge of the conditions the organism experiences in its natural environments.

Expression of *S. pombe mip1* is slightly induced by osmotic stress (NaCl)

A number of MIP genes appear to be affected by osmotic stress and their expression patterns may vary according to the growth phase of the organism (Flick *et al.*, 1997). In this study, the expression of *S. pombe mip1* was monitored by Northern blotting using the *S. pombe ura4* as a control. A decrease in the *S. pombe mip1* mRNA level was observed as cells entered stationary phase (Fig. 13). However, growing *S. pombe* cells in media containing 5% NaCl resulted in a 2-3 fold induction of *S. pombe mip1* (Fig. 15). This is in contrast to other osmoregulated genes such as *gpd1* (Ohmiya, *et al.*, 1995; Aiba *et al.*, 1998) whose expression can be induced up to 20-fold by an increase in external osmolarity.

Fission yeast cells respond to increased osmolarity via a mitogen-activated protein (MAP) kinase cascade, involving the Wis1 MAP kinase kinase and the Sty1 MAP kinase (for review, Banuett, 1998). The Sty1 MAP kinase transduces a signal to a target gene via a basic leucine zipper (bZIP) transcription factor, Atf1. Several stress responsive genes such as *fbp1*, *ctt1*, and *gpd1* have upstream activating sequences (UAS) on which Atf1 binds directly and then trigger gene expression (Wilkinson *et al.*, 1996). The signaling pathway controlling the observed induction in the *S. pombe mip1* is unknown. Analysis of the upstream sequences of *S. pombe mip1* revealed three potential Atf1 binding sites (CRE motif) at nucleotide -859 to -852 (CAACGTTC), -448 to -441 (AAACGTTC), and -180 to -187 (ACACGTTG) relative to the translation initiation codon (ATG). These sequences suggest that *S. pombe mip1* could be controlled in Atf1-Wis- Sty1 dependent way. However, this observation requires experimental confirmation.

MIP channels can be regulated both at gene and protein level. Phosphorylation sites controlling the activity of a significant number of eukaryotic MIP channels have been observed by many workers (Weaver *et al.*, 1994; Johnsson *et al.*, 1996; Maurel *et al.*, 1997). To date, little is known about MIP channel phosphorylation in microorganism but putative phosphorylation sites occur in the *S. pombe mip1*. Recently, a domain required to regulate the closure of the yeast Fps1p glycerol facilitator was identified (Tamás *et al.*, 1999). The Fps1p regulatory domain is located in the N-terminal extension, which is an unusual feature among the MIP family proteins. Interestingly, *S. pombe mip1* also exhibits an extended N-terminal extension but unlike its closest homologue Yfl054p, it has no sequence that shows some similarity to the regulatory domain of Fps1p (Fig. 4A). It is thus likely that *S. pombe mip1* is regulated in a way different from that of Fps1p.

Concluding remarks

Although *in silico* analysis suggests that the *S. pombe mip1* encodes a glycerol facilitator, *in vivo* experiments did not indicate its involvement in glycerol transport across the plasma membrane. It is still unknown whether the protein is involved in transport of yet unidentified solute. However, the observation that *S. cerevisiae* cells overexpressing *S. pombe mip1* leak glycerol during osmotic stress tempts one to believe that the gene may encode for ungated glycerol channel. Possibly the protein permeates glycerol with a low turn over insufficient to make cells sensitive to hyper-osmotic stress or to alleviate the downshock sensitivity of *fps1Δ* mutants. While the expression studies point to a role in osmoregulation, the mutant does not show phenotypes related to osmoregulation. Similarly, the closest homologue from *S. cerevisiae YFL054* is induced by osmotic stress in a Hog-dependent way suggesting a role in osmoregulation but the mutants also show no osmotic stress related phenotypes (Tamás, 1999). Furthermore, *YFL054* shows a 5.6 fold induction during a diauxic shift from fermentation to respiration (DeRisi *et al.*, 1997) but the significance of this induction is still obscure as there is no evidence for its involvement in metabolism. Therefore, the physiological role as well as the transport properties of these MIP channels are still intriguing questions. Localization studies on *S. cerevisiae* MIP proteins indicate that Yfl054p might be a vacuole protein (Tamás, 1999b) whereas aqy2-1p is abundant on microsomal vesicles (Meyrial and Tacnet, 2000). Localization studies in homologous systems and heterologous expression in other systems such as *Xenopus* oocytes might provide clues on the physiological roles of *S. pombe mip1* as well as its transport properties.

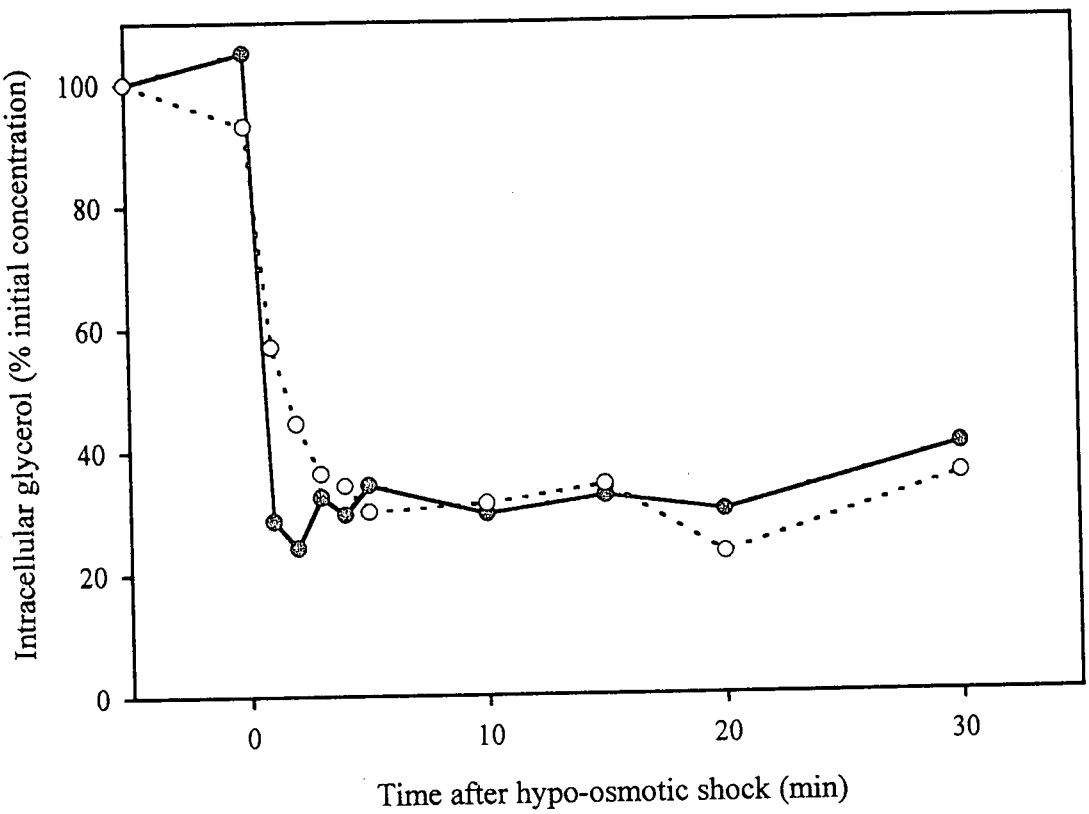


Figure 3. Glycerol release from *S. pombe* CBS 5682 (CSIR Y457) during hypo-osmotic stress. Cells were grown at 30°C YEPD broth until mid-exponential growth phase, resuspended in YEPD + 5% NaCl and cultured for 3 hours, harvested and then resuspended in YEPD without NaCl (osmotic downshock) held at 0°C (○) or at 28°C (●).

```
spombeMIP       MSVPLRFSTPSS----SPSASDN--------ESVHDDGPTTELDT--------------- 33
YFLO54C         MSYESGRSSSSSESTRPPTLKEEPNGKIAWEESVKKSRENNENDSTLLRRKLGETRKAIE 60
                **     *:.**    .*: .::        ***:..  ..* *:

spombeMIP       --------FNTTDVPRRVNTTKARQMRPKN-TLKVAFSSPNLKGLDNTADSDSQPWLGGY 84
YFLO54C         TGGSSRNKLSALTPLKKVVDERKDSVQPQVPSMGFTYSLPNLKTLNSFSDAEQARIMQDY 120
                        :.:    ::*   :  .::*:  :: .::* **** *:. :*::.   : .*

spombeMIP       LAGRLEDISGQSRRNYVDPYYEELN--AGRRPNKPVWSLNGPLPHVLGNSVVEKISQKNQ 142
YFLO54C         LS---RGVNQGNSNNYVDPLYRQLNPTMGSSRNRPVWSLNQPLPHVLDRGLAAKMIQKNM 177
                *:   ..:.  . .***** *.:**   *   *:****** ******...:. *: ***

spombeMIP       EARSRANSRVNSRANSRANSSVSLAGMDGSPNWKRKMKSAVFGSRVKLNDEEAQLPRNKS 202
YFLO54C         DARSRASSRRGSTDISRGGSTTSVK------DWKRLLRGAAPGK--KLGDIEAQTQRDN- 228
                :*****.** .*   **..*:.*:       :*** ::.*. *.  **.* ***  *::

spombeMIP       SVSIAEQAASRPKVSFSLQSSRQPSIAEEQPQTQRKSSAITVEHAENAEPETPRNNVSFS 262
YFLO54C         --TVGADVKPTKLEPENPQKPSNTHIENVSRKKKRTSHNVNFSLGDESYASSIADAESRK 286
                  ::. :. .    . . *.. :. * : . :.:*.*  :... .::: ..:  :  * .

spombeMIP       RKPSIAEQDSSQDITMPPNEIIAEES-----LDS---GSDTET-----LYLNYWCKIRHF 309
YFLO54C         LKNMQTLDGSTPVYTKLPEELIEEENKSTSALDGNEIGASEDEDADIMTFPNFWAKIRYH 346
                 *   : :.*:   *  *:*:* **.     **.   *:. :       : *:*.***:.

spombeMIP       FREGFAEFLGTLVLVVFGVGSNLQATVTNGAGGSFESLSFAWGFGCMLGVYIAGGISGGH 369
YFLO54C         MREPFAEFLGTLVLVIFGVGGNLQATVTKGSGGSYESLSFAWGFGCMLGVYVAGGISGGH 406
                          TMD1                          TMD2
                :** ***********:****.*******:*:***:****************:********

spombeMIP       VNPAVTISLAIFRKFPWYKVPIYIFFQIWGAFFGGALAYGYHWSSITEFEGGKDIRTPAT 429
YFLO54C         INPAVTISMAIFRKFPWKKVPVYIVAQIIGAYFGGAMAYGYFWSSITEFEGGPHIRTTAT 466
                                    TMD3
                :*******:******** ***:**. ** **:****:****.********** .***.**

spombeMIP       GGCLYTNPKPYVTWRNAFFDEFIGTAVLVGCLFAILDDTNSPPTQGMTAFIVGLLIAAIG 489
YFLO54C         GACLFTDPKSYVTWRNAFFDEFIGASILVGCLMALLDDSNAPPGNGMTALIIGFLVAAIG 526
                             TMD4                                TMD5
                *.**:*:**.***************:::*****:*:***:*:** :****:*:*:*:****

spombeMIP       MALGYQTSFTLNPARDLGPRMFAWWIGYGPHSFHLYHWWWTWGAWGGTIGGGIAGGLIYD 549
YFLO54C         MALGYQTSFTINPARDLGPRIFASMIGYGPHAFHLTHWWWTWGAWGGPIAGGIAGALIYD 586
                                                              TMD6
                **********:*********:**  ******:*** ***********.*.*****.****

spombeMIP       LVIFTGPESPLNYPDNGFIDKKV--------HQITAKFEKEEEVENLEKTDS--PIENN- 598
YFLO54C         IFIFTGCESPVNYPDNGYIENRVGKLLHAEFHQNDGTVSDESGVNSNSNTGSKKSVPTSS 646
                :.**** ***:******:*:::*        **  .....*. *:. .:*.*  .: ..
```

Figure 4A. Sequence alignment of *S. pombe mip1* and its closest homologue Yfl054p from *S. cerevisiae*. The highly conserved NPA motifs are indicated in bold and the amino acid residues in Yfl054p that shows homology to the regulatory domain of Fps1p are shown in a box. The six transmembrane domains (TMD) as predicted using the TopPred2 programme (http://www.biokemi.su.se) are underlined. Stars and dots indicate where there is perfect or imperfect match respectively.

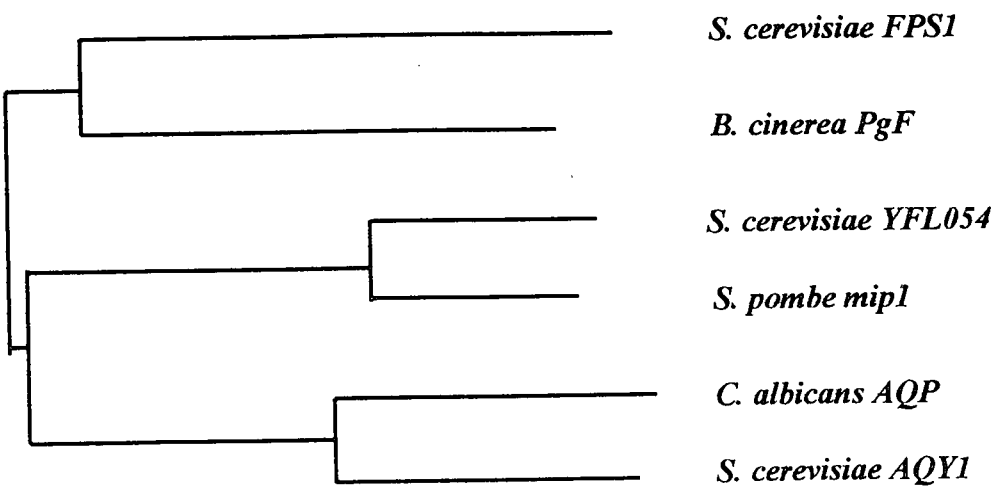
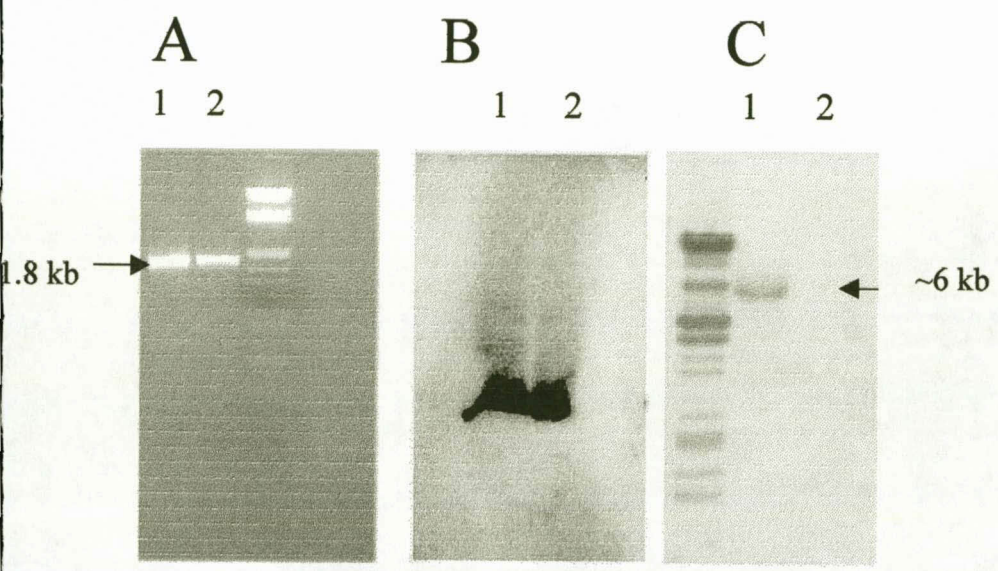


Figure 4B. Phylogenetic tree of fungal MIP channels. The tree was constructed using the Neighbor-Joining (NJ) method after a clustalW aligned amino acid sequences; *S. cerevisiae* glycerol facilitator *FPS1* (P23900), *YFL054* (P43549), aquaporin *AQY1* (AAC69713), *Candida albicans* putative aquaporin *AQP* (contig4-2389), *Botrytis cinerea* putative glycerol facilitator (AL112633) and *S. pombe mip1* (SPAC977.17).



gure 5. Isolation and verification of the *S. pombe mip1*.

1.8 kb *S. pombe mip1* PCR product was isolated from *S. pombe* strains (Panel A), labeled and ed in Southern blots (not shown) and Northern blot hybridization (Panel B) to confirm the igin and expression of the gene from *S. pombe* CBS 5682 wild-type strain (1) and *S. pombe* 2h⁻ laboratory strain (2). The two *S. pombe* strains were also verified by Southern blotting ing *ura4* as a probe on yeast genomic DNA digested with *EcoR*1 (Panel C).

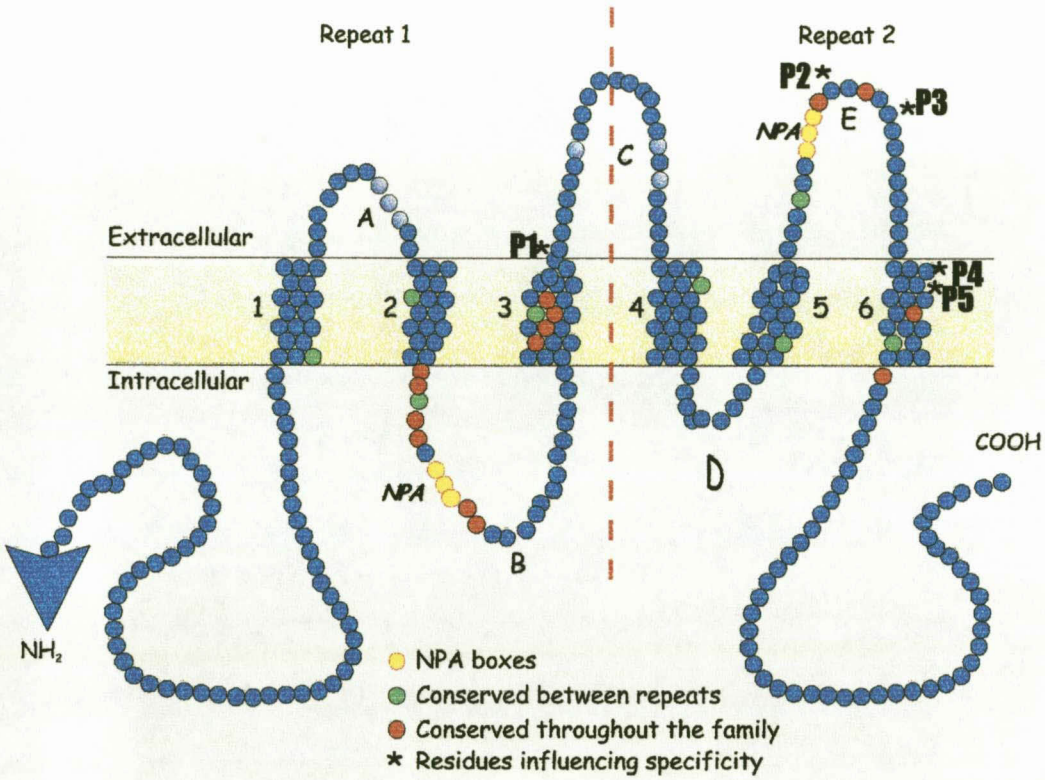


Figure 6. Schematic representation of the membrane topology for *S. pombe* mip1. The extended NH_2-terminus (about 310 amino acids) is indicated by a blue arrow. The putative membrane channel protein is predicted to have six transmembrane domains (1-6) and five connecting loops (A-E). The highly conserved residues common to most MIP family members as well as those residues that influence channel activity are highlighted (Froger *et al.*, 1998; Hohmann *et al.*, 2000).

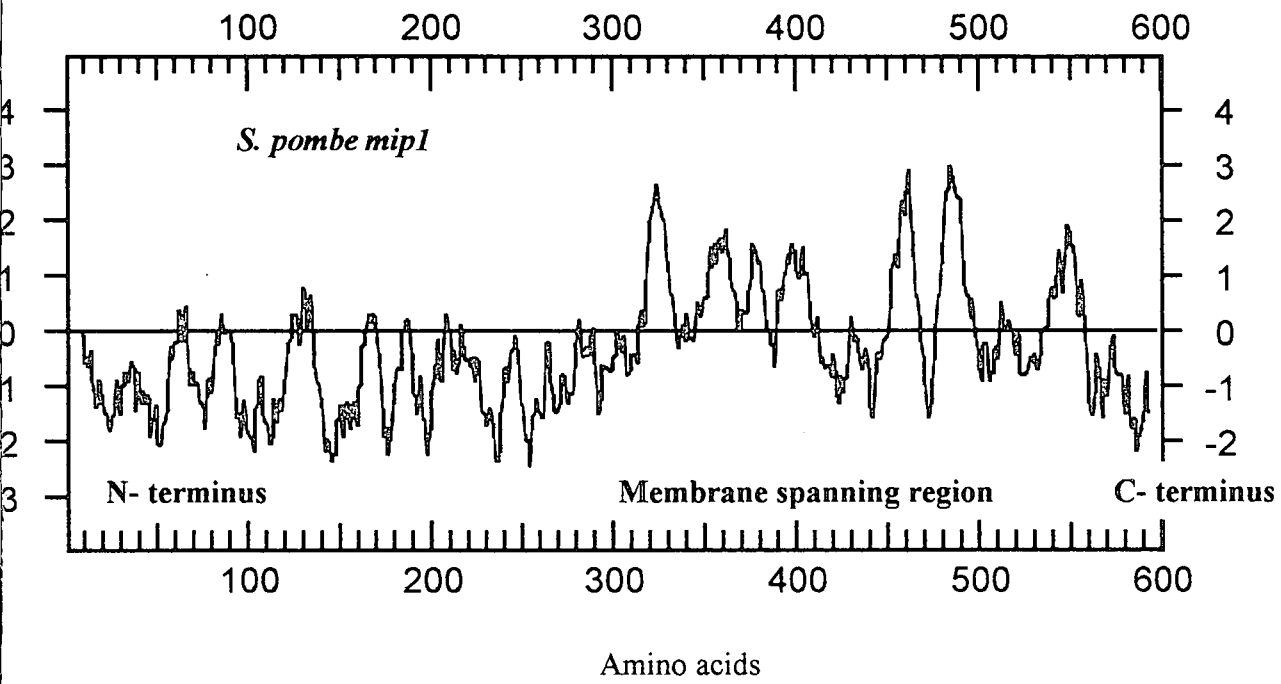


Figure 7. Hydropathy profiles of the *S. pombe* mip1 channel protein calculated according to Kyte and Doolittle (1982).

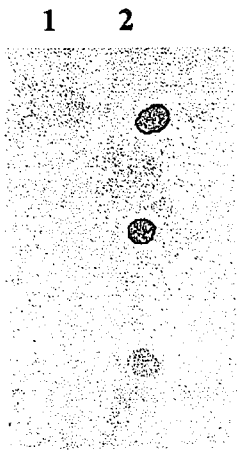


Figure 8. Analysis of expression of the *S. pombe mip1* in *S. cerevisiae* by Dot blotting
Total RNA from the *fps1Δ* strain + *pPGK* vector only (1) and *fps1Δ* + *pPGK-Spmip1* (2) was diluted, spotted on a nylon membrane and probed by the digoxigenin labeled fragment of *S. pombe mip1*

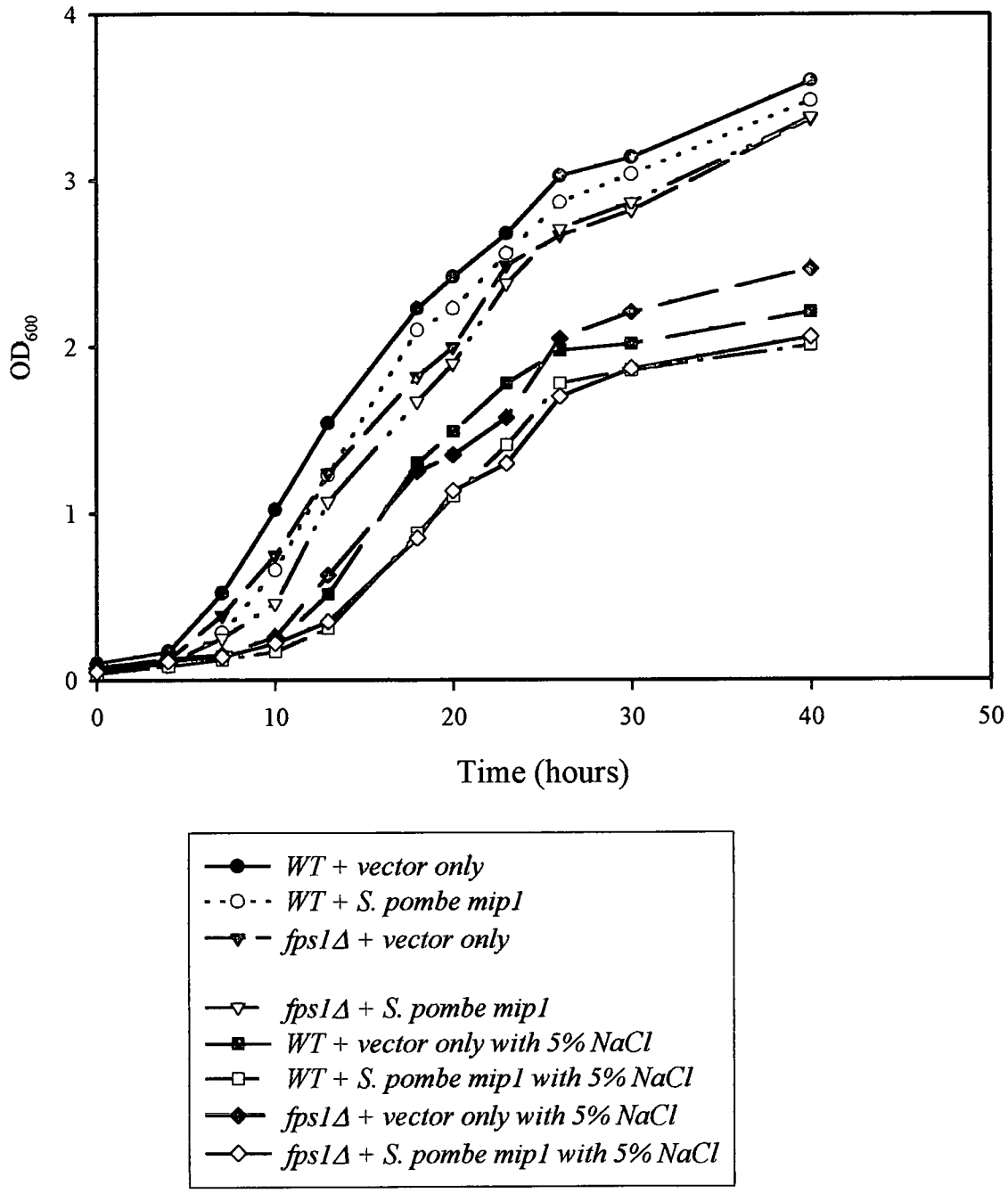
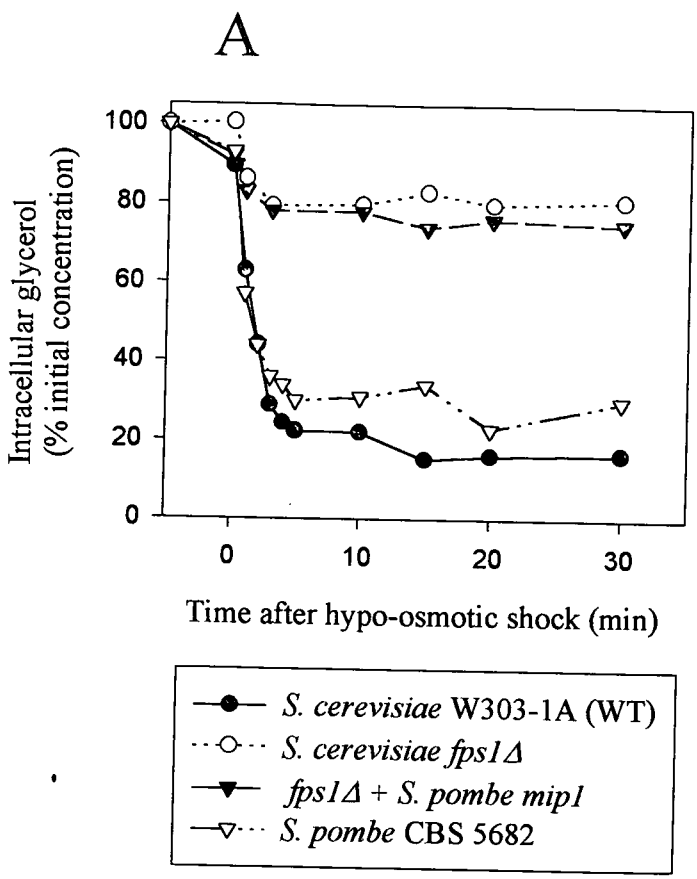


Figure 9. Growth of *S. cerevisiae* W303-1A and *fps1Δ* strains transformed with the pPGK vector with (open circles) or without (closed symbols) the *S. pombe mip1* gene in glucose YNB containing 0% (circles, triangles) or 5% NaCl (squares, diamonds)

Table 2. Distribution of intra-and extracellular glycerol content in *S. cerevisiae* W303-1A and *fps1*Δ transformed with pPGK vector with or without *S. pombe mip1* when cultivated in glucose -YNB with 5% NaCl till mid-exponential growth phase (OD_{600} = 1).
Values represent mean ± standard deviations of triplicate determinations.

Sample	Intracellular glycerol g/l	Extracellular glycerol g/l	% Intracellular glycerol
fps1Δ + vector only	0.195 ± 0.011	0.222 ± 0.009	46.76 ± 0.22
fps1Δ + *S. pombe mip1*	0.168 ± 0.006	0.274 ± 0.029	38.01 ± 1.65
WT + vector only	0.241 ± 0.018	0.39 ± 0.001	38.19 ± 1.70
WT+ *S. pombe mip1*	0.114 ± 0.005	0.261 ± 0.004	30.44 ± 0.75



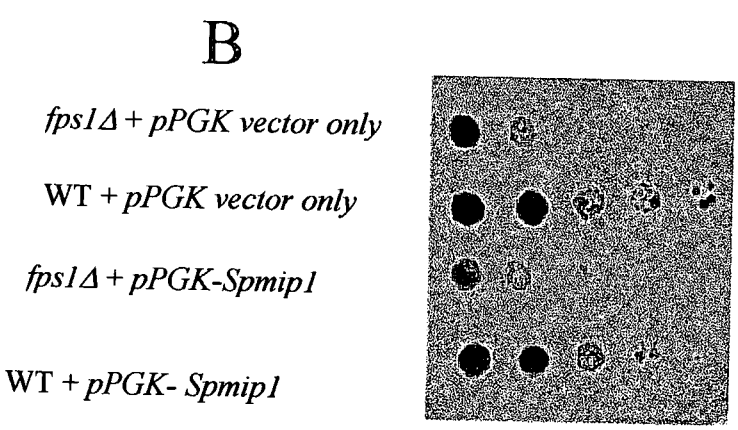


Figure 10. Effect of the expression of of *S. pombe mip1* on glycerol release (A) and survival (B) in *S. cerevisiae* W303-1A and *fps1Δ* strains during hypo-osmotic stress. Cultures were grown in glucose-YNB containing 5% NaCl for 3 hours followed by a downshock in glucose-YNB lacking NaCl.

WT + *pPGK vector only*

WT + *pPGK-Spmip1*

fps1Δ + *pPGK vector only*

fps1Δ + *pPGK-Spmip1*

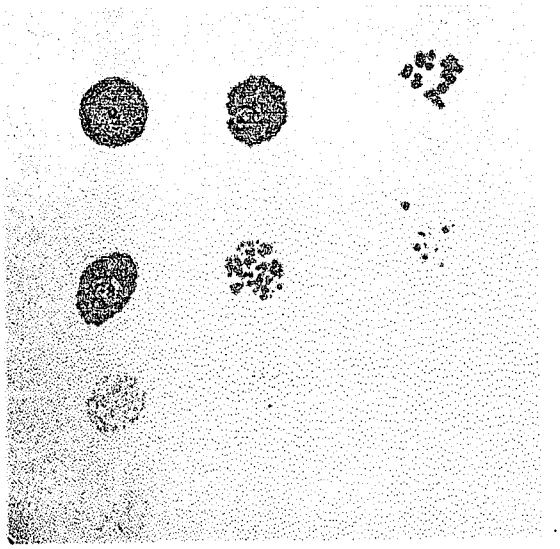


Figure 11. Growth of *S. cerevisiae* W303-1A and *fps1Δ* strains with or without *S. pombe mip1* cultivated on glucose-YNB under anaerobic conditions.

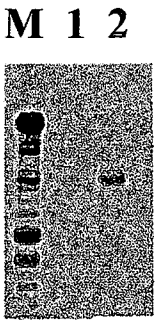
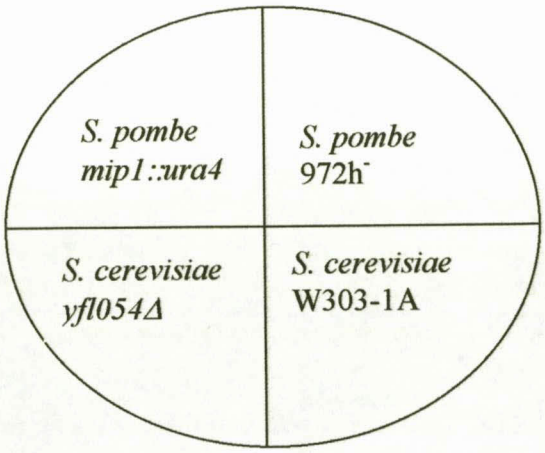


Figure 12. Southern blotting analysis of the deletion mutant.

Genomic DNA from the *S. pombe* 972h (lane 1) and YGK200 (lane 2) was digested with *Xba*1, transferred to a nylon membrane and probed with the *ura4* Dig labeled fragment as indicated in the materials and methods



A

B

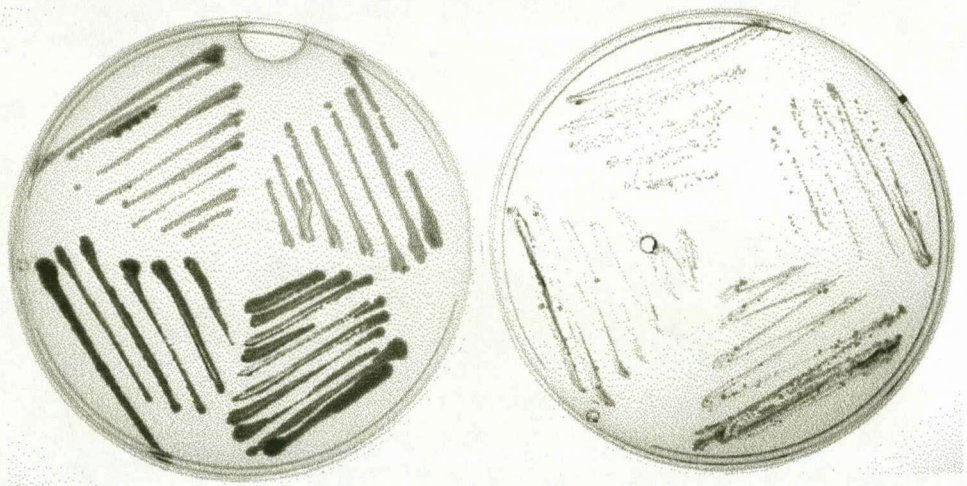


Figure 13. Growth of the *S. pombe* and *S. cerevisiae* strains on YNB with 2% glucose (A) or with 3 % glycerol (B).

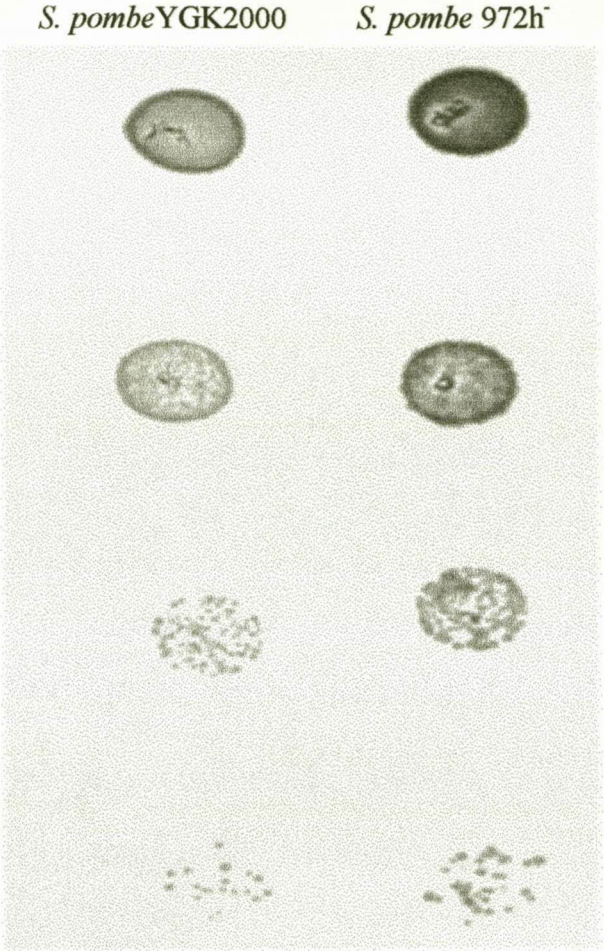


Figure 14. Growth of the *S. pombe mip1:: ura4* (YGK2000) mutant and its isogenetic wild-type *S. pombe* 972h⁻ on YEPD agar plates containing 3% NaCl.

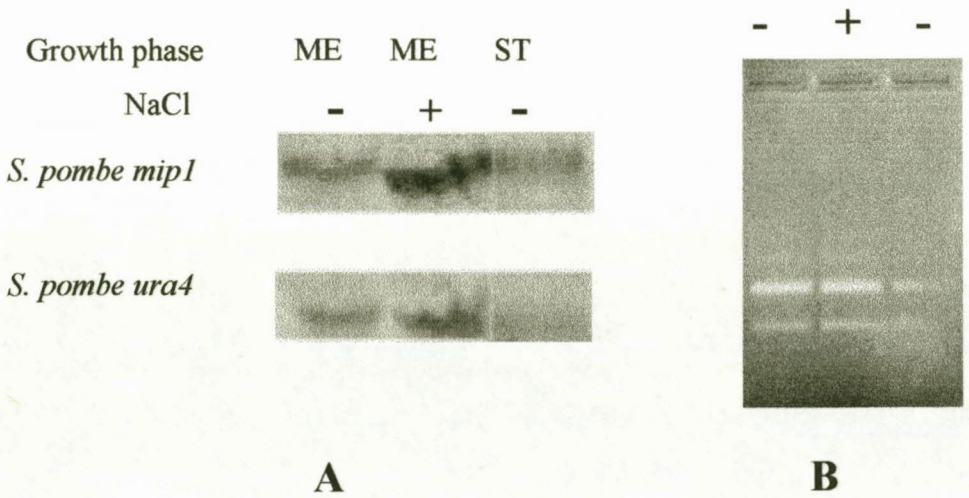


Figure 15. Northern Blot analysis (A). *S. pombe* CBS 5682 was grown at 30°C in YEPD medium with or without 5% NaCl until A_{600} reached approximately 0.5 (mid-exponential phase ME) or 3.0 (stationary phase ST). Total RNA was isolated and subjected to Northern hybridization analysis using *S. pombe mip1* and *ura4* as probes. An ethidium blomide stained gel (B) was used to check the quality and quantity of RNA prior to Northern blotting.

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CHAPTER 8

SUMMARY

This study examined the responses of yeasts to hypo-osmotic stress with special emphasis to osmolyte export and facilitating proteins. All yeast strains studied (*Z. rouxii*, *P. sorbitophila*, *S. cerevisiae* and *S. pombe*) rapidly release their intracellular osmolytes upon a decrease in external osmolarity (osmotic downshock or hypo-osmotic shock). Osmolyte release is very rapid, specific and is not affected at reduced temperatures neither inhibited by the channel blocker gadolinium or the protonophore CCCP. The export process is well controlled and the amount of osmolyte released is proportional to the shock intensity. Osmolyte release occurs with minimal cell lysis and thus the survival as well as the subsequent growth of yeast cells is largely unaffected after hypo-osmotic shock. The patterns and export kinetics suggested the involvement of channel proteins similar to that of Fps1p previously reported in *S. cerevisiae*. However, search for *FPS1* homologues from other yeasts using PCR and DNA probes resulted in weak hybridization signals suggesting that the putative glycerol channel encoding genes might have low sequence similarity to *FPS1*. It appears that although the mechanism of osmolyte release is conserved among yeasts, the proteins involved in this release might be divergent. This finding was in contrast to the general view that most of the genes involved in glycerol metabolism and stress responses such as *GPD1* (NAD^+-dependent glycerol-3-phosphate dehydrogenase), *DAK1* (dihydroxyacetone kinase) and *HOG1* (MAP kinase of the HOG pathway) are well conserved in all yeasts. Isolation and cloning of the corresponding gene(s) involved in osmolyte export will shed more light on the molecular nature and physiological roles of these exporters.

In yeast, osmolyte transport across the cell membrane occurs via active transport, mediated by channel proteins and by passive diffusion. The extent to which osmolytes permeates the cell membrane may then be influenced by the membrane lipid composition. In this study, the role of ergosterol (the most abundant sterol in yeast membranes) in osmolyte release and survival of yeast cells during hypo-osmotic stress was investigated. Cells lacking a glycerol facilitator (the *fps1Δ* strain) grow very poorly upon an osmotic downshock, but apparently survived the shock better and recovered more rapidly if ergosterol was supplied. Furthermore, the rate and amount of glycerol release was markedly enhanced in the *fps1Δ* mutant when exogenous ergosterol was supplied. The *erg-1* disruption mutant which is unable to synthesise ergosterol, survived and recovered from the osmotic shock more successfully at the higher ergosterol

concentration. Although the mechanism by which ergosterol improves glycerol release and survival of yeast cells is not well understood, it could presumably be related to the membrane stabilizing effects of ergosterol and the associated improvements in membrane fluidity. The polyene antibiotic nystatin, which affects membrane permeability in an ergosterol dependent way, caused *S. cerevisiae* cells to release a large amount of glycerol and equally inhibited the growth of wild-type and *fps1* deletion strains in medium containing 5% (w/v) NaCl. This study demonstrated the role of ergosterol in glycerol efflux and survival in *S. cerevisiae* after an osmotic downshock and provided additional evidence for the significance of membrane permeability and glycerol con servation in yeast osmoregulation.

The ability to regulate water and solute flux across cell membranes is critical in ensuring a constant turgor pressure as well as the proper functioning of biochemical processes. In most organisms, this process appears to be mediated by the MIP family transmembrane channel proteins, most of which have been characterized in higher animals. An *in silicio* phylogenetic analysis of microbial MIP channels revealed two major groups, the glycerol facilitators and the water channels (aquaporins), but further divided the glycerol facilitators into two subfamilies. Water channels seem to be important for growth after drastic changes in medium osmolarity, especially to lower osmolarity. Glycerol facilitators appear to exist in all microbial groups where they function in the uptake of glycerol and related compounds for their catabolism. The *S. cerevisiae* glycerol facilitator has been shown to be involved in osmoregulation by controlling the accumulation and release of glycerol. The occurrence of glycerol facilitators in other yeasts and their role in osmoregulation were investigated in this study. Blast searches in the *S. pombe* data bases revealed three putative glycerol transport proteins one of which shows considerably structural similarities to known MIP family glycerol facilitators. However, heterologous expression and subsequent functional analysis of this *S. pombe mip1* did not indicate its involvement in glycerol transport across the plasma membrane. It is still unknown whether the protein is involved in glycerol transport across other organelle membranes or whether it is involved in transport of a yet unidentified solute. The expression of *S. pombe mip1* is induced by osmotic stress suggesting a role in osmoregulation. However, deletion of *S. pombe mip1* does not cause any observable effects on growth of *S. pombe* cells during osmotic stress. Therefore, the physiological role of the *S. pombe mip1* as well as the actual transporter(s) controlling glycerol flux in *S. pombe* remains to be elucidated.

OPSOMMING

Hierdie studie het die respons van giste op hipo-osmotiese spanning ondersoek, veral osmoliet transport en die fasiliterende proteïene. Die studie het getoon dat al die giste- bestudeer *(Z.rouxii, P. sorbitophila, S. cerevisiae* and *S. pombe*) intrasellulêre osmoliete vinnig vrystel na dat die eksterne osmolariteit verlaagis (afwaardse osmotiese skok of hipo-osmotiese skok). Die vrystelling van osmoliete vind vinnig en spesifiek plaas en word nie deur verlaagde temperatuur beinvloed. Verder word die proses nie deur die kanaalblokkeerder ganadolium of die protonofoor CCCP geinhibeer nie. Die vrystelling van osmoliete word gereguleer sodat die hoeveelheid osmoliet wat vrygestel word korreleer met die verandering in eksterne osmolariteit. Omdat osmoliet vrysteling met minimale lise van selle plaasvind, word die oorlewing en groei van selle nie deur hipo-osmotiese skok geafekteer nie. Die kinetiese parameters van osmoliet vrystelling was soortgelyk aan die van die *FPS1* proteien van *S. cerevisiae*. Hibridisasie eksperimente (PCR en DNA peilers) het egter getoon dat daar 'n laë ooreenkoms tussen DNS volgordes van *FPS1* homoloë en 'n moontlike gliserol-kanaal-geen was. Dit blyk dus dat alhoewel die metodes om osmoliete vry te stel in giste gekonserveerd is dat die proteiene betrokke by die vrystelling verskil. Hierdie bevinding verskil van die algemeen aanvaarde opvatting dat die gene, betrokke by gliserol metabolisme en skok respons (bv. *GPD1; NAD*$^+$-dependent glycerol-3-phosphate dehydrogenase, *DAK1*; dihydroxyacetone kinase and *HOG1*; MAP kinase of the HOG pathway), gekonserveerd is in giste. Isolasie en klonering van die geen wat wel betrokke by vrystelling van osmoliete is, sal meer lig werp op die molekulêre aard en fisiologiese rol van transport-proteiene.

In giste word osmoliete opgeneem deur aktiewe transport deaur kanaal proteiene, asook passiewe diffusie oor die selmembraan. Die mate waar toe die selmembraan deurlaatbaar is, vir osmoliete, mag bepaal word deur die lipied samestelling van die selmembraan. In hierdie studie is die moontlike rol van ergosterol (die mees algemene lipied in gis selmembrane) in die vrysteling van osmoliete tydens hipo-osmotiese spanning ondersoek. Selle sonder 'n gliserol fasiliteerder (*fps*1Δ stain) het swak gegroei na 'n afwaardse osmotiese skok, maar indien ergosterol in die medium ingesluit is het sulke selle beter oorleef en vinniger herstel. Verder het die tempo waarteen gliserol deur die *fps1* Δ stain vrygestel is merkbaar verhoog in die teenwoordigheid van eksterne ergosterol. Die *erg*-1 disrupsie mutant, wat nie ergosterol kan sintetiseer nie, het ook beter oorleef en herstel in die teen woordigheid van eksterne ergosterol. Alhoewel die meganisme van bogenoemde effek van ergosterol op gliserol vrystelling onbekend is, is dit waarskynlik a.g.v. die stabiliseering en verbetering is

selmembraan vloeibaarheid. Die antibiotikum nystatin, wat membraan deurlaatbaarheid op 'n ergosterol afhanklike wyse beinvloed, het veroorsaak dat *S.cerevisiae* selle groot hoeveelhede giserol vrystel en het dus die groei van wilde tipe en *fps1Δ* delesie mutante in 5% (w/v) NaCl geinhibeer. Hierdie studie het getoon dat ergosterol 'n rol speel in die vrylating van gliserol uit selle asook in die oorlewing van *S. cerevisiae* na osmotiese skok. Dit verskaf ook verdere bewys vir die belangrikheid van membraam deurlaatbaarheid en gliserol bewaring in osmoregulering van giste.

Dit was duidelik dat die vermoë van selle om die beweging van water en ander oplosmiddels oor selmembrane te beheer belangrik is in die handhawing van korrekte turgordruk en biochemiese funksionaliteit. In meeste organismes blyk dit dat die proses deur transmembraanproteiene van die MIP familie beheer word. 'n "*in silico*" filogenetiese analise van mikrobiese kanaalproteiene het getoon dat daar twee hoofgroepe is nl. gliserolfasiliteerders en waterkanale (aquaporins). Die gliserol fasiliteerders was onderverdeel in twee groepe. Die waterkanale blyk belangrik te wees vir groei na verandering in medium osmolaritiet, veral verandering na laë osmolariteit. Gliserol fasiliteerdes kom waarskynlik in alle mikrobe organismes voor en speel 'n rol in die opname van gliserol en verbindings betrokke by gliserol katabolisme. Die gliserol fasiliteerder van *S. cerevisiae* beheer osmoregulering deurdat dit die opname of vrystel van gliserol kontroleer. Die voorkoms van gliserolfasiliteerders in ander giste en hulle rol in osmoregulering is in hierdie studie ondersoek. "BLAST" ondesoeke van DNS data van *S. pombe* het drie moontlike gliserol fasiliteerders van die MIP familie getoon. Heteroloë uitdruking en funksionele analises van *S. pombe mip1* kon egter geen bewys lewer dat dit betrokke is in gliserol transport oor die plasma membraan. Dus is dit nog onbekend of die proteïen betrokke is by gliserol transport oor ander organel membrane en of dit betrokke is by die transport van ander oplosmiddels. Uitdruking van S. pombe mip1 word deur osmotiese spanning geinduseer, wat 'n rol daaarvoor tydens osmoregulering aandui. Delesie van *S. pombe mip1* het egter nie die groei van *S. pombe* tydens osmotiese spanning geaffekteer nie. Die fisiologiese rol van *S. pombe mip1* en die werklike transporter(s) wat gliserol opname/vrystelling beheer in *S. pombe* moet dus nog ontrafel word.