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Glycerol transport in yeasts: physiological, molecular and phylogenetic approaches

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STATEMENT:

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Maria Luisa Vieira das Neves

A espantosa realidade das cousas

É a minha descoberta de todos os dias.

Cada cousa é o que é,

E é difícil explicar a alguém quanto isso me alegra,

E quanto isso me basta.

In O guardador de rebanhos

Alberto Caeiro, 1915

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Sumário

O glicerol é um composto fundamental na regulação de vários processos celulares em diferentes leveduras. Em *Saccharomyces cerevisiae*, uma parte dos genes envolvidos no seu consumo e produção, bem como no seu transporte, estão presentemente identificados. Nesta levedura, estudos prévios demonstraram que o glicerol pode permear a membrana através de dois processos independentes, um simporte com protões e um canal. Enquanto que o simportador se apresenta regulado pela glucose, induzido por crescimento em fontes de carbono não fermentáveis, o gene que codifica para o canal, *FPS1,* é constitutivamente expresso, sendo responsável pela difusão do glicerol e pelo seu efluxo/retenção em situações de choque osmótico. Os genes *GUP1* e *GUP2* foram associados ao transporte activo do glicerol. No entanto, só combinando a sua delecção com a do gene *GUT1*, que codifica para a primeira enzima da via catabólica do glicerol, é que se consegue abolir o transporte deste substrato em células cultivadas em condições de desrepressão.

Neste trabalho foi medido transporte activo de glicerol no mutante triplo, *gup1gup2gut1*, desde que cultivado em glucose com 1M NaCl, particularmente activo na fase diauxica de crescimento. Este novo resultado foi confirmado em outros mutantes, com diferentes combinações das delecções nos genes *GUP1/2, GUT1* e *FPS1*. Mostrou-se a indução do transportador pela conjugação da desrepressão/fase diauxica com o stress osmótico.

Concomitantemente, foi realizada uma pesquisa nas bases de dados de genomas de leveduras, de ortólogos dos genes de *S. cerevisiae GUP1*/2, *FPS1* e *GUT1* acima mencionados. Foram ainda incluídos nesta pesquisa os isogenes *GPD1/2,* que codificam para glicerol 3-fosfato desidrogenases citoplasmáticas. Posteriormente, uma grande parte das ORFs encontradas foram clonadas e usadas para complementar fenotipicamente os respectivos mutantes de *S. cerevisiae*. A análise filogenética realizada sugeriu que as proteínas codificadas pelos genes *GUP* deviam ser incluídos na extensa família de proteínas MBOAT (membrande bound *O*-acyl tranferases), que agrupa enzimas, acil-transferases, associadas à membrana plasmática. Dada a forte semelhança entre as sequências encontradas e o grau de conservação das proteínas desta família, considera-se não ser válido continuar a incluir as Gup1/2p na família de proteínas transportadoras que lhes tinha sido atribuída anteriormente.

Foi também feita uma análise de Southern para identificar ortólogos desses genes em leveduras para as quais o transporte de glicerol tinha sido fisiologicamene caracterizado, mas cujo genoma não se encontra disponível ou não estava ainda sequenciado na sua totalidade à altura do trabalho. Desses

resultados, salientamos os que dizem respeito à sonda *FPS1*. Tendo em consideração que todas as leveduras estudadas apresentaram entrada linear de glicerol para além do transporte activo, era de esperar a existência em todas elas de proteínas análogas a Fps1p. A sonda hibridou, tal como esperado, contra o genoma de *Z. rouxii, K. marxianus*, e *P. angusta*, mas não contra o genoma de *C. halophila, P. sorbitophila, D. hansenii, C. albicans* e *C. tropicalis*, sugerindo que a permeabilização de glicerol se fará nas leveduras deste segundo grupo por proteínas muito diferentes de Fps1p. A clarificação das razões subjacentes a este resultado necessita de mais trabalho experimental.

Uma das leveduras cuja base de dados sugeria a presença de um gene homólogo ao *GUP1* foi *Candida albicans*, um conhecido patogénico da espécie humana. Nesta, a síntese e acumulação de glicerol foi recentemente associada à sua patogenicidade, tendo desta forma adquirido um interesse especial. Por esta razão, fomos caracterizar o transporte de glicerol nesta levedura. Os resultados revelaram a presença de um simportador glicerol/protão específico, constitutivo e não afectado pelo sal. A proteína CaGup1, que apresenta um elevado grau de semelhança a ScGup1p, não pode ser responsável por este transporte, pois o mutante deleccionado nesse gene apresentava o mesmo tipo de transporte da estirpe selvagem. Para além disso, o efluxo de glicerol observado após choque hipo-osmótico foi semelhante ao que foi descrito para *S. cerevisiae*, sugerindo o envolvimento de um canal mediador. No entanto, o único gene com alguma similaridade com o *FPS1* de *S. cerevisiae*, o gene *AQY1*, não parece estar envolvido neste processo.

Como os resultados anteriores sugeriam que o transportador activo de glicerol não era codificado pelos genes *GUP1/2*, e no intuito de prosseguir com a pesquisa do gene codificante do transportador, foram utilizadas duas estratégias. A primeira consistiu em usar uma biblioteca de transposões para mutagenizar uma estirpe de *S. cerevisiae* deleccionada no gene *FPS1*, com o intuito de isolar clones com fenótipo em glicerol. Esta estratégia não resultou devido às dificuldades em transformar a referida estirpe. A segunda consistiu na pesquisa de fenótipo em glicerol da colecção de mutantes simples da EUROSCARF. Foram isolados clones presumivelmente envolvidos no consumo de glicerol, mas não no seu transporte, dado que todos demonstraram ter capacidade para acumular glicerol contra o seu gradiente químico.

Tendo em consideração todos os resultados obtidos, pode-se inferir que os genes *GUP1* e *GUP2*, apesar de envolvidos no transporte de glicerol não codificam para o simportador de glicerol. Para além disso, mostrou-se que o

transporte activo do glicerol em *S. cerivisiae*, além de induzido por fontes de carbono não fermentáveis também é induzido pelo stresse salino, em particular durante a fase diauxica de transição fermento/respirativa. Ficou ainda evidente o envolvimento de vários genes no transporte de glicerol, apesar dos seus papéis ainda não serem completamente compreendidos. Deste trabalho ressalta ainda a noção de que a análise filogenética é uma ferramenta fundamental em proteómica, mas que as conclusões que daí se podem retirar têm de ser interpretadas com algum cuidado, porque nem sempre as semelhanças em termos de sequências correspondem a semelhanças funcionais.

Abstract

Glycerol is a key compound in the regulation of several metabolic pathways in yeasts. In *Saccharomyces cerevisiae*, most of the genes involved in glycerol consumption, production and transport are now available. Previous studies evidenced that in *S. cerevisiae* glycerol could permeate the plasma membrane by two simultaneous and independent processes. A channel encoded by the gene *FPS1*, is responsible for glycerol diffusion as well as for its efflux/retention upon osmotic shock. A proton symporter, derepressed by growth on non-fermentable carbon sources, is responsible for glycerol active transport. This was associated with *GUP1* and *GUP2* genes. It was demonstrated that the combined deletion of *GUP1, GUP2* together with *GUT1* (glycerol kinase) was necessary to abolish active transport in cells cultivated on ethanol, *i.e.* on derepression conditions.

As the previous results were not conclusive in relation to the affiliation of the function of Gup1and Gup2p, we focused on further clarifying glycerol transport mechanisms in yeasts. Considerable symporter activity was measured in *gup1gup2gut1* mutants grown on glucose under salt-stress, particularly high in cells collected during diauxic-shift. This result was confirmed in other mutant combinations of the genes *GUP1/2, GUT1* and *FPS1.* These results showed that the transport regulation could be activated by the combination of derepression/diauxic shift with osmotic stress.

Concomitantly, a search for *GPD1/2*, *GUT1, GUP1/2* and *FPS1* orthologues in a series of hemiascomycetous yeasts was performed. All the genes cloned were able to complement *S. cerevisiae* mutant phenotypes and presented a high degree of similarity to the corresponding genes in this yeast. Phylogenetic analysis suggested the allocation of Gup proteins in the MBOAT (Membrane Bound *O*-Acyl Transferases) family, which includes enzymes from the plasma membrane. This is suggested as more valid than their inclusion in the TC-DB/Glycerol Uptake family.

Besides, a Southern blot analysis was done to identify the glycerol related genes mentioned above in yeasts, besides *S. cerevisiae*, from which glycerol transport had been physiologically characterized and which genome was either unavailable or just partially sequenced. From these we stress the results from *FPS1* probe. Considering that all the studied yeasts presented linear glycerol entry besides active transport, the presence of Fps1p-like channels in all yeasts was predicted. Results were positive, as expected, in *Z. rouxii, K. marxianus* and *P. angusta*, but negative in *C. halophila, P. sorbitophila, D. hansenii, C. albicans* and *C. tropicalis*, suggesting that glycerol permeation must occur in these yeasts through very different proteins. The clarification of the reason underlying this difference will need further approaches.

Recently, glycerol accumulation has been associated with *Candida albicans* pathogenecity/infectious ability. We consequently chose to characterize glycerol transport in *C. albicans*. Experiments revealed the presence of a specific and constitutive proton/glycerol symport, not affected by osmotic shock. Gup1p is not responsible for this active transport, since the mutant strain presents the same kind of glycerol transport of the wild type. The kinetics and patterns of glycerol export are similar to *S. cerevisiae'*s, suggesting the involvement of a channel mediated transport. Yet, the only *C. albicans* gene *AQY1* that is homologous to *S. cerevisiae FPS1* is apparently not involved in the observed glycerol efflux.

As the obtained results suggested that Gup1p and Gup2p were not glycerol transporters, two strategies were developed in order to find further genes involved in glycerol transport. The first corresponded to the use of a transposon-mutagenized library to transform *S. cerevisiae fps1* mutant in order to isolate clones deficient in glycerol utilization. This strategy was unsuccessful due to difficulties in the *fps1* strain transformation. The second corresponded to a screen for glycerol defective growth of the EUROSCARF mutant collection. Several genes putatively involved in glycerol consumption were isolated, but not on transport, since they all proved to be able to accumulate glycerol against its chemical gradient.

Taking into consideration all the results here presented and discussed, we can state that *GUP1* and *GUP2*, despite their involvement in glycerol transport, do not encode the glycerol carrier. Furthermore, we showed that glycerol active transport in *S. cerevisiae*, besides being induced by non-fermentable carbon sources, is also induced by salt stress, particularly during diauxic shift, *i.e.,* during the transition from fermentative to respiratory metabolism. It became evident that several genes are involved in glycerol uptake, though their roles are not yet completely understood. Besides, it outstands from this work the notion that phylogenetic analysis is a powerful tool for suggesting protein roles but that it cannot be taken de *per se* as an indication of protein function.

Publications within the thesis context

Papers published in peer-reviewed journals

Neves, L., Lages, F. and Lucas, C. (2004) New insights on glycerol transport in *Saccharomyces cerevisiae*. FEBS Letters 565:160-162

Neves, L., Oliveira, R. and Lucas, C. (2004) Yeast orthologues associated with glycerol transport and metabolism. FEMS Yeast Research (in press)

Kayingo, G., Neves, L., Martins, A., Lucas, C., and Wong, B. (2004) Glycerol transport in *Candida albicans*. Evidence for a constitutive active glycerol uptake system independent of CaGup1p, and a glycerol efflux system independent of Fps1p-like Aqy1p. (submitted)

Publication of nucleotide sequences of genes at the GeneBank database of NCBI (National Center for Biotechnology Information)

Nucleotidic sequence of a gene encoding a glycerol 3-P dehydrogenase of *Pichia angusta*, *PaGPD*, with the accession number AY289714 (Jun 2003)

Nucleotidic sequence of a gene encoding a glycerol 3-P dehydrogenase of *Kluyveromyces thermotolerans, KtGPD*, with the accession number AY289713 (Jun 2003)

Nucleotidic sequence of *a GUP 1* orthologue from *Candida tropicalis*, *CaGUP1*, with the accession number AY299512 (August 2003)

Nucleotidic sequence of *a GUP 1* orthologue from *Pichia sorbitophila*, *PsGUP1*, with the accession number AY338363 (August 2003)

Nucleotidic sequence of *a FPS1* orthologue from *Zygosaccharomyces rouxii*, *ZrFPS1*, with the accession number AY299513 (August 2003)

Nucleotidic sequence of *a FPS1* orthologue from *Kluyveromyces lactis*, *KlFPS1*, with accession number AY338364 (August 2003)

Nucleotidic sequence of *a FPS1* orthologue from *Kluyveromyces marxianus*, *KmFPS1*, with the accession number AY541009 (March 2004)

Nucleotidic sequence of *a GUT1* orthologue from *Pichia sorbitophila*, *PsGUT1*, with the accession number AY391774 (October 2003)

Publications in conference procedures

Neves, L., Lages, F., Oliveira, R., Brandt, A., Kielland-Brandt, M. and Lucas, C. (2003) Glycerol uptake in *Saccharomyces cerevisiae*. New insights. Yeast. Vol 20, S248

Scope and outline of the thesis

A better understanding of glycerol movements across cell membranes as well as its metabolism can help to unravel many cell homeostasis mechanisms. Additionally, it can contribute to the improvement of biotechnological processes, as glycerol properties make it useful in numerous industrial, therapeutic and diagnostic applications. The studies presented on this thesis aimed to contribute to some extent to a better understanding of glycerol transport, using yeasts as eukaryotic model organisms.

After a general introduction to this theme (Chapter I) results obtained with our research are presented and discussed.

Availability of partially or completely sequenced genomes in databases, like the one from the Génolevures Programme, allowed us to search and clone orthologues of *Saccharomyces cerevisiae* genes involved in glycerol transport and metabolism. Following, a phylogenetic analysis was done, which allocated Gup1/2p, primarily identified as involved in glycerol uptake in *S. cerevisiae*, in the Membrane Bound O-Acyl-Transferases (MBOAT) family, in agreement with previous suggestions in the literature (Chapter II).

Considering that the phylogenetic results indicate for Gup1/2p functions other than glycerol transport, we proceeded by further assaying glycerol uptake in *S. cerevisiae* mutants deleted in *GUP1*, *GUP2* and *GUT1*, *i.e.*, in cells previously shown to be devoid of glycerol active transport when exponentially growing under induction conditions (Chapter III). Results obtained suggest that glycerol proton symport transport in this yeast operates independently of Gup1p and Gup2p.

In chapter IV, evidence is presented for the existence of a specific and constitutively expressed glycerol/H^+ symport, function in the human pathogen *Candida albicans*. CaGup1p was shown not to determine glycerol active transport, since the CaGup1 mutant strain presents the same kind of glycerol transport of the wild type, with identical K_m.

Results presented in previous chapters leaded us to conclude that *GUP1* and *GUP2*, despite its involvement in glycerol transport, do not encode the real glycerol transporter(s), and prompted us to delineate several strategies in order to search for other genes related to glycerol transport. Despite that these strategies did not allow us to achieve the ultimate goal, *i.e.,* the identification of the gene encoding for glycerol transporter, they allowed the identification of several genes putatively involved in glycerol consumption regulation, which roles will need further studies (Chapter V).

Finally, the most relevant results are highlighted and future perspectives for continuing these studies are outlined.

Chapter I

General Introduction

The importance of glycerol for living organisms and biotechnological processes

Glycerol, a main product of yeast metabolism, is a precursor for several cell compounds and a regulator of various metabolic pathways. Some studies suggest that glycerol metabolism appeared very early in the evolutionary process, namely in prebiotic era (Weber, 1987) and the pathways in which glycerol is involved have been preserved throughout evolution, demonstrating their fundamental importance.

Glycerol is an important substrate in several species' energy metabolism and lipid synthesis (Bortz *et al.,* 1972; Nevoigt and Stahl, 1987; Holms, 1996) and plays an important role in the balance of cell redox potencial and inorganic phosphate recycling (Ansell *et al.,* 1997; Alonso-Monge *et al.,* 2003). In prolonged fasting, glycerol can be used as the only source for gluconeogenesis (Baba *et al.,* 1995), an important evolutionary adaptation in the hibernation process (Yeh *et al.,* 1995; Berrada *et al.,* 2000).

In humans, glycerol seems to be involved in thermoregulation, resistance to high temperatures and endurance to physical activities (Robergs and Griffin, 1998; Wagner, 1999), and is involved in the neuronal response to glycemia variations (Yang *et al.,* 1999). It has been shown to be involved in several physiopathological mechanisms. For instance, higher glycerol concentrations, with increased turnover rates are generally observed in obese persons (Bortz *et al.,* 1972) and glycerol 3-phosphate shuttle is involved in tumor cell growth and apoptosis (MacDonald *et al.,* 1990; McCabe, 1995; Mazurek *et al.,* 1997).

Nowadays, glycerol is related with several therapeutic and diagnostic applications. It is used in the treatment of cerebral oedemas, intraocular and intracranial hypertension (Bayer *et al.,* 1987), as a purgative agent (McCabe, 1995) and also in cancer therapy (Ohnishi *et al.,* 1999). Glycerol can also be used in the diagnosis of carbohydrate metabolism disorders, like glucose intolerance (Gaudet *et al.,* 2000).

In laboratory technologies glycerol has proved to be one of the most efficient compounds for the cryoprotection of sperm, skin and other fibrous membrane samples, as well as of microorganisms, enzymes and nucleic acids (Tsuruta *et al.,* 1992; Izawa *et al.,* 2004a,b; Hubálek, 2003). The applications in biotechnological industries are very diverse. For example, glycerol is used as a softener for cough syrups, emollients and ointments (McCabe, 1995). In red wines and in *cachaça* (sugar cane aqua-vitae) glycerol viscosity adds smoothness to the product without

affecting its aromatic properties (Remize *et al.,* 2000; Rogélio Brandão, personal communication).

In eukaryotic microorganisms it is the main compatible solute produced to counterbalance the low water availability in high-osmotic stressed environments (Rep *et al.,* 1999; Wang *et al.,* 2001).

Thus considering, the homeostasis of glycerol levels is essential in the most diverse organisms/tissues/cells. A full understanding of the ways glycerol crosses the cell membrane, how it is involved in diverse metabolic pathways and cell responses, is crucial. Yeasts, in particular *Saccharomyces cerevisiae*, can be utilized as a model and help to understand important physiopathological mechanisms (Brisson *et al.,* 2001; Hara and Verkman, 2003). The knowledge derived from glycerol metabolism, transport and regulation processes has a strong technological potential for industry and agriculture applications, putatively providing a major breakthrough for interesting biotechnological applications, like tools for the genetic engineering of other organisms (Serrano, 1996).

Transmembrane transport of glycerol in yeasts

Transmembrane transport of glycerol is the first step for glycerol utilization, but is also very important for other things, like the efflux of glycerol after hypoosmotic shock, or the capture of glycerol from the medium for osmotic balance purposes.

For many years, glycerol was considered as a lipossoluble molecule able to cross cell membranes by simple diffusion (Gancedo *et al.,* 1968, Heredia *et al.,* 1968). Yet, this was not consistent with the fact that yeasts retain and accumulate glycerol inside the cell (Blomberg and Adler, 1989). To explain this, some authors hypothesized changes in the composition and concentration of fatty acids in cell membrane of yeasts grown at reduced water activity, which may decrease the membrane permeability (Tunblad-Johansson *et al.,* 1987; Watanabe and Takakuwa, 1987), while others proposed the presence of active transport systems responsible for the reuptake of glycerol that leaked to the medium by simple diffusion (Lucas *et al.,* 1990; van Zyl *et al*, 1990). The impermeability of the cell membrane to glycerol has been stated for *Fusarium oxisporum* (*Castro & Loureiro-Dias, 1991*). Recently, Oliveira and co-workers (2003) presented evidence sustaining the low probability of glycerol to cross freely *S. cereviae* membrane. This is consistent with the fact that, under anaerobic conditions, when glycerol is produced for redox regulation, the lack of Fps1p, a glycerol channel which controls

glycerol retention and efflux according to osmotic hyper or hypo-osmotic shocks, results in dramatic glycerol accumulation and in growth defect (Tamás *et al.,* 1999).

Glycerol transport in Saccharomyces cerevisiae

In *Saccharomyces cerevisiae,* Albertyn and co-workers (1994b) observed that upon salt stress a *gpd1* strain, *i.e.* a strain deleted in one of the genes encoding for glycerol 3-phosphate dehydrogenase, is able to increase the intracellular glycerol level by taking up extra cellular glycerol against a concentration gradient, indicating that the cell has an active uptake system for glycerol. Consistently, Ansell and Hohmann (personal communication) found that small amounts of glycerol in the medium lowers the osmo-sensivity of strains deficient in glycerol synthesis.

Evidence of mediated transmembrane transport of glycerol in *S. cerevisiae* was first reported in the early 1990's. Van Aelst and co-workers (1991) cloned *FPS1* gene as a multicopy suppressor of the growth defect on fermentable sugars of the yeast *fdp1* (*FDP1* is also known as *GGS1*, for component of the proposed General Glucose Sensor) (Thevelein, 1994, Van Aelst *et al*, 1991). Fps1p is a MIP family permease reported to be required for glycerol uptake by facilitated diffusion (Luyten *et al*, 1995). Nevertheless, the utilization of glycerol as carbon source on osmotically stressed conditions, pointed to the existence of more transport systems. In fact, glycerol uptake in a *fps1* mutant incubated under osmotic stress was two times higher than in the same strain under normal conditions, pointing to other uptake systems functioning under osmotic stress to capture glycerol from the medium. Also, Fps1p does not seems to be involved in glycerol utilization since no growth defect on glycerol was observed in a *fps1* mutant (Luyten *et al.*, 1995). Actually, results obtained by these researchers with biochemical and genetic experiments demonstrated the role of Fps1p in glycerol efflux. Its possible role in glycerol influx was just inferred from accumulation experiments. The authors proposed a transport model with two components: one independent of *FPS1* and probably due to free diffusion through the lipid bilayer and a second of higher capacity apparently due to facilitated diffusion by the FPS1 channel protein.

Subsequently, Lages and Lucas (1997) described an active transport system for glycerol in *S. cerevisiae*. Experiments were done in the strain IGC 3507. Exponential cells grown on glycerol, ethanol or acetic acid, both in minimal or rich media, present glycerol active uptake, while exponential glucose mineral medium grown cells only present simple diffusion. No glycerol/H^+ uptake induction was obtained by starvation of cells up to 24 hours in mineral media without carbon source. On the other hand, ethanol grown cells incubated with glucose for crescent

periods of time leaded to disappearance of the uptake. This was not reversible after glucose removal. In YPD grown cells in diauxic phase glycerol uptake followed saturable kinetics of lower affinity, but no accumulation or proton uptake. Cells grown in minimum medium with glucose in the presence of several NaCl concentrations and collected in different growth phases didn't present proton symport. The presence of NaCl in the assay of cells grown either in glucose, ethanol or glycerol, didn't affect uptake. In glucose grown cells simple diffusion measured in presence of 1M NaCl was 3 times lower than in the absence of salt. Taking into account these results it was proposed that *S. cerevisiae* presents, besides simple and facilitated diffusion, a symport of glycerol with protons subjected to glucose repression, gluconeogenic induction and apparently not involved in osmotic stress (Lages and Lucas, 1997).

By the same time, Sutherland and co-workers (1997), using a different *S. cerevisiae* strain (W303-1A) confirm the presence of a glycerol/proton symport system in derepressed cells, independent of Fps1p-mediated uptake. This was characterized as a constitutive facilitated diffusion system. Furthermore, the presence of passive diffusion was measured using higher glycerol concentrations. The authors also point a function of Fps1p in the control of passive diffusion, as the diffusion constant (*K*d) in YPD grown cells was considerably lower in a *fps1* mutant compared to the wild type. This was attributed to differences observed in the phospholipids and glycolipid fractions of the total lipid concentration of the cell membrane between the wild type and the *fps1* mutant (Sutherland *et al.,* 1997).

In 1999, Tamás and co-workers stated that the physiological role of Fps1p appeared to be glycerol export rather than uptake. They confirm previous results (Luyten *et al*, 1995) that proved *fps1* mutants to be sensitive to hypoosmotic shock and osmolyte export to be required for recovery from a sudden drop in external molarity, and demonstrated that glycerol efflux after a hyposomotic shock was almost instantaneous and fully dependent on Fps1p.

Later, the active transport system described by Lages and Lucas (1997) was associated with the genes *GUP1* and *GUP2* (Holst *et al,* 2000). *GUP1* was picked up in a screen done in a glycerol synthesis impaired strain (*gpd1gpd2*), by its involvement in the capability of this strain to grow in media with glycerol as sole carbon and energy sources and in YPD media supplemented with 1 M NaCl and 10 mM glycerol. *GUP1* disruption in a wild type background, also provoked an osmosensitive phenotype that was not revertible with supplementation with 50 mM glycerol in glucose-based medium. Gup1p presented twelve predicted transmembrane domains, which was compatible with a transporter role. *GUP2* was

identified in databases for presenting a very high homology with *GUP1*, despite its disruption did not present any phenotype concerning glycerol utilization and osmotic stress, under the same growth conditions. Furthermore, Gup1p was shown to be active under derepression conditions, while Gup2p seems to be active under salt stress, provided glycerol production was impaired. Active transport could be, nevertheless, quantified in mutants deleted in both *GUP1/2* genes, under derepression conditions. This was attributed to transport measurements artefacts, created by the first enzyme from glycerol catabolism, glycerol kinase (Gut1p), and actually *gup1gup2* mutants also deleted in *GUT1* show no measurable glycerol uptake in ethanol grown cells (Holst *et al,* 2000).

In a recent work about the control of Fps1p glycerol channel, Tamás and co-workers (2003) confirm previous results that reported the influx of glycerol to be significantly reduced after hyperosmotic shock (Tamás *et al.,* 1999). Furthermore, a significant hyperosmotic shock-induced reduction of glycerol influx was also observed in cells expressing hyperactive Fps1p alleles. However, glycerol influx after osmotic shock is still much higher in these cells than in cells expressing wild type Fps1p. This suggests that even hyperactive Fps1p lacking the N-terminal regulatory domain retains an ability to reduce channel activity upon hyperosmotic shock albeit not sufficiently to support normal glycerol retention and growth in high osmolarity medium. The regulatory domain seems to perform its restricting function both under standard conditions as well as under high osmolarity. Truncation or mutation of the domain causes higher glycerol uptake both in the presence and in the absence of hyperosmotic stress. Part of the reduction of glycerol flux upon a hyperosmotic shock is independent of Fps1p, as is also observed in cells lacking *FPS1*. This could be due to other transport proteins or putative passive diffusion through the lipid bilayer, which could be affected by altered membrane tension. It also appeared that Fps1p-dependent glycerol flux across the hyperactive channel is partially diminished upon hyperosmotic shock, but remains at much higher level than wild type. While these data is in line with the notion that Fps1p is controlled by gating, it appeared that the regulatory domain is not the only determinant for a possible gating mechanism. It rather seems as if the regulatory loop N keeps Fps1p in conformation that allows it to readily restrict glycerol transport both in presence and in the absence of osmotic stress. In the absence of this domain transmembrane glycerol flux is too high to allow efficient accumulation of glycerol produced by the cell and hence sensitivity to high osmolarity. A stepwise opening/closing, like a transition from a fully open state and a expanded state, mechanism may make sense given the various roles of Fps1p: rapid glycerol

export upon hypo-osmotic shock but also for moderate, sustained glycerol export during growth under anaerobic conditions (Tamás *et al.,* 1999) and for turgor control during cell fusion of mating yeast cells (Philips and Herskowitz, 1997).

In accordance with this putative functional model, evidence was presented (Oliveira *et al.*, 2003) that kinetics of the facilitated diffusion, attributed to Fps1p, is an artefact created by glycerol kinase activity and that Fps1p, instead, is responsible for mediating the major part of glycerol diffusion. Based on glycerol's chemical properties the spread concept of its supposed liposoluble nature is discussed and data is shown which supports the putative passive diffusion to be channel mediated. Besides Fps1p, another channel responsible for glycerol simple diffusion seemed to be the one encoded by the YFL054c gene.

As stated before, *GUP1* allows the recovery of salt stress phenotype in spite of cells being cultivated on glucose, which has been shown to repress glycerol active transport (Holst *et al.,* 2000). *GUP1* and *GUP2* expression studies have shown these genes to be very low and constitutively expressed and no correlation was found between *GUP1* and *GUP2* expression, glycerol intracellular levels and glycerol active transport (Oliveira and Lucas, 2004). The high similarity between *GUP1*-like sequences from different yeast species (Chapter II, Neves *et al.,* 2004b) is not consistent with the detection of glycerol active transport in the same species (Lages *et al.*, 1999). All this data raised doubts concerning the role of *GUP1* and *GUP2* in glycerol uptake and led to new glycerol uptake experiments in a *gup1 gup2 gut1* strain (Chapter III, Neves *et al.*, 2004a). These, showed that, regardless of the deletions in *GUP1*, *GUP2* and *GUT1* genes, cells cultured under salt stress and collected during diauxic shift, *i.e.* under derepression conditions, present glycerol active uptake, with similar kinetic parameters of the one published before. This pointed to the possibility of Gup1p and Gup2p not being glycerol carriers, but instead regulating proteins, since they interfere with transport activity (Holst *et al*, 2000).

Taking all the data described above, the model proposed for glycerol transmembrane transport in *S. cerevisiae* is the one described in fig I1.

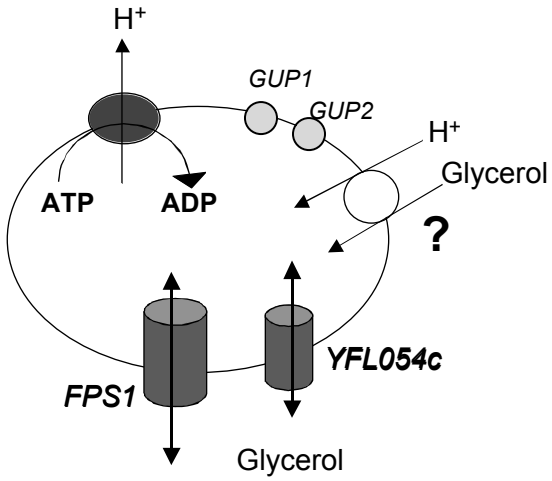


Fig. I1. Glycerol transport in *S. cerevisiae*. Fps1p is the main responsible for glycerol diffusion, as well for glycerol export upon hypoosmotic shock, and closes under hyperosmotic shock. Yfl054cp is responsible for a minor percentage of glycerol diffusion. The gene that encodes the glycerol/H^+ symport system, repressed by glucose and inducible by gluconeogenic conditions and salt stress, is still unknown. Gup1 and Gup2p influence glycerol uptake in a yet unveiled fashion.

Differences obtained in different *S. cerevisiae* strains could be due to the fact that glycerol assimilation by *S. cerevisiae* is considered a strain dependent phenomenon (Barnet *et al.*, 2000). The apparent reduction of glycerol flux in cells under osmotic stress could be explained by the lower glycerol kinase (Gut1p) activity in those conditions, as Gut1p has been described in *S. cerevisiae* as being reduced by approximately 50% when cells were incubated in 1M salt (Albertyn *et al.,* 1994a).

Independently of the active transport mechanism, the involvement of Fps1p in osmotic stress response is made through the control of intracellular levels of glycerol by regulating its efflux. The long N-terminal domain of the protein was demonstrated to be involved in this process of opening/closing of the channel, which is in accordance with the hypo-osmotic shock sensitivity of *fps1* mutant strains (Tamás *et al*., 1999; Bill *et al.,* 2001). Recently, a short conserved region in this domain was identified to be responsible for regulation of glycerol transport through Fps1p, contributing to its retention under hyper-osmotic stress (Tamás *et al*., 2003; Karlgren *et al.,* 2004), as well as a regulatory domain in the C-terminal extension (Hedfalk *et al.*, 2004).

Interestingly, deletion of *FPS1* has effects in other cellular processes, some of which are not directly involved in osmotic stress. Besides the effect of suppressing the defect of *GGS1* mutants (van Aelst *et al.,* 1991), as also mentioned above, the proportion of glycolipids and phospholipids in plasma membrane composition is

affected by *FPS1* (Sutherland *et al*., 1997; Toh *et al.,* 2001). Another effect caused by *FPS1* deletion is cell fusion deficiency (Philips and Herskowitz, 1997). This phenotype was explained with the need for proper osmotic balance in fusion during mating due to the process of local degradation and remodelling of the cell wall. The molecular mechanism proposed for this effect is the higher glycerol content of *fps1* mutant cells that would activate the PKC (protein kinase C) pathway, which inhibits cell fusion (Wojda *et al.,* 2003). However, this effect and the reported influence of Fps1p in glycerol production (Luyten *et al*., 1995; Tamás *et al*., 1999; Remize *et al*., 2001) may not be a direct influence of Fps1p, but an indirect consequence of altered glycerol content. Also, it was recently published that a *fps1* mutant which accumulates glycerol inside the cell without an exogenous supply of glycerol into the medium acquired tolerance to freeze stress and retained high leavening ability in dough after frozen storage (Izawa *et al.,* 2004a). A remarkable example of an effect of *FPS1* deletion that does not involve directly glycerol is the resistance to the trivalent metalloids arsenite and antimonite by a mechanism independent of the intracellular glycerol (Wysocki *et al*., 2001). These observations and the hypersensitivity to these metalloids of cells expressing a truncated version of Fps1p with unregulated open channel provide support to the proposed function of Fps1p directly in transport of arsenite and antimonite. Fps1p is also required for cells to delocalize Hrp1p (a RNA binding protein which shifts from nucleus to the cytoplasm) after exposure to hyperosmotic conditions (Henry *et al.,* 2003).

On the other hand, *GUP1/2* roles and its relation to glycerol transport are still undisclosed. Yet, some clues are available in literature and a discussion about their possible roles is done in Chapter II. They have been included in the membrane bound *O*-acyl transferases (MBOAT) superfamily (Hofmann, 2000, Neves *et al.,* 2004; Chapter II) and were shown to be highly similar to diacyl glycerol *O*-acyl transferase (Oelkers *et al.,* 2000) and to a bacterial predicted membrane protein involved in the incorporation of D-alanine into lipoteichoic acids (Perego *et al.,* 1995). A temperature sensitive phenotype and a significant increase in triglyceride synthesis with a concomitant decrease in phospholipids synthesis were associated with a *gup1* mutant strain (Oelkers *et al.,* 2000). These observations together with its constitutive and low expression (Oliveira and Lucas, 2004) permit to infer their involvement in transference of chemical groups to the membrane or even to the cell wall and thus leading to modifications in those cell components properties. Cell wall may be a target of the osmotic stress response but also part of the osmotic stress-induced signalling events (Mager and Siderius, 2002). This could have an effect in the function of the glycerol transporter, namely

through the HOG pathway. They also could be acting as sensors or regulators of that same transport. Furthermore *GUP1* deletion mutants exhibit a number of additional phenotypes, like defects in bipolar bud site selection (Ni and Snyder, 2001), defects in sorting of carboxypeptidase Y, proteinase A and alkaline phosphatase (Bonangelino *et al*, 2002) and changes in telomere length (Askree *et al*, 2004). Further efforts have to be made to clear up this imbroglio.

We would conclude by stressing that the apparently pleiotropic events related to *FPS1* and to *GUP1* and *GUP2* may equally be an indirect consequence of a more general glycerol-mediated regulation phenomen.

Glycerol transport in non-Saccharomyces yeasts

Active transport systems for glycerol have been reported for several fungi, even before than for *S. cerevisiae*. Yet, little is known about its genetics.

In 1990, Lucas and co-workers reported an active transport in *Debaryomyces hansenii*, involved in glycerol accumulation under osmotic stress. It was characterized as a specific and constitutive sodium-glycerol symporter system, also able to accept potassium as a co-substrate. NaCl is the driving force for glycerol accumulation, which varied as a function of the extra cellular sodium chloride concentration. A similar transport system, also with osmoregulatory functions, was described in *Zygosaccharomyces rouxii* (van Zyl *et al,* 1990).

An H^+/glycerol symporter, similar to the one described above for *S. cerevisiae*, was first characterized in *Pichia sorbitophila* (Lages and Lucas, 1995). Although its main function seemed to be osmoregulation, this is a constitutively expressed transport system, not needing induction by salt or glycerol. Consistently, *P. sorbitophila* grows well on glycerol. Simultaneously, first-order kinetics of glycerol entry was measured and, at that time, it was attributed to simple diffusion. This was affected by the presence of salt in the growth medium, decreasing with increasing salt concentrations, identically to what happens in *S. cerevisiae* Fps1p-mediated glycerol entry.

Later, a study involving 42 yeast species, concluded that active glycerol uptake is a mechanism underlying halotolerance in yeasts (Lages *et al*, 1999). It was suggested a relation between the constitutive expression of active glycerol transport systems and a higher level of salt stress resistance. This could be an evolutionary advantage for growth under such conditions. In fact, constitutive glycerol active uptake and accumulation against gradient was only present in strains belonging to classes resistant to 3 and 4 M NaCl. *Candida halophila*, *C. nodaensis*, *P. sorbitophila*, *Stephanoascus ciferrii* and *Wingea robertsiae* present

an H^+/glycerol symport, while *C. cacaoi*, *C. halonitratophila*, *C. versatilis*, *Sterigmatomyces halophilus*, *C. famata*, *C. magnoliae*, *C. silvicurtix*, *C. matrietensis*, *D. hansenii*, *P. etchellsii*, *P. farinosa*, *P. membranaefaciens*, *Torulaspora delbrueckii* and *D. castellii* evidenced sodium-dependent glycerol transport identical to *D.hansenii* (Lucas *et al.,* 1990). Strains from lower NaCl classes, like*. Z. rouxii*, *C. tropicalis*, *Kluyveromyces marxianus*, *P. angusta* and *S. cerevisiae* (2 M), show glycerol active uptake induced by growth on glycerol, suggesting its involvement in glycerol catabolism. *Schizosaccharomyces pombe*, belonging to the 1M NaCl class did not present glycerol accumulation even when glycerol was present in the growth medium. The diffusion constant (K_d) was affected by salt, being 0.011 in its absence and 0.003 l h^{-1} in its presence. A contradiction with previous results was observed in *Z. rouxii*, which transport system was said to be constitutive (van Zyl *et al*, 1990). But different strains were used in those two studies and this species is known for their strain heterogeneity (Tokuoka and Ishitani, 1991), and for exhibiting different salt tolerance in relation to the medium pH (Watanbabe *et al.,* 1993). So, different strains and different conditions could explain different results.

Glycerol transport was also characterized in *C. versatilis* (syn *C. halophila*) (Silva-Graça and Lucas, 2003). Results point to the presence of a highly specific H^+/glycerol symporter, constitutively expressed, as suggested before (Lages *et al.,* 1999). First-order kinetics of glycerol uptake, indicating passive entry, could not be measured despite computer regression analysis of uptake raw data suggested its existence (Silva-Graça and Lucas, 2003).

The human pathogenic yeast *C. albicans* also exhibits a specific glycerol/proton symport constitutively expressed and unresponsive to NaCl (Chapter IV).

Total or partial genomes sequences from some of those yeasts were made available in databases by different sequencing projects. *C. albicans* (http://www-sequence.stanford.edu/group/candida/index.html), *D. hansenii* (http://natchaug.labri.u-bordeaux.fr/Genolevures/index.php) and *Schiz. pombe* (http://www.geneb.org/genedb/pombe/index.jsp) genomic sequences are complete. *Z. rouxii, C. tropicalis, K. marxianus* and *P. angusta* genomic sequences are partially available in Génolevures Database.

C. albicans present a Gup1p homologue, which, as expected, is not responsible for active glycerol uptake (Chapter IV) and none *FPS1* homologue (Neves *et al*, 2004b, Chapter II). When osmotically stressed cells were exposed to a decrease in external osmolarity, the intracellular glycerol was rapidly exported to the external medium, in a similar way to *S. cerevisiae*, and the export process was independent

of *AQY1*, the only known *C. albicans* gene with similarity to glycerol export channels (Chapter IV).

Schiz. pombe also present a Gup1p homologue, but as mentioned before this yeast does not present active glycerol uptake (Lages *et al.,* 1999). This could be understandable since, as stated above, *GUP1*, despite its involvement in glycerol uptake (Holst *et al.,* 2000) does not encode the H^+/glycerol symport observed both in *S. cerivisiae* and *C. albicans* (Neves *et al.,* 2004a, Chapter III, Chapter IV). Release of glycerol in *Schiz. pombe* is similar to *S. cerevisiae* and is independent of the MIP channel Spac977.17p, the only gene which present homology to glycerol channel proteins of the MIP family, as it did not complement the *S. cerevisiae fps1* mutant nor its deletion affects glycerol accumulation or release in *Schiz. pombe* (Kayingo *et al.,* 2004).

C. tropicalis, D. hansenii and *P. sorbitophila* genomes have a *GUP1* homologue, but no *FPS1* homologues, as can be deduced from the databases and also by the negative results obtained in Southern blot analysis using a *S. cerivisiae FPS1* probe (Chapter II). *P. sorbitophila* and *C. tropicalis GUP1*s complement the correspondent *S. cerevisiae* mutant phenotype (Neves *et al.,* 2004b, Chapter II). Yet, no studies had been done so far in an attempt to relate glycerol transport observed in these yeasts with those or other genes available. The same is true for *D. hansenii*.

Z. rouxii, K. lactis, K. marxianus and *P. angusta* present *FPS1* homologues (Neves *et al.,* 2004b; Chapter II), as well as *GUP1* homologues. *FPS1* from the first three yeasts complements the correspondent mutant phenotype from *S. cerevisiae* (Neves *et al.,* 2004b; Chapter II), but further studies are needed in order to establish a relation between Fps1p or Gup1p and glycerol transport in these yeasts.

C. halophila has never been subjected to a genetic approach or sequencing program and so nothing is known about the genes involved in its described glycerol uptake system (Silva-Graça and Lucas, 2003). Southern blot analysis has shown this yeast not to present a *FPS1* homologue (Chapter V), which could explain the fact that simple diffusion could not be measured in this yeast (Silva-Graça and Lucas, 2003).

Inferences from phylogeny comparisons must be taken carefully, as similar sequences not always mean similar function, as is the case of Gup1p and Gup2p, and the absence of a certain gene does not mean that function is not performed by another unrelated gene, as seems to be the case of glycerol efflux upon osmotic shock in both *Schiz. pombe* and *C. albicans* (Kayingo *et al.,* 2004; Chapter IV).

Glycerol utilization in yeasts

Many yeasts are able to grow on glycerol as a sole carbon source (Barnett *et al.,* 2000). In *S. cerevisiae*, glycerol catabolism involves transmembrane transport and two further enzymatic steps: phosphorylation catalysed by cytosolic glycerol kinase, encoded by *GUT1*, and dehydrogenation catalysed by mitochondrial glycerol 3-phosphate dehydrogenase, encoded by *GUT2*.

The remaining catabolic steps are included in gluconeogenesis. The product formed by glycerol kinase, glycerol 3-phosphate, can be used either as a precursor for lipid biosynthesis or for conversion to dihydroxyacetone phosphate, which can be transformed in glyceraldehyde-3-phosphate by a triose phosphate isomerase into the central metabolic pathway or can serve as a substrate for the synthesis of other metabolites (Grausland *et al.,* 1999).

Detection of glycerol kinase in *S. cerevisiae* was done by Wieland and Suyter, (1957), but the pathway above referred was only confirmed by Sprague and Cronan (1977). It was observed that mutants defective in glycerol kinase and glycerol 3-phosphate dehydrogenase activities were unable to use glycerol as sole carbon and energy source, and that Gut1p was repressed, but not inactivated, by glucose and derepressed in non-fermentable carbon sources. Also, Gut1p has been described in *S. cerevisiae* as being reduced by approximately 50% when cells were incubated in 1M NaCl (Albertyn *et al.,* 1994a). The gene *GUT1* was cloned (Pavlik *et al.,* 1993) and expression studies showed it to be regulated by carbon source and controlled by the positive regulators Adr1p, a transcriptional activator of genes repressed by glucose like *ADH2* encoding catabolic alcohol dehydrogenase (Bemis and Denis, 1988), Ino2p and Ino4p and the negative regulator Opi1p, DNA binding proteins required for the regulation of genes encoding proteins involved in phospolipid biosynthesis like inositol 1-phosphate syntethase (Hoshizaki *et al.,* 1990; Nikoloff *et al.,* 1992; White *et al.,* 1991; Pavlik *et al.*, 1993; Grauslund *et al.,* 1999).

GUT2, which encodes for a FAD-dependent glycerol 3-phosphate dehydrogenase located at the inner mitochondrial membrane by hydrophobic interactions (Janssen *et al.,* 2002), was cloned by Rønnow and Kielland-Brandt (1993) as a low-copy suppressor of a previously isolated *gut2* mutant deficient in mitochondrial glycerol 3-phosphate dehydrogenase activity (Sprague and Cronan, 1977). The expression of the FAD-dependent glycerol 3-phosphate dehydrogenase is, in accordance with the expression of glycerol kinase, repressed, but not inactivated, by glucose and derepressed by non-fermentable carbon sources like

ethanol, glycerol, and lactate (Sprague and Cronan, 1977). This enzyme is also involved in the glycerol 3 phosphate redox shuttle (Larsson *et al.,* 1998), which couples cytosolic with mitochondrial redox sinks. *GUT2* expression is, as *GUT1,* repressed by Opi1p, but is derepressed by the protein kinase Snf1p, a central regulatory component in glucose repression, and the heteromeric complex Hap2/3/4/5, a protein complex responsible for the activaction of several nuclear genes encoding mitochondrial proteins (Grauslund and Rønnow, 2000). Recently, Gut2p was shown to be a substrate for the inner membrane peptidase, Imp1, as failure to cleave the Gut2p precursor may contribute to the impairment to grow in glycerol of certain *imp* mutants (Esser *et al.,* 2004).

A glycerol dissimilation pathway similar to *S. cerevisiae* was also described for *Candida utilis* (Gancedo *et al.,* 1968), *D. hansenii* (Adler *et al.,* 1985), and *C. glycerolgenesis* (Wang *et al.,* 2000). Nothing was published so far about its genetics, but they probably are differently regulated, as it was found that Gut1p in *Candida utilis* is inducible by glycerol in growth medium (Gancedo *et al.,* 1968), while in *S. cerevisiae* is subject to catabolite repression (Sprague and Cronan, 1977). Interestingly, *C. glycerolgenesis* hardly assimilate glycerol without glucose (Wang *et al.,* 2000), as was also observed in *S. cerevisiae* (Lages and Lucas, 1997). It is like glucose functions as a stimulus for glycerol dissimilation.

Another pathway was reported in yeasts, namely in *Schiz. pombe* (May and Sloan, 1981; 1982). No glycerol kinase activity could be detected in glycerol grown cells of two strains of *Schz. pombe*, but a significant activity of an NAD^+-linked glycerol dehydrogenase was observed, which was subjected to catabolite repression (May and Sloan, 1981). It was also found that those strains could phosphorylate dihydroxyacetone, and an alternative pathway for glycerol utilization was proposed, which involved glycerol oxidation to dihydroxyacetone by the NAD^+-linked glycerol dehydrogenase, followed by phosphorylation of dihydroxyacetone. Both enzymes are subjected to catabolite repression and may also be inducible but are not co-ordinately regulated since the enzyme levels in glycerol grown cells when compared with glucose grown cells increased differently (18- to 35-fold for glycerol dehydrogenase, and just 4- to 7-fold for dihydroxiacetone kinase) (May *et al.*, 1982).

This pathway was also detected in the osmotolerant yeasts *D. hansenii* (Adler *et al.,* 1985) and *Z. rouxii* (van Zyl *et al.,* 1991). DHA kinase and glycerol dehydrogenase activities were also reported in species of *Candida*, *Lodderomyces*, *Endomycopsis*, *Hansenula, Pichia* and *S. cerevisiae* (Hofmann and Babel, 1981; Tikhomirova *et al.,* 1988; Blomberg, 1997; Norbeck and Blomberg, 1997; Luers *et*

al., 1998; van der Klei *et al.,* 1998) but its importance in glycerol dissimilation was not established. In *S. cerevisiae* the genes *GCY* and *DAK1/DAK2* encoding the two steps of this pathway are present (Norbeck and Blomberg, 1997; Molin *et al.,* 2003), and might play a role in the regulation of the glycerol concentration during osmotic stress (Blomberg, 2000). *Schiz. pombe* and *Z. rouxii DAK* genes have been cloned and sequenced (Kimura *et al.,* 1998; Itoh *et al.,* 1999; Wang *et al.,* 2002). The *GCY1* from *Z. rouxii* has also been cloned (Iwaki *et al.,* 2001).

If we look in databases no *GUT1* homologue is found in *Schiz. pombe* genome, which is in accordance with the inexistence of a functional glycerol kinase. Nevertheless, difficulties of this yeast to grow in glycerol-based media cannot be fully understood due to the existence of the dihydroxiacetone glycerol dissimilation pathway. Eventually, this could be related to the absence of a mechanism for active uptake of glycerol. A *GUT1* homologue was cloned from the osmotolerant yeast *P. sorbitophila*, which was able to complement the correspondent *S. cerevisiae* mutant phenotype (Neves *et al.,* 2004; Chapter II), but no other experiments were done in order to clarify the glycerol catabolic pathway in that yeast. Probably it will be similar to *S. cerevisiae*, but differently regulated as it is known from previous works that *P. sorbitophila* grows much better in glycerol than *S. cerevisiae* (Lages *et al.,* 1999).

Glycerol synthesis in yeasts

In *S. cerevisiae* glycerol is synthesized from the glycolytic intermediate dihydroxyacetone phosphate in two steps catalyzed by glycerol-3-phosphate dehydrogenase (Gpd) and glycerol 3-phosphatase (Gpp). Two isoenzymes are present for each case, which are coded by *GPD1/2* and *GPP1/2* (Larsson *et al.,* 1993; Albertyn *et al.,* 1994b; Ericksson *et al.,* 1995). This metabolic pathway seems to be common to a number of yeasts since *GPD1* and/or *GPD2* genes from yeasts like *Schiz. pombe*, *C. glycerolgenesis*, *Z. rouxii* and *D.hansenii* have been cloned and activity of glycerol 3-phosphate dehydrogenase was reported (Nilsson and Adler, 1990, Ohmiya *et al.,* 1995, Wang and Zhuge, 1999; Wang *et al.,* 2000; Iwaki *et al.,* 2001; Thomé, 2004). Moreover, *GPD* from *P. angusta*, *K. thermotolerans* and *D. hansenii*, as well as *Z. rouxii GPD1*, are able to complement *S. cerevisiae gpd1gpd2* phenotype (Neves *et al.,* 2004b; Chapter II; Thomé, 2004; Watanabe *et al.,* 2004).

Phylogenetic analysis shows *GPD*s from different yeast to be closely related (Neves *et al.,* 2004b; Thomé, 2004). Yet regulation of these genes expression, as

well as the regulation of the correspondent enzymes can be quite different. For instance, *Z. rouxii* and *C. halophila* glycerol 3-phosphate dehydrogenases are able to use as co-factors both NADH and NADPH, while the same enzyme in *S. cerevisiae* only utilizes NADH (Oshiro and Yagi, 1996; Silva-Graça *et al.*, 2003). In contrast to *S. cerevisiae*, *D. hansenii GPD* RNA and Gpdp levels are low under normal growth conditions (Thomé and Trench, 1999) and is possible that in this yeast a single gene codes for two isoenzymes (Thomé, 2004). Also, and despite that Zrgpd1p is the primary enzyme involved in glycerol production (Watanabe *et al,* 2004), *ZrGPD1* and *ZrGPD2* expression is not stimulated by osmotic stress (Iwaki *et al.,* 2001), while in *S. cerevisae GPD1* expression is increased by osmotic stress (Larsson *et al.,* 1993; Albertyn *et al.,* 1994b; Ansell *et al.,* 1997; Rep *et al.,* 2000; Gash *et al.,* 2000; Causton *et al.,* 2002; Yale and Bohnert, 2001) and Gpd2p is under redox control (Björkqvist *et al.,* 1997; Ansell *et al.,* 1997; Valadi *et al.,* 2004). So, one should be carefully in inferring function from sequence comparison, as mentioned above, but also from heterologous complementation, as it is not demonstrated that similar genes from different species once in their native genetic environment, correspond to identical molecular/physiological responses to the same stimulus.

Whether the dihydroxyacetone route operates in yeasts for the synthesis of glycerol besides other putative roles, is unclear. In *S. cerevisiae* the *gpd1gpd2* mutant has been reported as being unable to accumulate intracellular glycerol (Ansell *et al.,* 1997), but recent studies have shown some intracellular glycerol in this strain (Oliveira and Lucas, 2003), which could corroborate this hypothesis.

Roles of glycerol in yeasts metabolism

The function related to glycerol production in yeasts most widely discussed is the one of main compatible solute to counteract the low a_w in high osmolarity environments (Brown *et al.*, 1978; Yancey *et al.*, 1982; Reed *et al.*, 1987; Blomberg and Adler, 1989; Andre *et al.*, 1991; Tokuoka and Ishitani, 1993; van Eck *et al.*, 1993, Albertyn *et al.*, 1994a; Hohmann, 2002). Under osmotic stress glycerol synthesis increases (Blomberg and Adler, 1989; André *et al.*, 1991; Albertyn *et al.*, 1994a, Varela *et al.*, 1992; Norbeck and Blomberg, 1997; Norbeck *et al.*, 1996) and plasma membrane-related processes allow glycerol retention. In *S. cerevisiae* while synthesis is regulated at transcriptional level (Albertyn *et al.*, 1994b; Ansell *et al.*, 1997; Rep *et al.*, 2000; Gasch *et al.*, 2000; Causton *et al.*, 2001; Yale and Bohnert, 2001), retention is achieved mainly by the regulation of the glycerol

channel Fps1p, which closes under osmotic stress (Tamás *et al.,* 1999). Actually, glycerol concentrations ratios (intra/extracellular) greater than 1000-fold have been reported in yeasts such as *Z. rouxii* (van Zyl and Prior, 1990).

Glycerol has for long also been connected to lipid synthesis. The precursors for biosynthesis of the key metabolic intermediate for lipid metabolism, phosphatidic acid, are glycerol 3-phosphate and dihydroxyacetone phosphate and glycerol can be directly utilised for glycerophospholipid, and triacylglycerol biosynthesis from its derivatives glycerol 3-phosphate and dihydroxyacetone phosphate (Racenis *et al.,* 1992; Christiansen, 1978). Also, in the regulation of transcription of *GUT1* and *GUT2*, the transcription factors Ino2p, Ino4p, Opi1p, and the complex Hap2/3/4p (Grauslund *et al*., 1999; Grauslund and Rønnow, 2000) are shared with many genes of phospholipid and sterol metabolism.

Another crucial role of glycerol metabolism is linked to redox potential balance (van Dijken and Scheffers, 1986) and inorganic phosphate turnover. Glycerol metabolic pathway branches from the upper part of glycolysis at the level of dihydroxyacetone phosphate, suggesting that changes in glycerol metabolism can have direct effects in the glycolytic flow. Glycerol synthesis can act as a electron or hydrogen sink during fermentation (Ansell *et al.,* 1997; Bjorkqvist *et al.,* 1997; Påhlman *et al.,* 2001). Respiratory deficient cells become strictly dependent on the Gpd2p for providing NAD^+ (Valadi *et al.,* 2004). This protein presents a mitochondrial pre-sequence which allows it to be localized in the outer layer of the mitochondrial membrane, providing for redox balance under non-respiratory conditions (Valadi *et al.,* 2004). Gpd1p is localized in the cytosol and in the peroxisomes, being mostly in the peroxisomes in respiratory deficient mutants. The distinct intracellular localization of Gpd1p and Gpd2p is compatible with a different contribution to redox-driven glycerol production (Valadi *et al.,* 2004).

Glycerol metabolism is also involved in oxidative stress response, which is due to high intracellular concentrations of free oxygen radicals resulting from several metabolic reactions as incomplete reduction of oxygen to water in the respiration process, or from stress agents as radiation, metals and drugs (for a review see Ames *et al*., 1993). Under these conditions several enzymes related to glycerol pathways, such as glycerol dehydrogenases and glycerol phosphatases are over induced (Norbeck and Blomberg, 1997; Godon *et al*., 1998, Påhlman *et al*., 2001) and mutants defective in some of those enzymes showed increased sensitivity to redox-active compounds (Chaturvedi *et al*., 1997; Påhlman *et al*., 2002). Nevertheless, expression of the correspondent genes in oxidative stress response remains to be elucidated.

Moreover, glycerol appears to play a protective role against heat shock. Mutants like *gpd1gpd2* and *gpp1gpp2*, unable to produce glycerol, present temperature sensitive phenotype (Siderius *et al*., 2000), which is reverted by the presence of glycerol in the growth media. Also, *GPD1* transcription is induced by exposure to 42ºC (Rep *et al*., 1999).

Recently, it was shown that the HOG (high-osmolarity glycerol) pathway, which regulates glycerol production, regulates adaptation to citric acid stress (Lawrence *et al.,* 2004) and is essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress through the activation of 6-phosphofructo-2-kinase (Dihazi *et al.,* 2004). Yet, surprisingly, HOG pathway exerts only limited control over *GPD1* expression and glycerol production during wine fermentation (Remize *et al.,* 2003).

The multiple roles of glycerol in the metabolism of the cells subjected to different kinds of stress suggest an interplay and cross-activation of stress responses. Response to heat shock and high osmolarity involves connection between the HOG and PKC pathways, which, together, regulate intracellular levels of glycerol (Wojda *et al*., 2003). Either heat or salt shock can cause cross adaptation to heat, salt and freezing stress. When protein synthesis is blocked with cycloheximide, cross adaptation is still observed between heat and salt stresses although to a lesser extent (Lewis *et al*., 1995). Accordingly, the expression of the genes *HSP12* and *HSP26*, encoding heat shock proteins (Praekelt and Meacock, 1990; Petko and Lindquist, 1986; Susek and Lindquist, 1989), is induced with osmotic stress (Varela *et al*., 1992). Five target genes of the repressor Sko1p, a mediator of the HOG (High Osmolarity Glycerol) signal transduction pathway (Brewster *et al*., 1993), are known or predicted to be involved in oxidative damage repair (Rep *et al*., 2001). One of the possibilities to explain the osmotic stress-driven regulation of genes involved in oxidative damage repair implicates directly glycerol metabolism. Under osmotic stress, the high metabolic flux towards glycerol production could cause NAD^+ surplus produced in the reduction of dihydroxyacetone phosphate by NADH-dependent glycerol 3-phosphate dehydrogenase. The redox unbalance generated by depletion of reduction equivalents in the form of NADH could cause oxidative stress (Rep *et al*., 2001). These unexpected features of interplay of stress responses suggest that adaptation to stressful conditions are much more complex than anticipated and the involvement of specific stress response mechanisms like glycerol accumulation in osmotic adaptation might be common for many other stress conditions.

Roles of glycerol in yeast pathogenicity

The identification of virulence mechanisms of pathogenic fungi is essential for understanding fungal adaptation to a complex and changing environment of their hosts and also for identifying potential novel targets in antifungal therapy, as well as in its diagnosis. Polyol production and accumulation has been associated to fungal pathogenicity and so, as being involved in such virulence mechanisms.

Candida albicans, a dimorphic yeast able to colonize the human body and seriously compromise human health, particularly in immuno-depressed individuals has been used as a model pathogen (Odds, 1988; Fox, 1993). It synthesizes and accumulates large amounts of D-arabinitol, as well as considerable amounts of glycerol, in culture and in the tissues of infected animals (Kiehn *et al.,* 1979; Bernard *et al.,* 1981; Wong *et al.,* 1982a,b; San José *et al.,* 1996). Glycerol appears to be the main protective solute with which *C. albicans* cells maintain osmotic homeostasis and glycerol synthesis is under the control of *HOG1* gene (San José *et al.,* 1996). *C. albicans* mutants, *hog1*, that under produce glycerol are extremely susceptible to environmental stresses and are proved to be much less virulent than wild type (Alonso-Monge *et al.,* 1999, 2003). Dimorphic transition corresponds to cell differentiation, allowing yeast to display hyphal growth. This has long been suspected to play a role in *C. albicans* pathogenesis, relating to important infection phenomena such as adhesion, escape from phagocytic cells and invasion (Ryley and Ryley, 1990; Calderone, 1993; Odds, 1994; Kobayashi and Cutler, 1998). Hyphal growth has also been associated with the HOG1 pathway (Alonso-Monge *et al.,* 1999; Ochiai *et al.,* 2002; Hube, 2004). Yet, results from Alonso-Monge and collaborators (1999) demonstrated that hyphal formation is apparently not enough to determine strain virulence, since a functional *in vitro* hyphal development does not correlate with the ability to infect mice. Results from these investigators also suggest a link between cell wall metabolism and the HOG pathway in *C. albicans*. Moreover, *C. albicans hog1* mutants are not impaired in the invasion of epithelial cells, but displayed an increased sensitivity to agents producing reactive oxygen species (ROS), such as oxidants (menadione, hydrogen peroxide or potassium superoxide) and UV light. Consistently, Hog1p was activated not only in response to osmotic stress but also in response to hydrogen peroxide. *HOG1* gene also plays a role in chlamydospore formation, another oxygen-related morphogenetic event (Alonso-Monge *et al.,* 2003). So, it seems that Hog1p behaves like a general stress kinase, essential for successful infection in the host, and the identification of this signal transduction pathway is of

fundamental importance in the search for novel antifungal targets. The HOG response pathway allows adaptation to high osmolarity conditions and participates in a pleiotropic response that enables a correct and rapid adaptation to different types of stress and seems to be especially important in terms of environmental adaptation. Thus, having efficient mechanisms for regulating glycerol accumulation may be important for pathogenic fungi to colonize and survive in their mammalian host where a plethora of environmental stresses such as nutrient availability, osmolarity, temperature, oxygen levels and oxidative killing by host phagocytes are frequently encountered (Vazquez-Torres and Balish, 1997).

Besides *C. albicans*, *C. tropicalis* and *C. parapsilosis*, also medically important species, produce mainly arabinitol, but also glycerol in infected tissues. Detection of polyols is used in the diagnosis of fungal infections (Kiehn *et al.,* 1979; Bernard *et al.,* 1981; Wong *et al.,* 1982a,b; Switchenko *et al.,* 1994).

Cryptococcus neoformans and *Aspergillus fumigatus* produce mannitol in culture and in infected animals (Wong *et al.,* 1989, 1990). *C. neoformans* mutant that under produces and under accumulates mannitol presents an increased susceptibility to oxidative killing by normal human neutrophils and by cell-free oxidants. Besides, it is much less virulent in mice and more susceptible to osmotic and heat stress in comparison to the correspondent wild type strain (Chaturdevedi *et al.,* 1996a,b). Results obtained by Chaturdevedi and co-workers (1997) have shown that expression of bacterial *mtl*D, which encodes the mannitol-1-phosphate dehydrogenase, in *S. cerevisiae* results in mannitol synthesis and protects a glycerol-defective mutant from salt and oxidative stress. This is consistent with a role of polyols production by fungi in the scavenging oxidants generated by the phagocyte oxidative burst. This will allow fungi to resist to mammalian host defences.

Several phytopathogenic fungi, like, *Aspergillus nidulans*, *Eremothecium/Ashbia gossypii* and *Magnaporthe grisea*, depend on glycerol for appressorial turgor allowing invasiveness (de Jong *et al*., 1997). *M. grisea* causes a serious disease of cultivated rice and infects rice leaves by producing an *appressorium*, which is a dome-shaped cell that differentiates from the end of a short germ tube after the attachment and germination of a spore on the leaf surface. Penetration of the rice leaf results from the exertion of mechanical force against the plant cuticle. The *appressorium* generates considerable cellular turgor, which allows the fungus to send a narrow penetration peg through the leaf cuticle (Howard *et al.,* 1991). Interestingly, the glycerol produced for appressorial turgor seems to be independent of glycerol-3-phosphate dehydrogenase and glycerol dehydrogenase,

being instead the result of triacylglycerol and glycogen degradation (Thines *et al.,* 2000). In this fungus hyperosmotic stress leads predominantly to arabinitol production, under the control of *OSM1*, an osmosensory MAPK-encoding gene, functionally homologous to *HOG1* (Dixon *et al.,* 1999).

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Chapter II

Yeast orthologues associated with glycerol transport and metabolism

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Abstract

Glycerol is a key compound in the regulation of several metabolic pathways in *Saccharomyces cerevisiae*. Most of the genes from this yeast involved in glycerol consumption, production and transport are now available. Some of the mechanisms involving glycerol metabolism and transport are common to other yeasts. This work presents a search for *GPD1/2*, *GUT1, GUP1*/*2* and *FPS1* orthologues in a series of hemiascomycetous yeasts. All the genes cloned were able to complement *S. cerevisiae* mutant phenotypes and presented a high degree of similarity to the corresponding genes in this yeast. A phylogenetic analysis is presented. The allocation of *GUP* genes in the Membrane Bound *O*-Acyl Transferases (MBOAT) family is suggested as more consistent than their inclusion in the TC-DB/Glycerol Uptake family.

Key words

Hemiascomycetous yeasts; glycerol-related genes; *GPD1/2, GUT1, GUP1, FPS1* orthologues

Introduction

Glycerol plays a central role in yeast metabolism. Besides acting as compatible solute under low a_w stress environments (Blomberg and Adler, 1989; Tokuoka and Ishitani, 1991; van Eck *et al.,* 1993), glycerol is involved in different metabolic pathways. Its production relates to glycolytic flux (van Dijken and Scheffers, 1986; Björkqvist *et al.,* 1997; Larsson *et al.,* 1998; Overkamp *et al.,* 2000; Blomberg, 2000; Pählman *et al.,* 2001; Pählman *et al.,* 2002) and is involved in lipid metabolism (Christiansen, 1978; Racenis *et al.,* 1992; Grauslaund and Rønnow, 2000) as well as in the regulation of inorganic phosphate turnover (van Aelst *et al.,* 1991; Luyten *et al.,* 1995). Presently, most of *S. cerevisiae* genes involved in glycerol consumption and production are known. Furthermore, three genes have been described as involved in glycerol uptake and export, respectively, *GUP1* and *GUP2* (Holst *et al.,* 2000) and *FPS1* (Luyten *et al.,* 1995; Tamás *et al.,* 1999; Tamás *et al.,* 2000). Altogether, these genes regulate intracellular glycerol levels according to several stimuli in a yet not completely unveiled fashion. Some of these mechanisms, like the stimulation of glycerol production and accumulation under

stress growth conditions and the presence of active transport for glycerol, are common to a large series of other yeasts (Reed *et al.,* 1978; Yagi, 1992; Lages and Lucas, 1997; Lages *et al.,* 1999). But, despite all the similarities in what concerns glycerol uptake and metabolism in yeasts (Lages *et al.,* 1999), it is also known that different yeasts deal differently with respect to their needs for glycerol (van Eck *et al.,* 1993; Silva-Graça and Lucas, 2003).

In an attempt to explore similarities in glycerol metabolism and transport at gene level, we decided to search for genes similar to *GUP1/2* and *FPS1*, as well as to *GPD1* and *GPD2*, isogenes for glycerol 3-phosphate dehydrogenase, the first enzyme of glycerol production pathway in *S. cerevisiae* (Larsson *et al.,* 1993; Albertyn *et al.,* 1994; Ericksson *et al.,* 1995) and *GUT1*, encoding for glycerol kinase, the first enzyme of glycerol assimilation pathway also from *S. cerevisiae* (Sprague and Cronan, 1977; Pavlik *et al.,* 1993). Another metabolic pathway has been described before in *Schizosaccharomyces pombe* (May *et al.,* 1982), from which genes encoding for similar enzymatic steps have been shown to exist also in *S. cerevisiae* (Norbeck and Blomberg, 1997). Nevertheless, the metabolic role and importance of this pathway is still not understood (Molin *et al.,* 2003).

We began by using the partial sequencing data available in the Génolévures Program (Feldmann, 2000), for a series of hemiascomycetous yeast species with biomedical or industrial interest (Souciet *et al.,* 2000), *i.e*. *Candida tropicalis, Kluyveromyces lactis, K. marxianus, K. thermotolerans, Pichia angusta, P. sorbitophila and Zygosaccharomyces rouxii*. Besides, we included in our search *C. albicans*, of which the full genome sequence is available.

Genomic sequences retrieved showed a high degree of similarity to those of *S. cerevisiae* and were able to complement the correspondent mutant phenotypes. *GPD1/2, GUT1, GUP1/2* and *FPS1* orthologues were used to enlarge gene families, in which we also included genomic sequences available from other databases. A phylogenetic analysis is presented and *FPS1* and *GUP1/2* allocation in relation to their putative function is discussed.

Materials and methods

Yeast strains

Yeast strains used were: *C. albicans* IGC 3436, *C. tropicalis* IGC 3097, *K. lactis* CBS 2359, *K. marxianus* IGC 3886, *K. thermotolerans* CBS 137, *P. angusta* IGC 4129, *P. farinosa* (syn. *P. sorbitophila*) CBS 7064 and *Z. rouxii* CBS 732.

P. sorbitophila was recently renamed as *P. farinosa* (Barnett *et al.,* 2000; Kurtzam and Fell, 1998). Nevertheless, it is known from previous work (Lages *et al.,* 1999) that the type strain of *P. farinosa* (IGC 2459) presents phenotypes different from *P. sorbitophila* strain used in this work, previously considered type strain. For this reason we kept the previous designation.

Besides, the following *S. cerevisiae* strains were used: W303-1A, *MATa leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100* (Thomas and Rothstein, 1989), BHY54, isogenic to W303-1A but *gup1::His5*$^{+}$*,* CLy1, isogenic to W303-1A but *gut1*, YSH642, isogenic to W303-1A but *gpd1::TRP1 gpd2::URA3*, BHY61-1A, isogenic to W303-1A, but *fps1::HIS* (Holst *et al.,* 2000).

Media and growth conditions

Cells were grown in YPD liquid medium [1% (w/v) yeast extract; 2% (w/v) peptone; 2% (w/v) glucose] at 30ºC, 160 rpm, and growth was monitored by spectrophotometry at 640 nm. Cultures were maintained in YPDA. Transformants selection was performed in YNB w/o amino acids (DIFCO) 0.67% (w/v), supplemented with 2% (w/v) glucose, 2% (w/v) agar and the required amino acids. Phenotype selection media plates were prepared with YPD with: 1 M NaCl and 10 mM glycerol (for *GUP1*) (Holst *et al.,* 2000); 1.4 M NaCl (for *GPD1/2*) (Albertyn *et al.,* 1994) or replacing glucose for glycerol as carbon and energy source in a gut-diagnostic media (for *GUT1*) (Rønnow and Kielland-Brandt, 1993). For *FPS1* phenotypic analysis, cells were grown on YPD with1M sorbitol and then transferred to YPD plates (hypoosmotic shock) (Tamás *et al.,* 1999). Drop tests were done from 10^{-1} to 10^{-5} dilution of overnight-grown cultures (O.D. 640 = 1).

DNA, RNA and plasmid manipulation

Genomic DNA and RNA isolation were done by standard methods as described by Ausubel and co-workers (1996). Plasmids were amplified from one single

colony of *Escherichia coli* transformants, by growth in LB (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 1 N NaOH to adjust to pH 7.5) supplemented with 100 µg/ml ampicillin (Sigma) (LBamp) and isolated by the alkaline lysis method (Ausubel *et al.,* 1996; Sambrook *et al.,* 1989). The concentration and purity of the nucleic acid samples were determined by spectrophotometry. Integrity of RNA was determined by agarose gel electrophoresis under denaturing conditions with formaldehyde. DNA fragments obtained by PCR were gel eluted using High Pure PCR product Purification Kit (Roche Cat nº1732668) prior to utilization.

Bacterial and yeast transformation

Escherichia coli strain DH5 [F- 80dlacZ M15 (lacZYA-argF)U169 endA1 recA1 hsdR17(r_k^- m_k^+) deoR thi-1 phoA supE44 -gyrA96 relA1] was used for plasmid maintenance and amplification of isolated clones. Bacteria were grown in LB liquid medium or plates with additional 2% (w/v) agar. Cultivations were done at 37ºC with aeration at 200 r.p.m. for liquid media. *E. coli* was transformed with a $CaCl_2$-based protocol (Sambrook *et al.,* 1989). Each transformation was done with 100 ng of plasmid DNA and 100 µl of competent cells prepared according to Inoue and co-workers (Inoue *et al.,* 1990). Selection of transformants was done by plating cells on agar plates containing LBamp with X-gal/IPTG (Gibco/Amersham).

S. cerevisiae cells were transformed with the lithium acetate method (Ausubel *et al.,* 1996). Cells were plated on selective YNB according to strains and constructions demands and incubated at 30ºC.

Obtaining genomic sequences

A search for genomic sequences similar to *S. cerevisiae* genes associated to glycerol transport and metabolism, *GUP1/2, FPS1, GPD1/2* and *GUT1*, was made by BLAST (Altschull *et al.,* 1997) against the databases from Génolévures Program and *C. albicans*. The clones containing the more promising sequences were obtained from Génolévures Program: AYOAA011A02TP1 (*K. thermolerans*), BBOAA025DO1TP1 (*P. angusta*), BAOABO23BO7LP1 and BAOABOO5B0LP1 (*K. lactis*), AZOAA009E0371 (*K. marxianus*), BDOAAOO9A10TP1 (*C. tropicalis*), AROAAOO8B09LP1 and AROAAOO3BO7TP1 (*Z. rouxii*), AXOAAO18FO8TP1 and AXOAA026DO8TP1 (*P. sorbitophila*). Inserts were sequenced by primer walking, starting from the correspondent partial sequence available in the database. The inserts which did not contain the complete genomic sequence of the desired homologue, were subjected to RNA ligase mediated rapid amplification of

cDNA ends (RLM-RACE) (Maruyama and Sugano, 1994; Schaefer, 1995), using FirstChoice™ RLM-RACE (Ambion) according to the manufacturer's instructions. Sequencing was done by Genómica STAB Vida, Lda (Portugal).

Subsequently, PCR reactions were designed to retrieve the complete genomic sequences. These were performed using 100-200 ng of genomic DNA as template, with 1,5 to 3 mM magnesium chloride, 0,2 mM dNTP mix, 0,5 µM of each primer, 1 U Taq DNA polymerase (Fermentas) or 2U of *Pfu*Ultra™High-Fidelity DNA Polymerase (Stratagene). A hot start was done for all PCR reactions and the program was 3 min at 94°C, 35 cycles: 30 sec at 94°C, 30 sec at the corresponding annealing temperature (55 to 62°C), 30 sec at 72°C, and finally 7 min at 72°C. The specific primers used are listed in Table II1. The amplified sequences were inserted in an *E. coli* p-GEM easy vector (Promega) and sequenced again. The inserts were further ligated into two sets of shuttle expression vectors, CEN/ARS- and 2µm-based plasmids, containing GPD promotor sequence, p413GPD, p416GPD and p423GPD, p426GPD, respectively. The two plasmids in each set differ in the auxotrophic marker (Mumberg *et al.,* 1995). Before ligation, vectors were treated with alkaline phosphatase (Roche Cat nº 405612). Gene sequences were registered in GeneBank at NCBI (National Center for Biotechnology Information).

Table II1. Primers used in PCR reactions.

KlGPD-f	5'- GACAGCTGCTCCAACAGAAA - 3'
KlGPD-r	5'- CCTTCCGCTGTTGGTTTATG - 3'
PaGPD-f	5'- TAGTCCTCAGATGCCTCAAG - 3'
PaGPD-r	5' - GCAGGTAAACGTCATACTGC - 3'
CtGUP-f	5' - CGTCAGATGGTTGATTATCG - 3'
CtGUP-r	5' - GTTGTACTGAAGGGTTTGGG - 3'
PsGUP-f	5' - GTGGAATACTTGGGAATTTC - 3'
PsGUP-r	5' - CACTGATGTGCACTCATGCT - 3'
CaGUP-f	5' - CTCCCCACCTTTGAAAAACA - 3'
CaGUP-r	5' - CCTTACATCAATGGCTCTTC - 3'
KlFPS1-f	5' - TTGTTCTCATGCGCTTCTGT - 3'
KlFPS1-r	5' - AACCGTCAATCTTCTTGCCA - 3'
KmFPS1-race	5' - TATGTACAGCAATATCTCGACGAAGGTTCCTATTTTCCAG - 3'
KmFPS1-f	5' – ATGGAAATCTTAAGAACCAT – 3'
KmFPS1-r	5' – TGAAAGAAAGATCACTGAAA – 3'
ZrFPS1-f	5' - TTAGGTGTTCTGGAGACAGG - 3'
ZrFPS1-r	5' - GATAGTGAGGTTAACGAACC - 3'
PsGUT-race	5' - GTTGCCTTGGTGATCAGTCT - 3'
PsGUT-f	5' - TATGTCCTATATTTGACGGG - 3'
PsGUT-r	5' - GTTGTTTTACAGTGCCAGTG - 3'

Yeast strains nomenclature: *Ca - C. albicans, Ct - C. tropicalis, Km - K. marxianus, Kt - K. thermotolerans, Pa - P. angusta, Ps - P. sorbihophila, Kl - K. lactis, Zr - Z. rouxii*.

Genomic sequence analysis

Amino acid sequences were deduced using programs available at Expasy Molecular Biology Server and NCBI. Comparison of the deduced amino acid sequences was done using BLAST (Sequence Similarity Search) at NCBI, Clustal (Thompson *et al.,* 1994, 1997) and Multalin (Corpet, 1998) programs freely available at the WWW. MacBoxshade2.15 program was used to edit the alignments and to do the identity/similarity matrixes. Transmembrane profiles were compared for predicted proteins using different programs, namely TMHMM (Sonnhammer *et al.,* 1998; Krogh *et al.,* 2001) and TMPRED (Hofmann and Stoffel, 1993) transmembrane helices prediction programs.

Results

Sequences retrieval

The complete sequences of *K. thermotolerans* and *P. angusta GPD*-like ORFs, *K. lactis* and *Z. rouxii FPS1*-like ORFs and *C. tropicalis* and *P. sorbitophila GUP*-like ORFs were obtained by primer walking of clones from the Génolévures Program. The sequence of a *C. albicans GUP*-like ORF was taken directly from the correspondent database. Besides, *P. sorbitophila GUT1*-like ORF and *K. marxianus FPS1*-like ORF complete sequences were obtained by using RACE after primer walking. *K. lactis GUP1* (*KlGUP1*) was subjected to a similar approach but the complete sequence was meanwhile published (LangkjÆr *et al.,* 2003). Sequence analysis of these ORFs revealed a high degree of homology with the correspondent genes from *S. cerevisiae*, reason why they were named accordingly: *KtGPD, PaGPD, KlFPS1, ZrFPS1, CtGUP1, PsGUP1, CaGUP1, PsGUT1* and *KmFPS1*, respectively. The sequences were deposited in GeneBank, with the following accession numbers: AY289713 (*KtGPD*), AY289714 (*PaGPD*), AY338364 (*KlFPS1*), AY299513 (*ZrFPS1*), AY299512 (*CtGUP1*), AY338363 (*PsGUP1*), AY391774 (*PsGUT1*), AY541009 (*KmFPS1*).

Complementation analysis of S. cerevisiae *mutants*

Complementation analysis for *GPD1/2* putative orthologues (Fig. II1) was done based on the fact that the double mutant strain *gpd1gpd2* is unable to grow in media with NaCl concentrations above 1.4 M due to its impairment to produce

enough glycerol to counterbalance osmotic stress (Albertyn *et al.,* 1994; Oliveira and Lucas, 2004). It is not possible, just based on sequence analysis, to distinguish if the cloned genes are either *GPD1* or *GPD2* orthologues. According to the high degree of homology between these two genes, Gpd1p and Gpd2p are isoenzymes, though they play different physiological roles. Gpd1p is induced under osmotic stress (Larsson *et al.,* 1993; André *et al.,* 1991), while Gpd2p is under redox control (Björkqvist *et al.,* 1997; Ansell *et al.,* 1997). The complementation test used is related to osmotic stress response, thus a Gpd1p-like phenotype, but it cannot be considered as a definitive answer, since the two isoenzymes can, in part, compensate for each other role (Björkqvist *et al.,* 1997; Ansell *et al.,* 1997; Remize *et al.,* 2001).

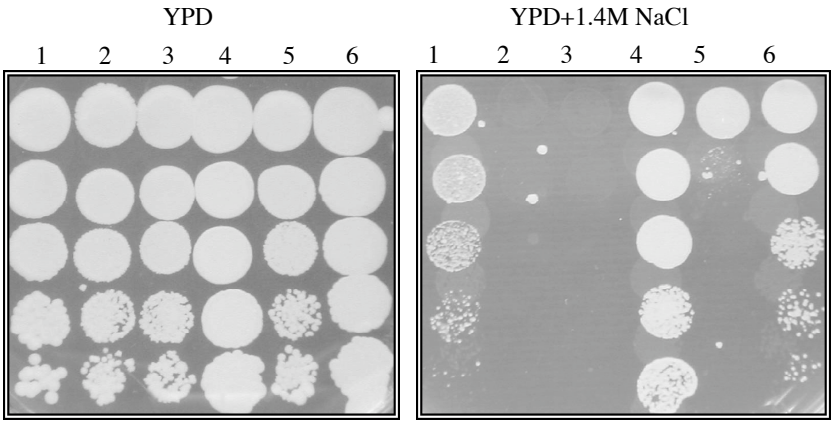


Fig.II1. Complementation of *S. cerevisiae gpd1gpd2* mutant strain. Lane 1, *S. cerevisiae* W303-1A. Lanes 2 and 3, *gpd1gpd2* strain transformed with multicopy p423GPD and single copy p413GPD (vectors without insert). Lane 4 and 5, *gpd1gpd2* strain transformed with p423GPD/*KtGPD* and p413GPD/*KtGPD*. Lane 6, *gpd1gpd2* strain transformed with p423GPD/*PaGPD*.

Complementation analysis for *GUT1*-like sequence was done using a gut-diagnostic medium (Rønnow and Kielland-Brandt, 1993), which is a semi-complex medium with glycerol as carbon source, in which *S. cerevisiae gut1* mutant strain is unable to grow. Cloning of *PsGUT1* in the deleted strain rescued growth in this medium (Fig. II2).

As *S. cerevisiae GUP1* presented a salt-stress associated phenotype (Holst *et al.,* 2000), complementation was assayed using *gup1* strain in salt-based medium. As previously observed with *S. cerevisiae GUP1* (*ScGUP1*), *PsGUP1, CtGUP1* and *CaGUP1* rescued growth from salt stress, when small amounts of glycerol are present in media, besides glucose as carbon source, while its over-expression does not lead to enhanced growth in the same medium (Fig. II3). As it is also known, *S. cerevisiae GUP2* does not complement *gup1* phenotype. For this reason it can be assumed that the *GUP*-like sequences hereby cloned are *GUP1*

orthologues. Actually, in the case of *C. albicans*, only one *GUP*-like sequence is present in the correspondent Genome Data Bank.

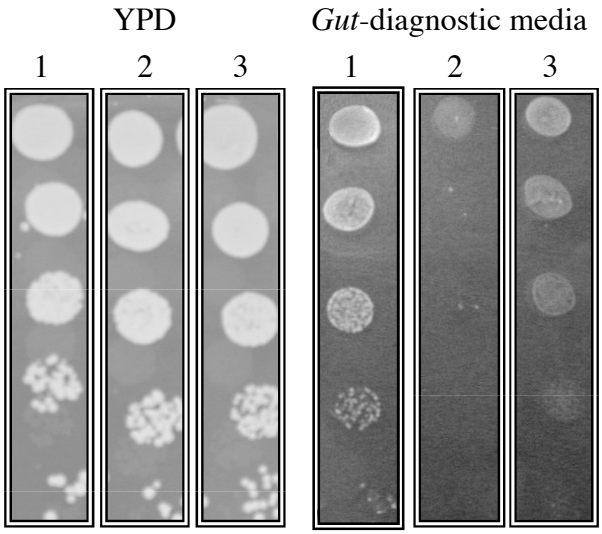


Fig. II2. Complementation of *S. cerevisiae gut1* mutant strain. Lane 1, *S. cerevisiae* W303-1A. Lane 2, *gut1* strain transformed with vector without insert. Lane 3, *gut1* strain transformed with p426GPD/*PsGUT1*.

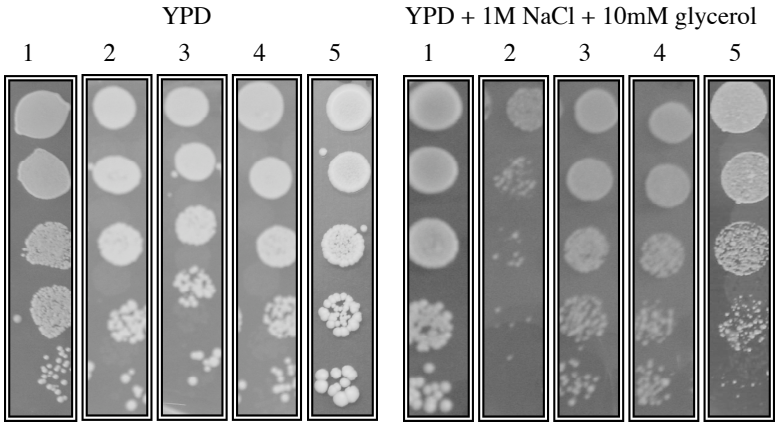


Fig II3. Complementation of *S. cerevisiae gup1* mutant strain. Lane 1, *S. cerevisiae* W303-1A. Lane 2, *gup1* strain transformed with vector without insert. Lane 3, *gup1* strain transformed with p426GPD/*PsGUP1*. Lane 4, *gup1* strain transformed with p426GPD/*CaGUP1*. Lane 5, *gup1* strain transformed with p426GPD/*CtGUP1*.

In the case of *FPS1*-like ORFS, the phenotypic complementation analysis was done based on the role of Fps1p in the exportation of glycerol during hypoosmotic shock. The assays were performed using cells pre-grown on 1 M sorbitol and plated on YEPD. *fps1* strain takes much longer to recover from shock (Tamás *et al.,* 1999). All the putative orthologues could restore partially the wild type capability to recover from hypoosmotic shock (Fig. II4).

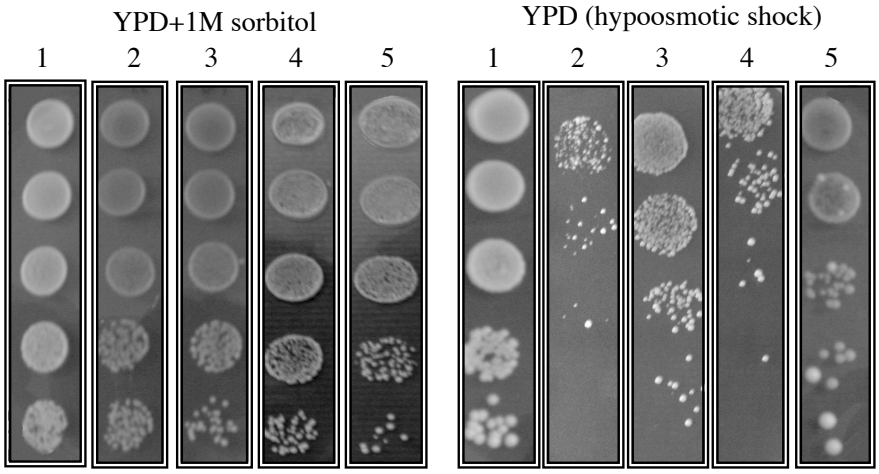


Fig. II4. Complementation of *S. cerevisiae fps1* mutant strain. Lane 1, *S. cerevisiae* W303-1A. Lane 2, *fps1* strain transformed with vector without insert. Lane 3, *fps1* strain transformed with p426GPD/*KlFPS1*. Lane 4, *fps1* strain transformed with p426GPD/*ZrFPS1*. Lane 5, *fps1* strain transformed with p426GPD/*KmFPS1*. Results after 48h incubation at 30ºC.

Summarizing, it can be said that all the cloned genes are able to do heterologous complementation of the *S. cerevisiae* strains deleted in the respective gene. No clear phenotypic differences were observed using of a centromeric or a multicopy plasmid (not shown), except for the *KtGPD* (Fig. II1). These results suggest that these genes might encode functional homologues of *S. cerevisiae* correspondent proteins, but do not necessarily prove that their function is the same in the original species from which they were obtained (see Discussion).

Sequence analysis

Alignments of the genes cloned in this work and the correspondent ones of other yeasts available in databases were done using the Clustal (see Supplementary Data) and Multalin programs. The programs use different algorithms, and so, small differences could be found, exclusively in the regions with lower similarity, but these were considered non-significant. Clustal was chosen to proceed. Identity/similarity matrixes were done (see Supplementary Data).

The deduced amino acid sequences of *GPD1/2* from different strains showed a similarity/identity that varied from a minimum of 47.9/ 36.9%, between *KtGPD* (this work) and *Schz. pombe GPD2*, to a maximum of 99.7/ 99.5% between *S. paradoxus* and *S. mikatae GPD*s. Between all the data some particular comparisons are relevant. The S*accharomyces* sensu lato *GPD*s showed the highest identity/similarity among all, being *S. castelli*'s the one that is more distinct

from this group. *Z. rouxii GPD1* and *GPD2* are very close to Saccharomyces group correspondent *GPD*s. Furthermore, *GPD* sequences of S*. cerevisiae*, *P. angusta* and *K. thermotolerans* share approximately 60 % identity.

For *GUT1* the highest similarity (96.2% between *S. cerevisiae* and *S. paradoxus*) is once more among the *Saccharomyces* species, being *S. castelli* the most different. *PsGUT1* is more similar to *C. albicans* (76%) than to *S. cerevisiae* (50.5%) orthologue.

Continuing with *GUP1/2*, the *Saccharomyces* sensu lato group presented the highest similarity/identity for the deduced amino acid sequences of those genes, being *S. castelli* once more the most different, and *S. paradoxus* and *S. mikatae* the most similar - 96.6% similarity. *P. sorbitophila* Gup1p was shown to be 70.7% similar to *C. albicans* Gup1p, and only 60.5% similar to *S. cerevisiae* Gup1p, 51.1% to *S. cerevisiae* Gup2p and 43.5% to *Schz. pombe* Gupp. *C. tropicalis* Gup1p was shown to be 72.2% similar to *C. albicans* homologous protein, 68.7% to ScGup1p and 56.6% to ScGup2p. CaGup1p is more similar to ScGup1p (65.1%) than to ScGup2p (54.1%). These data reinforce the above results concerning complementation analysis, which suggest that the genes cloned are *GUP1* orthologues.

Gup1p sequences were rather similar in what regards predicted transmembrane domains (TMM). All Gupp present 11 to 13 TMM, except for *PsGUP1*, which has only 7 to 9 predicted TMM. It is known from previous work that on *P. sorbitophila* as well as *C. albicans* and *C. tropicalis* the standard leucine codon CUG encodes a serine (Ohama *et al.,* 1993; Santos and Tuite, 1995; Sugita and Nakase, 1999; Suzuki *et al.,* 2002; Tekaia *et al.,* 2000). So, using this alternative codon usage, alignments with orthologues as well as search for predicted TMM, were repeated. The results obtained were identical, eventually because the CUG codon is rare enough not to contribute to significant differences in amino acid sequence (Tekaia *et al.,* 2000; Blandin *et al.,* 2000). Despite the different number of predicted TMM, *PsGUP1* complemented the *gup1* mutant phenotype of *S. cerevisiae* (Fig. II3). A sequence error cannot be disregarded, or the number of TMM is not determinant for the functions of Gup1p.

S. cerevisiae Fps1p is a member of the MIP family with 6 TMM and with unusually long N- and C-terminal extensions (van Aelst *et al.,* 1991; Luyten *et al.,* 1995; Tamás *et al.,* 1999; Bill *et al.,* 2001). The same was found for the predicted Fps1-like proteins cloned in this work (not shown). *FPS1* sequences are consistent with the high relatedness of the *Saccharomyces* strains. *Z. rouxii* Fps1p was

shown to be only 57.4% similar to ScFps1p and 45.2% to KlFps1p and 45.8% to KmFps1p.

Phylogenetic trees were built by the neighbor joining method. Bootstrap statistical procedure of resampling (Felsenstein, 1985), through the analysis method of one thousand replicates, gave the confidence values for the groups included in the consensus tree thus obtained (Fig. II5). The trees obtained for the deduced amino acid sequences of those genes confirm the phylogenetic positions of those yeasts determined by 18S and 28S rRNA sequence comparison (Souciet *et al.,* 2000) and reflect the identity/similarity relationships above mentioned.

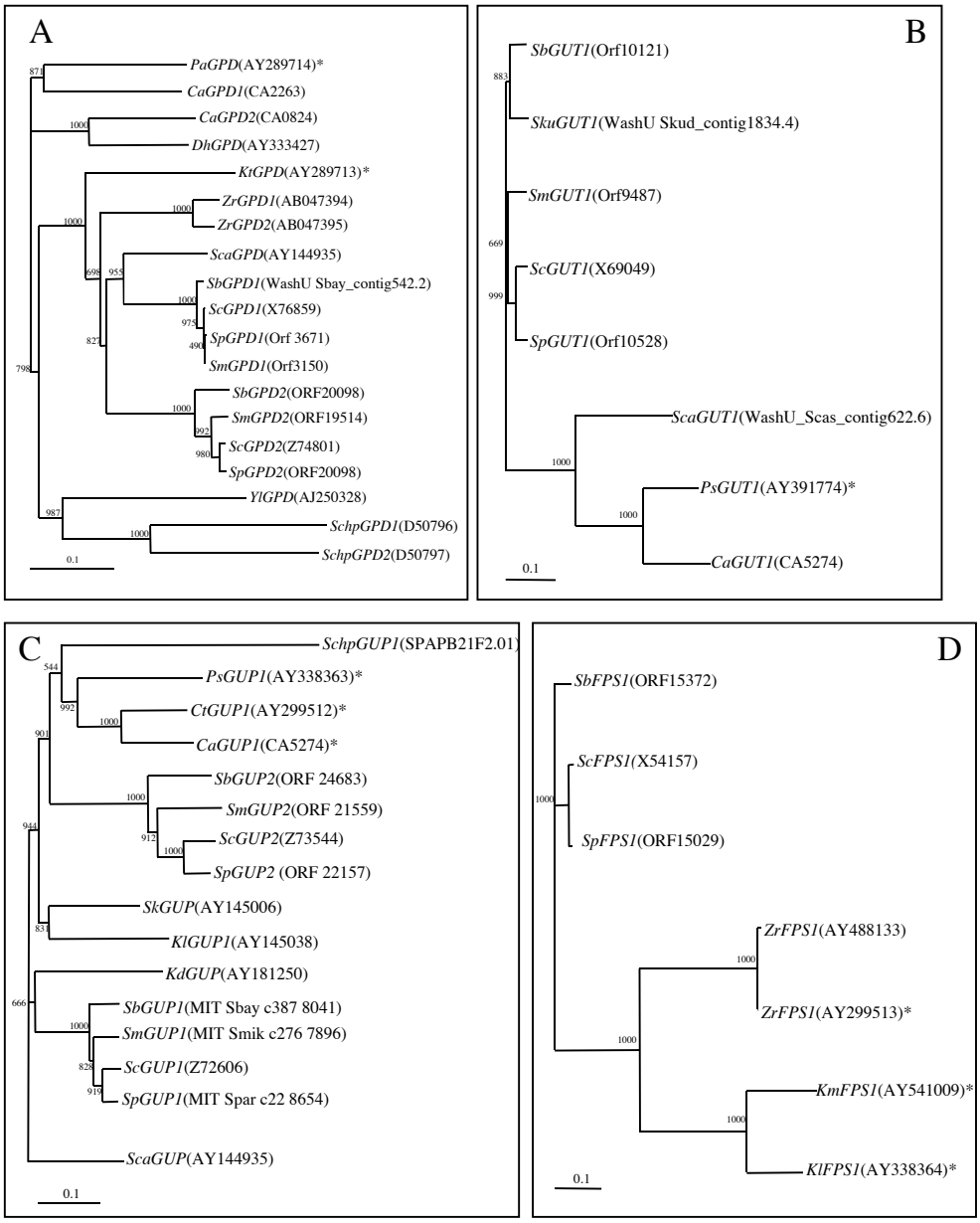


Fig II5. Phylograms for the predicted amino acid sequences of the genes: A - *GPD1/2*; B - *GUT1*; C - *GUP1/2*; D - *FPS1*. *Genes cloned and tested for phenotypic complementation in this work. Accession numbers are presented between brackets. Strains nomenclature: *Ca - C. albicans, Ct - C. tropicalis, Dh – D. hansenii, Kd – K. delphinensis, Kl – K. lactis, Kt – K. thermoltolerans, Pa – P. angusta, Ps - P. sorbitophila, Sb - S. bayanus, Sca – S. castelli, Sc - S. cerevisiae, Sk – S. kluyvery, Sku - S. kudriavzevii, Sm - S. mikatae, Sp - S. paradoxus, Schp – Sch. pombe, Yl – Y. lipolitica and Zr – Z. rouxii.*

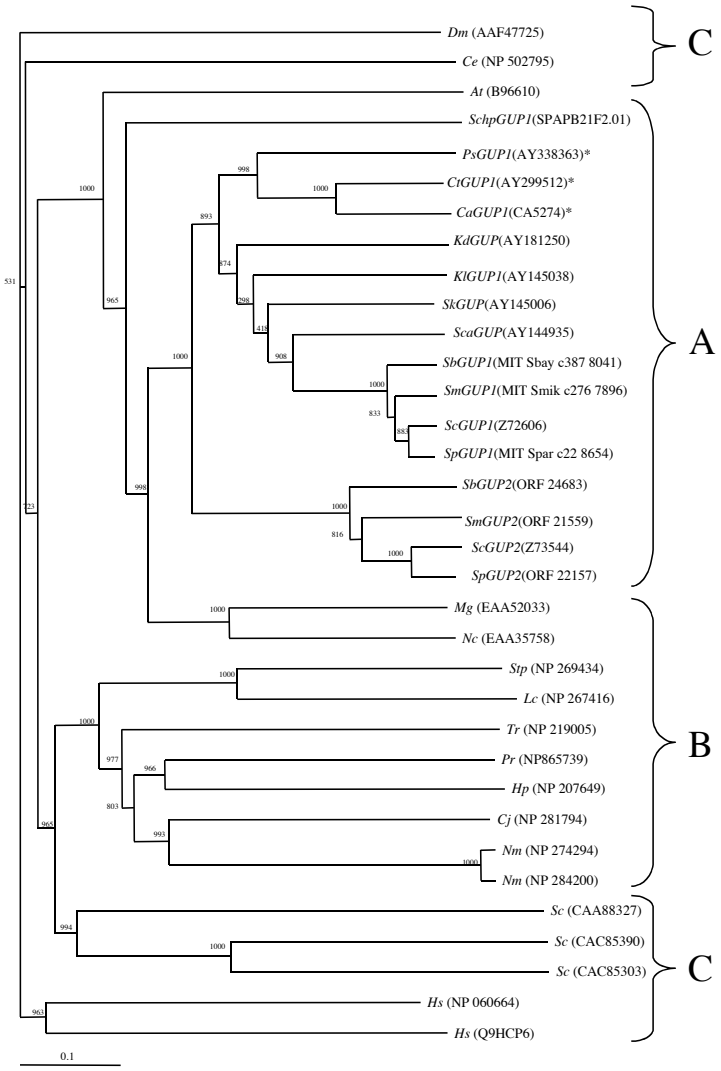


Fig. II6. Phylogram of Gup-related predicted aminoacid sequences. A – Gup-like sequences; B – proteins from MBOAT/DltB cluster; C – proteins from MBOAT/KOG3860 cluster. Accession numbers are presented between brackets. Only complete ORF sequences were used. Organisms nomenclature, other than yeasts: *At – Arabidopsis thaliana, Ce – Caenorhabditis elegans, Cj – Campylobacter jejuni, Dm - Drosophila melanogaster, Hp – Helicobacter pylori, Hs – Homo sapiens, Lc – Lactococcus lactis, Mg – Magnaporthe grisea, Nc – Neurospora crassa, Nm – Neisseria meningitides, Pr – Pirellula pyogenes, Stp - Streptococcus pyogenes and Tr – Treponema pallidum*. Information on sequences other than Gup-like ones: Ce(NP_502795) - skinny Hedgehog-like protein (KOG3860); Cj(NP_281794) - putative transmembrane protein (DltB); Dm(AAF47725) – Rasp (protein-cysteyne N-palmitoyltransferase – Hedgehog protein (KOG3860); At(B96610) - hypothetical protein (KOG3860); Hp(NP_207649) - alginate O-acetylation protein (DltB); Hs(NP_060664) - melanoma antigen recognized by T cells 2, required for palmtoylation of Hedgehog family (KOG3860); Hs(Q9HCP6) - O-acyltransferase (KOG3860); Lc(NP_267416) - peptidoglycan biosynthesis protein (DltB); Mg(EAA52033) - hypothetical ORF (DltB), Nc(EAA35758) - hypothetical protein (DltB); Nm(NP_274294) - alginate O-acetylation protein (DltB); Nm(NP_284200) - putative polysaccharide modification protein (DltB); Pr(NP_865739) - alginate O-acyltransferase (DltB); Sc(CAA88327, CAC85390, CAC85303) - O-acyltransferases (KOG3860); Sp(NP_269434) - putative membrane protein (DltB); Tr(NP_219005) - alginate O-acetylation protein (DltB).

Discussion

Comparative genomics can help to understand molecular evolution. It can also, to some extent, help to predict the function of proteins when these are allocated in one family. It can contribute to further understand up to which point amino acid sequence divergence among proteins can account for differences in physiological functions/responses. The fact that *GPD1/2, GUT1, GUP1/2* and *FPS1* orthologues cloned in this work could be used to enlarge the correspondent gene families already existent (Eriksson *et al.,* 1995; Bateman *et al.,* 2002; Hofmann, 2000; Park and Sayer, 1996) and were able to complement *S. cerevisiae* mutant phenotypes suggest that these may have conserved, in different species, a function close to the one described for their homologues in *S. cerevisiae* (Saier, 1994; Gaillardin, 2000). This way, phylogenetic relatedness inferred from sequence comparison could be translated into functional homology. Yet, the fact that the heterologous genes work in *S. cerevisiae* does not demonstrate that these genes, once in their native genetic environment, correspond to identical molecular/physiological responses to the same stimulus. For instances, the differences in the sequences of all the Gpdp analyzed were, in our opinion, too small to account for differences in the nature of the enzymatic reactions performed. Despite the close relatedness of *Z. rouxii* and *S. cerevisiae GPD1/2*, the *Z. rouxii* glycerol 3-phosphate dehydrogenase is able to use as co-factors both NADH and NADPH, while the same enzyme in *S. cerevisiae* only utilizes NADH (Oshiro and Yagi, 1996). Also, in spite of the importance of Fps1p in yeast physiology and metabolism (van Aelst *et al.,* 1991; Luyten *et al.,* 1995; Tamás *et al.,* 1999, Tamás *et al.,* 2000; Philips and Herkowitz, 1997; Toh *et al.,* 2001; Henry *et al.,* 2003), some yeast species like *C. albicans* and *Schz. pombe* do not present *FPS1* homologues. Yet, both *C. albicans* (personal communication from Gerald Kayingo, Yale University, USA, 2004) and *Schz. pombe* (Kayingo *et al.,* 2004) are able to rapidly release glycerol and recover from hypoosmotic shock like *S. cerevisiae*. Furthermore, considering the ability of Fps1p to mediate glycerol non-saturable uptake (Oliveira *et al.,* 2003) and that *Schz. pombe* takes up glycerol precisely in such a fashion (Lages *et al.,* 1999), it is likely that in some yeasts, the role of Fps1p might be performed by another structurally different protein.

Fps1p belongs to the MIP (Major Intrinsic Protein) family of channel proteins, that transport water, small molecules like glycerol, urea, NH_3, CO_2 or ions, through an energy independent mechanism (van Aelst *et al.,* 1991; Park and Saier, 1996). Depending on substrate specificity, three subgroups of the MIP family have been

proposed: aquaporins (water specific channels), aquaglyceroporins (water and other solutes) and glycerol facilitators (Froger *et al.,* 1998; Hohmann *et al.,* 2001). Fps1p has been considered an atypical member of the glycerol facilitator group, due to the long amino- and carboxyl-terminal extensions and the lack of the characteristic NPA motifs associated with the regulation of channel opening/closure by osmotic stress (Bill *et al.,* 2001; Park and Saier, 1996; Hohmann *et al.,* 2001; Tamás *et al.,* 2003; Hedfalk *et al.,* 2004). In the COG database, Fps1p belongs to COG0580, containing Glpf, the glycerol facilitator from *E. coli* and related permeases. In the same database, this gene is also present in another cluster, KOG0224, which contains aquaporins. If we look at blast scores between some of the proteins of this family, very high scores are found (low *e* values) between all the Fps1 proteins (ScFps1p/KlFps1p – $410/e^{-113}$; ScFps1p/ZrFPs1p – $564/e^{-159}$), while between ScFps1p and Glpf, it is just $94.7/9e^{-18}$; and between ScFps1p and HsAQ9, only $126/1e^{-27}$. Between ScFps1p and Yfl054cp blast score is $122/3e^{-26}$. As a consequence, the Fps1-like proteins cluster together and apart from the glycerol facilitators or the aquaporins (not shown). Yet, until more genes and more physiological data confirming their role(s) are available, it is not possible to create/rearrange families.

GUP1 and *GUP2* encode multimembrane-spanning proteins, which were first included in the major facilitator superfamily - MFS - Family number 7 of proteins of unknown function (Nelissen *et al.,* 1997). Later, due to the homology these proteins present to several acyltransferases, they were included in the MBOAT superfamily (Membrane-Bound O-Acyl Transferases) (Hofmann, 2000) . All biochemically characterized members of this superfamily encode enzymes that transfer organic acids to hydroxyl groups of compounds placed in the membrane. Consistently, a temperature-sensitive (*ts*) phenotype, as well as a significant modification in lipid composition was associated with a *gup1* strain (Oelkers *et al.,* 2000). Gup1p and Gup2p were further shown to present sequence similarity to DGAT (diacylglycerol O-acyltransferase), a mammalian acyltransferase, which belongs to the ACAT (acyl-coA cholesterol acyltransferase) family, which also includes yeast *ARE1* and *ARE2* (ACAT Related Enzymes). These two isoenzymes catalyze intracellular sterol esterification (Oelkers *et al.,* 2000). More recently, in the COG (Cluster of Orthologous Groups of Proteins) database at NCBI, which includes genomes of prokaryotes and unicellular eukaryotes, Gup1p and Gup2p were included in a cluster of bacterial predicted membrane proteins, COG1696, involved in the incorporation of D-alanin into lipoteichoic acids (DltB) (Perego *et al.,* 1995), which are also members of the MBOAT superfamily. According to the recently released

KOG (Eukaryotic Orthologous Groups) database (Tatusov *et al.,* 2003), Gup1p and Gup2p belong to KOG3860, a cluster of acyltransferases required for palmitoylation of Hedgehog signal transduction pathway family of secreted signaling proteins. Moreover, and according to published results, which suggest the involvement of these genes in glycerol active transport (Holst *et al.,* 2000), they constitute the only members of the Glycerol Uptake (GUP) family (2.A.50.1.1), in the TC-DB (Transport Protein Database).

Considering the results hereby obtained, *Saccharomyces* sensu lato strains, as well as *P. sorbitophila* and *C. tropicalis*, presented genes with a very high degree of identity/similarity to *S. cerevisiae GUP1* and *GUP2*. This is in accordance with the above mentioned putative function of these genes as involved in glycerol active transport, since this has been shown to be present in these yeasts (Holst *et al,,* 2000; Lages and Lucas, 1997; Lages *et al.,* 1999; Lages *et al.,* 1995). In the case of *C. albicans, K. delphinensis* and *K. lactis*, this correspondence cannot be made for the time being, due to the lack of physiological data. In what concerns *Schz. pombe*, this same relation is actually contradicted. A single *GUP* orthologue was found in this yeast. Yet, in *Schz. pombe* glycerol mediated transport has not been detected in either stress/non-stress, repression/derepression growth conditions (Lages *et al.,* 1999), corroborating the extremely poor growth this yeast presents on glycerol-based media.

Gup1p and Gup2p inclusion in MFS family was duly done according to the unquestionable transmembrane nature of the correspondent proteins (Nelissen *et al.,* 1997). On the other hand, the inclusion of these two proteins in the MBOAT family, in the clusters COG1696 (DltB) and KOG3860 (the blast score for *ScGUP1* in relation to KOG3860 is $472/1^{e-133}$), was based on sequence similarity (Hofmann, 2000), in relation to an extensively represented family of proteins with well established function, and is corroborated by the lipid and *ts* phenotypes observed in a *gup1* strain (Oelkers *et al.,* 2000). In opposition, the inclusion of *GUP1* and *GUP2* in the Glycerol Uptake (GUP) family is based exclusively on data concerning glycerol uptake related phenotypes (Holst *et al.,* 2000). Nevertheless, recently it has been published that *GUP1* and *GUP2* expression does not match glycerol transport regulation (Oliveira and Lucas, 2003). If we consider those results and we take together the fact that *Schz. pombe* has a *GUP* gene without presenting glycerol mediated transport, a question arises, whether *GUP1* and *GUP2* are actually genes encoding for transporter proteins or if, instead, they correspond to regulators influencing transport indirectly. Alternatively, the *ts* and lipid composition phenotypes mentioned above (Oelkers *et al.,* 2000) could mean that the function of

Gup1p and Gup2p could be connected to cell membrane and/or wall modifications affecting glycerol transporter function. Any of these hypotheses would be more compatible with the predicted functions of MBOAT members than with a transporter function.

To further evaluate the inclusion of *GUP1* and *GUP2* in the MBOAT family, a phylogenetic tree was built, using several complete ORFs belonging to the clusters or families mentioned above and belonging to different organisms (Fig. II6). Gup1p/Gup2p-like sequences are clustered together, but not distant from other clusters. This may reflect some functional differences between those proteins, but it can also be due to the fact that all the Gupp included in this tree belong to yeasts, which increases the probability of them clustering together since phylogenetic closeness of proteins is largely determined according to the phylum of the organisms of origin (Souciet *et al.,* 2000). In spite of the high degree of similarity between *GUP1* and *GUP2* (Holst *et al.,* 2000), Gup1p-like sequences group together and separately from the group of Gup2p-like sequences (Fig. II6), according to the different phenotypes associated so far with these proteins (Holst *et al.,* 2000; Oelkers *et al.,* 2000).

With the data presently available, we consider that the allocation of GUP genes in the MBOAT family appears more consistent than in the Glycerol Uptake (*GUP*) family. Yet, to reinforce this idea, clarification of these proteins functions must be undertaken.

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Supplementary data

Identity/similarity matrixes

Gpd1/2p-like sequences

PaGPD*	----	72.3	71.1	70.0	69.0	70.6	71.2	71.5	71.8	72.0	71.5	62.4	62.5	62.6	62.3	60.4	66.0	58.3	59.9
CaGPD1	62.4	----	66.8	65.3	64.8	67.5	68.3	68.3	68.3	68.1	68.9	62.7	62.8	62.9	61.9	62.2	64.1	54.4	55.3
CaGPD2	58.0	56.3	----	86.0	62.3	64.4	63.6	63.6	63.6	64.1	64.4	56.8	57.0	56.8	57.0	56.8	60.6	53.8	54.9
DhGPD	57.9	55.3	74.9	----	63.4	65.8	65.7	65.7	65.7	66.0	64.6	57.8	58.0	57.6	57.3	56.6	60.7	54.4	55.2
ZrGPD1	55.8	53.3	51.7	51.8	----	93.5	78.6	78.9	78.9	79.6	80.4	70.3	70.3	70.3	70.2	66.4	61.4	56.4	53.7
ZrGPD2	57.5	55.7	54.0	54.2	91.5	----	80.8	81.1	81.1	81.1	80.5	72.3	72.4	72.3	72.4	68.8	63.7	57.0	56.6
SpGPD1	58.8	57.5	54.0	55.9	70.6	72.6	----	99.7	99.5	98.5	84.1	72.6	72.3	72.3	72.5	66.1	63.9	54.3	56.2
SmGPD1	58.8	57.5	53.8	55.7	70.9	72.9	99.5	----	99.7	98.7	84.3	72.6	72.3	72.3	72.5	66.1	63.9	54.6	56.4
ScGPD1	59.3	57.5	54.0	55.9	70.9	72.9	99.5	99.5	----	98.7	84.3	72.6	72.3	72.3	72.5	66.1	63.9	54.6	56.4
SbGPD1	58.8	57.0	53.8	56.2	71.1	72.6	97.7	98.2	98.0	----	85.1	73.0	72.8	72.6	72.7	66.1	64.4	54.6	56.2
ScaGPD	57.9	58.2	54.5	54.4	71.5	72.2	78.8	78.8	78.8	79.5	----	72.9	72.9	72.9	72.8	66.9	63.1	54.2	55.5
ScGPD2	49.9	52.1	46.8	46.9	61.4	63.4	64.6	64.6	64.6	65.8	66.1	----	98.6	97.3	94.5	65.9	61.7	50.4	48.7
SpGPD2	49.8	52.0	46.6	47.2	61.2	63.3	64.4	64.4	64.4	65.3	65.8	98.2	----	97.3	94.5	66.1	61.8	50.2	48.8
SmGPD2	50.3	51.9	46.8	46.6	61.0	63.4	63.9	63.9	63.9	64.9	65.8	96.4	95.9	----	93.9	65.9	61.7	50.4	48.7
SbGPD2	49.3	51.8	46.2	47.0	60.4	63.8	64.5	64.5	64.5	65.5	65.5	91.8	92.3	91.1	----	65.4	61.1	50.3	48.5
KtGPD*	50.8	53.4	47.2	48.1	59.7	62.6	60.2	60.2	60.2	60.4	60.5	57.5	57.6	57.5	57.6	----	55.2	49.9	48.3
YlGPD	52.5	54.3	48.8	49.9	49.0	51.0	54.5	54.5	54.5	54.7	52.5	48.4	48.5	48.9	48.3	46.2	----	54.1	57.0
SchpGPD1	42.5	40.8	40.3	41.6	42.2	42.0	41.2	41.0	41.2	41.5	40.0	36.8	36.3	36.6	36.0	37.0	41.6	----	68.4
SchpGPD2	45.5	42.3	41.0	42.2	40.0	42.2	44.1	44.3	44.3	44.3	42.8	37.4	37.5	37.2	37.3	36.3	42.7	56.0	----

Gut1p-like sequences

SbGUT1	----	92.4	91.4	91.7	91.8	66.0	58.1	57.3
SkuGUT1	89.8	----	93.5	93.5	93.4	68.0	59.6	58.5
ScGUT1	88.4	90.8	----	96.2	93.8	67.5	59.8	58.8
SpGUT1	88.7	90.3	95.3	----	94.5	67.8	59.5	59.1
SmGUT1	88.9	91.0	91.8	92.4	----	68.2	59.9	59.0
ScaGUT1	57.4	58.9	59.3	59.0	59.1	----	56.9	56.7
PsGUT1*	49.3	50.9	50.8	50.1	50.4	47.6	----	83.3
CaGUT1	47.1	47.8	48.6	48.5	48.5	46.8	75.5	----

Gup1p/2p-like sequences

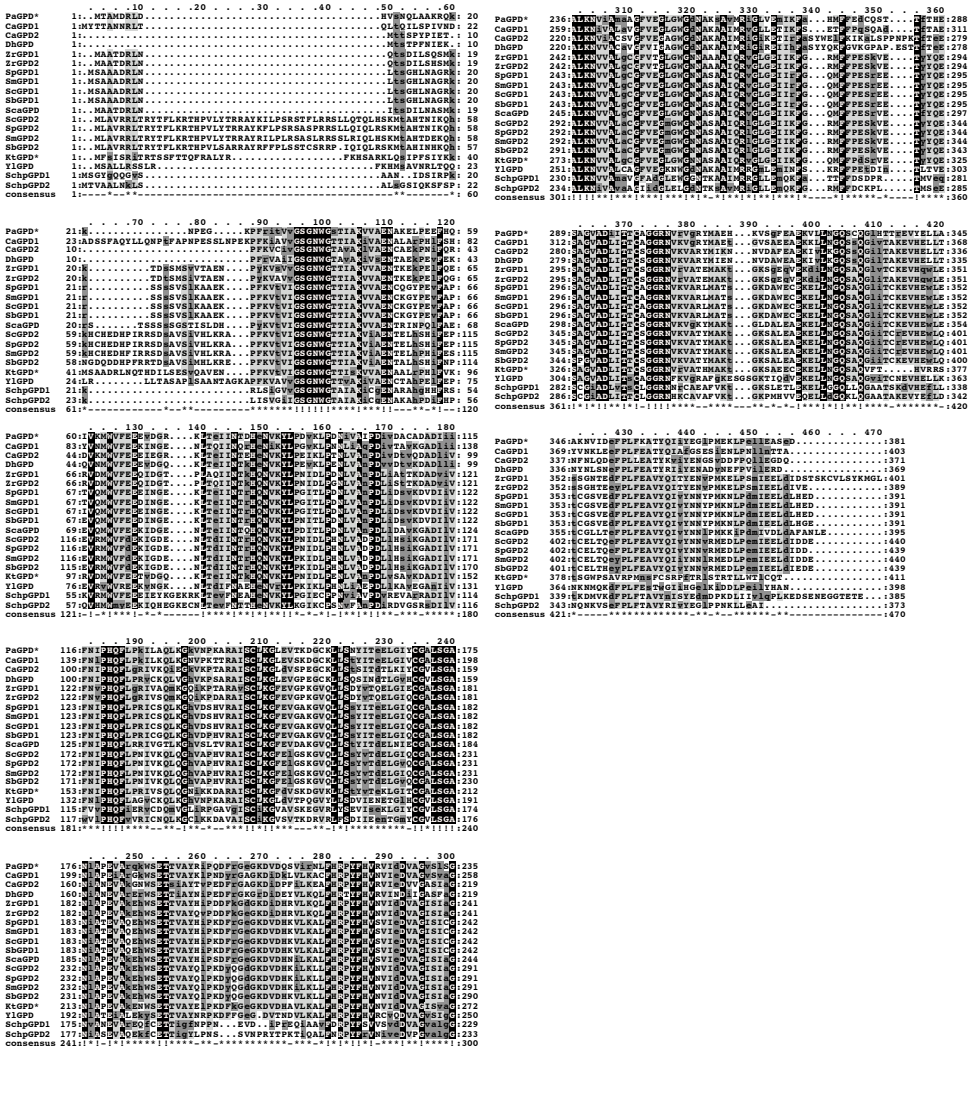
CtGUP1*	----	80.6	63.3	66.6	65.1	69.5	69.0	69.0	68.6	63.8	63.4	56.2	54.9	55.0	56.0	49.7
CaGUP1	72.5	----	63.4	68.1	67.8	69.3	69.3	69.0	69.0	65.2	63.3	58.6	54.9	55.3	57.3	49.3
PsGUP1*	52.6	52.8	----	60.4	57.1	60.5	60.3	59.9	60.5	57.6	54.6	51.1	47.3	49.0	51.4	43.1
SkGUP	54.8	55.6	50.9	----	77.7	74.1	73.4	74.1	74.3	70.7	74.4	64.4	60.5	63.0	64.7	46.1
KlGUP1	52.2	54.3	45.5	65.1	----	71.4	72.1	71.9	72.1	67.9	69.7	63.5	59.6	61.6	63.6	45.6
ScGUP1	54.8	54.9	47.6	63.2	58.0	----	97.3	95.0	93.2	76.7	71.9	64.6	60.1	61.5	64.8	49.1
SpGUP1	55.0	54.9	48.3	62.4	57.7	94.6	----	96.3	94.8	76.3	72.4	65.1	60.5	61.6	64.9	49.1
SmGUP1	54.3	54.7	47.5	62.7	58.3	90.7	93.0	----	94.3	76.5	72.1	65.0	60.4	61.8	65.1	49.3
SbGUP1	55.0	54.3	48.0	63.4	57.5	89.3	91.3	90.4	----	76.2	71.9	65.0	60.2	62.4	64.9	48.8
KdGUP	50.5	50.8	43.6	56.9	54.1	64.9	64.3	64.5	63.8	----	70.0	61.5	57.2	59.3	60.7	47.6
ScaGUP	51.2	52.1	44.1	63.9	59.0	64.8	64.8	64.4	63.8	58.1	----	68.3	64.3	64.0	68.0	45.6
ScGUP2	41.4	43.0	37.6	48.7	46.9	51.2	51.1	52.0	51.1	44.0	54.4	----	86.7	83.1	86.7	44.9
SpGUP2	40.1	40.4	35.0	45.7	43.9	48.1	48.0	48.1	47.2	41.7	51.3	82.8	----	82.8	79.8	42.0
SmGUP2	39.9	40.9	36.5	46.7	45.2	47.6	47.3	47.9	47.7	42.4	50.5	77.3	76.6	----	80.7	42.7
SbGUP2	41.1	43.3	37.9	50.6	49.1	51.6	51.5	51.9	51.5	45.5	55.3	78.7	72.6	73.2	----	44.0
SchpGUP1	34.5	33.9	29.4	33.7	30.8	33.8	34.4	34.6	34.9	32.6	31.1	30.7	28.5	28.5	30.3	----

Fps1p-like sequences

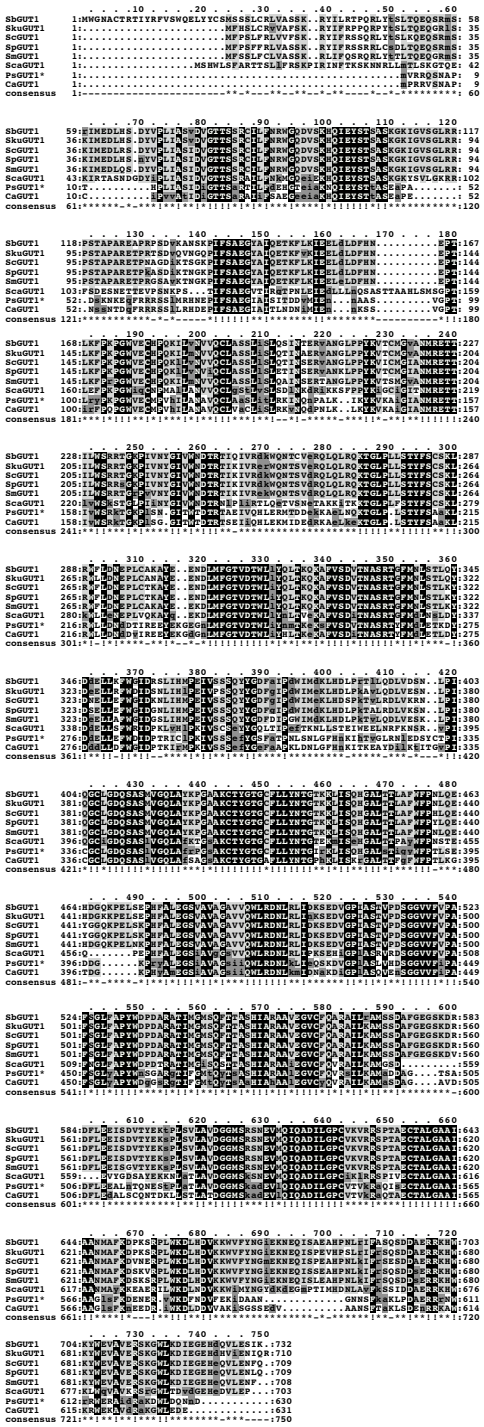
ScFPS1	----	97.8	92.7	58.6	57.4	47.4	46.5
SpFPS1	97.3	----	92.4	58.5	57.3	46.5	46.4
SbFPS1	90.6	90.7	----	58.7	57.5	47.0	46.7
ZrFPS1	48.1	48.4	48.5	----	94.2	41.1	42.4
ZrFPS1*	46.9	47.2	47.5	94.2	----	42.2	43.5
KmFPS1*	34.3	34.0	34.6	29.7	30.8	----	78.0
KlFPS1*	35.8	35.9	35.9	31.9	33.0	71.7	----

Alignments

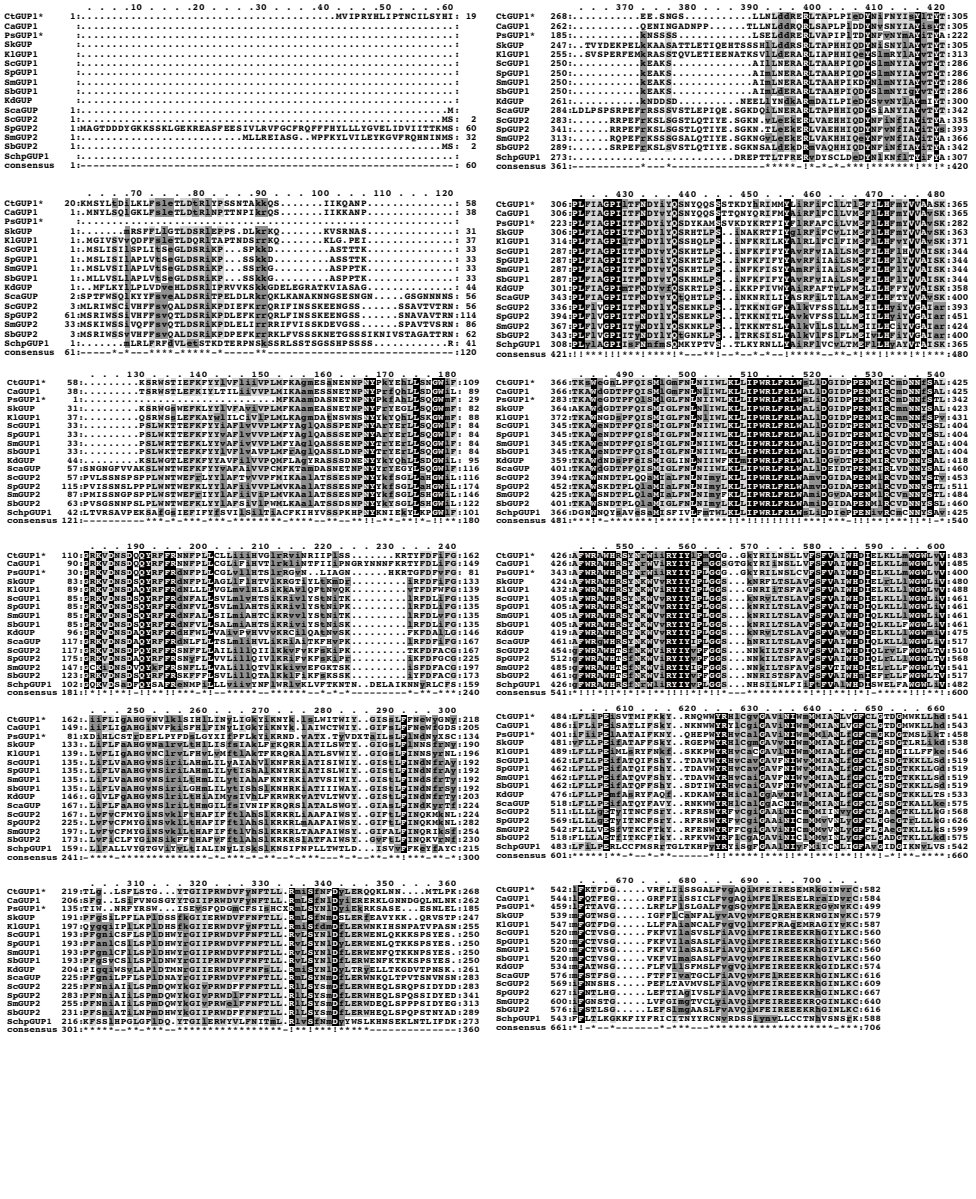
Gpd1/2p-related sequences



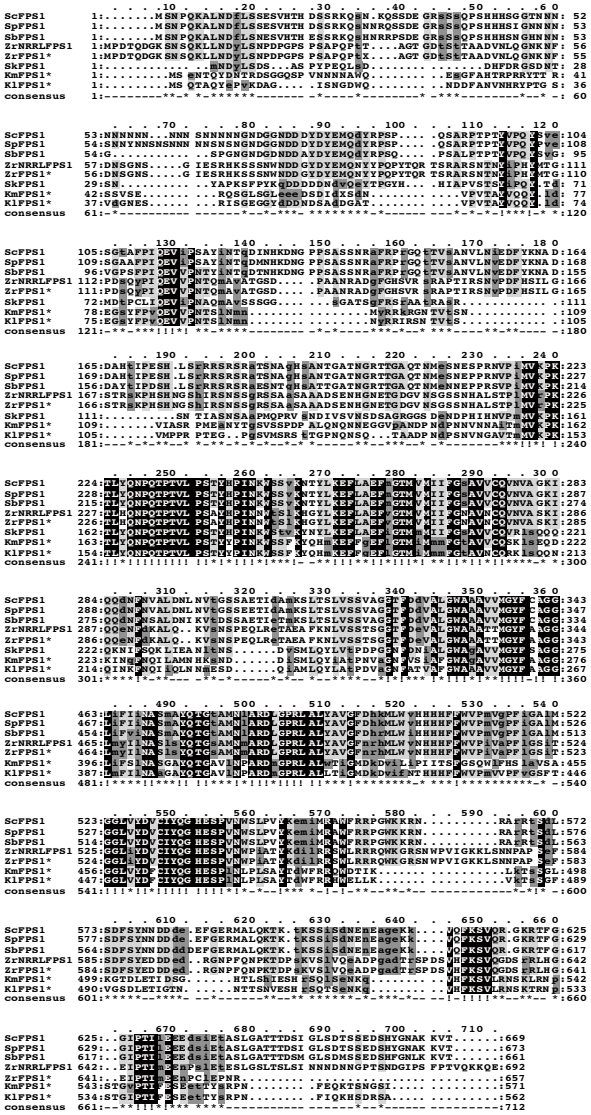
Gut1p-related sequences



Gup1/2p-related sequences



Fps1p-related sequences



Complementary data

Southern blot analysis

In order to complete the presented study, a different approach was used to enable sequence identification of desired orthologues in the yeasts mentioned before which genomes were only partially sequenced. Furthermore, we included *C. versatilis* (syn. *C. halophila*) CBS 4019 and *D. hansenii* CCMI 1941 (Amaral-Collaço *et al.,* 1989). These are very halo/osmotolerant yeasts with known specific features in what concerns glycerol metabolism and uptake (Lucas *et al.,* 1990; Neves *et al.,* 1997; Silva-Graça and Lucas, 2003; Silva-Graça *et al.,* 2003). *C. halophila* has never been subjected to a genetic approach or sequencing program and *D. hansenii* genome database from Génolévures Program was not yet completely available. Our objective was to establish a relation between the genes *GUT1*, *GUP1/2* and *FPS1*, the ones that so far have been related to glycerol transport in *S. cerevisiae* (Holst *et al.,* 2000), and the glycerol transport described in other yeasts (Lucas *et al.,* 1990; van Zyl *et al.,* 1990; Lages and Lucas, 1995; Lages *et al.,* 1999; Silva-Graça and Lucas, 2003), as well as to investigate the frequency of *FPS1* presence in yeasts that were described before as presenting glycerol diffusion and/or glycerol efflux similar to *S. cerevisiae* (Lages *et al.,* 1999; Kayingo *et al.,* 2004; G. Kayingo, personal communication).

Southern blot was done by standard procedures (Sambrook *et al.,* 1989) using genomic DNA digested with *Eco*RI or *Sau*3A immobilized on a nylon membrane (Gene Screen Plus - NEN- Life Sciences Products). Hybridization was done overnight at temperatures 45ºC, 55ºC, and 60ºC. Detection and labeling was performed using the Gene Images^{TM}AlKPhos Direct^{TM} system (Amersham Pharmacia Biotech). The probes were obtained by PCR using *S. cerevisiae* DNA as template. The primers were designed from sequences available at the *Saccharomyces* Genome Database (Table 1).

Table 1. Primer list

GPDf	5'CAAAACGTGAAATACTTGCC3'
GPDr	5'AGCAACAACGTTCTTCAAAGCAC3'
GUT1f	5'CCTACAGTCTTGCCCTCCAC3'
GUT1r	5´AACATTCCCGCAACACTTTC3´
GUP1f	5'CGTGGTTCCCTTGATGTTCT3'
GUP1r	5'TCGTTCCTTTTCCTCCAGAA3'
FPS1f	5'CCTACAGTCTTGCCCTCCAC3'
FPS1r	5'AACATTCCCGCAACACTTTC3'

All probes hybridized with *S. cerevisiae* DNA as expected. *GPD1* probe gave positive signal in all strains. *FPS1* probe gave a positive signal in *Z. rouxii, K.*

marxianus and *K. lactis*, as well as in *K. thermotolerans* and *P. angusta.* The signal was negative in *C. halophila, P. sorbitophila, D. hansenii, C. albicans* and *C. tropicalis*. *GUP1* and *GUT1* probes did not present detectable signal in any strain with all the hybridization temperatures used, eventually because they correspond to genes sequences less conserved. Being so, further efforts must be made to elucidate the presence or absence of these genes in the used strains.

The positive results given by the *FPS1* probe were obtained only when the hybridization was done at 45ºC. Thus, we could be facing a non-specific hybridization, for instance with aquoporins or aquaglyceroporins that supposedly are present in all eukaryotic genomes. Yet, the positive signal obtained in *K. lactis K. marxianus* and *Z. rouxii*, as well as the absence of signal in *D. hansenii* and *C. albicans* agree both with previous results and data available from sequencing projects, which are almost complete for these last two yeasts. Being so, one can conclude that the hybridization was specific and infer the existence of a *FPS1* homologue in *P. angusta* and its absence in *C. halophila, P. sorbitophila,* and *C. tropicalis*. This could explain the apparent absence of a first order kinetic for glycerol uptake reported for *C. halophila* (Silva-Graça and Lucas, 2003). In what relates glycerol export and channel closure, we cannot discard the possibility that the same functions might to be played by structurally different proteins. This is the case of *C. albicans* and *Schiz. pombe* that do not present *FPS1* homologues and in which cases glycerol export happens in a similar way to *S. cerevisiae* (Kayingo *et al.,* 2004; G. Kayingo, personal communication). In fact, in spite that first order kinetics for glycerol entry was observed in all the used yeasts (apart from *C. halophila* as discussed), *FPS1* proved to be present in less than half of these strains.

From the hybridization results obtained with the *GPD* probe, we chose *C. halophila GPD* putative sequence to follow. The reason for this choice layed in that glycerol 3-phosphate dehydrogenase from *C. halophila* has been reported as being able to utilize both NADH and NADPH as co-factors (Silva-Graça *et al.,* 2003). Two parallel gels were run, one of which was used for Southern hybridization. Fragments of digested gDNA correspondent to the hybridization results (not shown) were gel-eluted and ligated into the shuttle vector p423GPD. The mini-library thus obtained was tested for complementation of the S*. cerevisiae* double mutant *gpd1gpd2*. Several clones were able to complement the mutant phenotype. Nevertheless the attempts to sequence these were unfruitful, apparently due to plasmid instability in *E. coli*. This kind of yet unexplained instability has been

reported before for *P. sorbitophila,* another halotolerant yeast also used in this work (Montigny *et al.,* 2000a)

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Chapter III

New insights on glycerol transport in *Saccharomyces cerevisiae*

The work presented in this chapter has been published:
Neves, L., Lages, F. and Lucas, C. (2004) New insights on glycerol transport in *Saccharomyces cerevisiae*. FEBS Letters 565:160-162.

Abstract

Previous studies evidenced in *S. cerevisiae* the activity of a H^+/glycerol symport, derepressed by growth on non-fermentable carbon sources, latter associated with *GUP1* and *GUP2* genes. It was also demonstrated that only the combined deletion of *GUP1*, *GUP2* together with *GUT1* (glycerol kinase) abolished active transport in ethanol-induced cells. In this work we show that a glycerol H^+/symport, with identical characteristics to the previously described, was found in *gup1gup2gut1* grown under salt-stress, particularly high in cells collected during diauxic-shift. These results suggest different roles for Gup1/2p than glycerol transport. The gene encoding for glycerol active uptake is thus yet unknown.

Key words

Glycerol uptake; *GUP1*; *GUP2*; *GUT1*; *Saccharomyces cerevisiae*

Introduction

Glycerol, the main compatible solute used by yeasts under low a_w in high osmolarity environments (Hohmann, 2002), can permeate *Saccharomyces cerevisiae* plasma membrane through a constitutively expressed channel, Fps1p (van Aelst *et al.,* 1991; Luyten *et al.,* 1995; Oliveira *et al.,* 2003), and a H^+/symport, active in cells derepressed by growth on non-fermentable carbon sources (Lages and Lucas, 1997). This transport system was associated with the expression of the genes *GUP1* and *GUP2* (Holst *et al.,* 2000). *GUP1* was found in a two step screening: one corresponding to a glycerol mediated salt phenotype recovery in a *gpd1gpd2* background, *i.e.* a strain impaired in glycerol synthesis, and another corresponding to a *gut*-diagnostic medium phenotype (Holst *et al.,* 2000). *GUP2* was obtained searching *S. cerevisiae* genome database, according to its high degree of similarity to *GUP1*. Even so, its disruption did not present similar phenotypes and did not complement *gup1* mutation (Holst *et al.,* 2000). Furthermore, mutants characterisation as to glycerol active transport has shown that this could still be measured in cells deleted in both genes, provided they were cultivated under derepression conditions. This was attributed to transport measurements artefacts, created by glycerol kinase (Gut1p), the first enzyme from glycerol catabolism. Consistently, no glycerol active transport could be measured

on a *gup1gup2gut1* mutant grown on YPE (Holst *et al.,* 2000). On the other hand, as expected (Holst *et al.,* 2000; Oliveira *et al.,* 2003), Fps1p-mediated glycerol entry by first order kinetics was always present.

Still a contradiction remained, the fact that *GUP1* allows the recovery of salt-stress phenotype in spite of cells being cultivated on glucose, which has been shown to repress glycerol active transport (Holst *et al.,* 2000). Further studies on *GUP1* and *GUP2* expression, have shown these genes to be very low and constitutive, and no correlation was found between *GUP1* and *GUP2* expression, glycerol intracellular levels and glycerol active transport detection (Oliveira and Lucas, 2004). Moreover, the high similarity between *GUP1*-like sequences from different yeast species (Neves *et al.,* 2004) is not consistent with the detection of glycerol active transport in the same species (Lages *et al.,* 1999). Also, and despite that these genes were attributed an exclusive family in the Transport Protein database (TC-DB2.A.50.1.1), they were preferentially included in a superfamily of membrane-bound *O*-acyl transferases (MBOAT/DltB/KOG3860) (Hofmann, 2000).

Considering that genes may be of completely different value for the cells under different physiological conditions, to complement previous studies and to try to clarify *GUP1* and *GUP2* roles in relation to glycerol active transport, this was studied in strains combining mutations on *GUP1*, *GUP2* and *GUT1* genes cultivated under salt stress.

Materials and methods

Yeast strains, media and growth conditions

Saccharomyces cerevisiae strains used in this work were W303-1A (Thomas and Rothstein, 1989), CLy3, isogenic to W303-1A but *ygl084c::His5*$^{+}$ *ypl189w::KanMX* and BHY67, isogenic to W303-1A but *gut1 ygl084c::His5*$^{+}$ *ypl189w::KanMX* (Holst *et al.,* 2000). Cells were grown in rich media [YP: 1% (w/v) yeast extract; 2% (w/v) peptone] with 2% (w/v) glucose (YPD). Salt-stress media were based on YPD with 1 M NaCl, with or without 15 mM glycerol. Batch cultures were grown in Erlenmeyer flasks with air/culture proportion of 2/1, at 30ºC, 160 r.p.m. and growth was monitored spectrophotometrically, measuring $A_{640\ nm}$.

Quantification of extracellular solutes by HPLC

Carbon source consumption and metabolites present in the growth media were followed by high performance liquid chromatography according to Lages and Lucas (1997).

Glycerol transport studies

Initial rates of glycerol uptake, external alkalinisation upon glycerol addition, as well as in/out accumulation ratios, were determined as previously described (van der Brock *et al.,* 1982; Lages and Lucas, 1995; Lages and Lucas, 1997; Lages *et al.,* 1999). The intracellular volume values used to determine intracellular glycerol molarity have been determined by Lages and Lucas (1997).

Results

W303-1A, *gup1gup2* and *gup1gup2gut1* were grown on YPD and YPD supplemented with 1M NaCl, with or without 15mM glycerol. Specific growth rates during exponential growth phase, as well as the pattern of compound variation in the growth medium, were identical in the three strains for each medium (example in Fig. III1). Lag phase duration was slightly shortened by the addition of the mentioned small amount of glycerol to the growth media.

Glycerol uptake and accumulation experiments were performed in cells collected in: (1) mid-exponential growth phase, while fermenting glucose; (2) during diauxic shift, when glucose was exhausted from the media and cells were using ethanol as carbon source; and (3) closer to the stationary phase (arrows in Fig. III1).

Either strain cells, cultivated on YPD and collected during exponential growth phase, presented only a first order kinetics, indicative of channel-mediated entry (Holst *et al.,* 2000; Oliveira *et al.,* 2003). This is in accordance with previously published results that show the active transport system to be under glucose repression (Lages and Lucas, 1997; Holst *et al.,* 2000). When cells were collected during diauxic shift, W303-1A and *gup1gup2* strains presented accumulation of radiolabelled glycerol against gradient, not measurable in the *gup1gup2gut1* strain (Fig. III2). These results are also consistent with published ones, from ethanol-grown cells, which showed that transport was only missing when *GUT1* gene, besides *GUP1* and *GUP2*, were deleted .

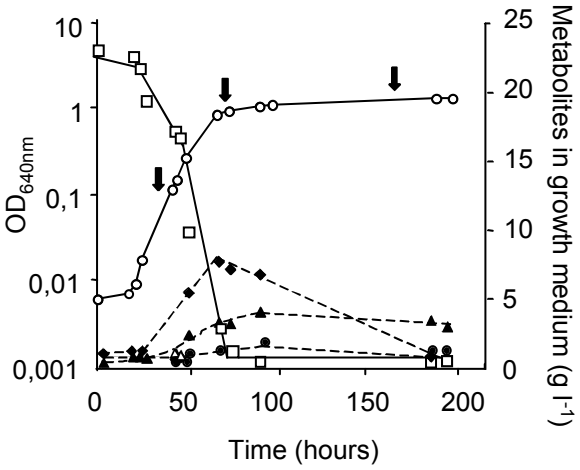


Fig III1. *gup1gup2gut1* growth curve in YPD + 1M NaCl + 15 mM glycerol at 30^{0}C (❍) and variation of metabolites in growth media (glucose (❑); ethanol (◆); glycerol (▲); acetate (●)). Arrows show sampling O.D. chosen for transport assays.

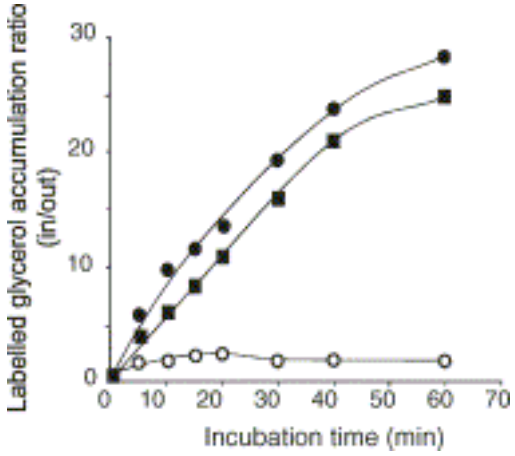


Fig III2. Accumulation ratios of [^{14}C] glycerol at pH5 and 30ºC, of cells cultivated in YPD and harvested during diauxic shift. W303-1A (●); *gup1gup2* (■); *gup1gup2gut1* (❍).

In cells grown in YPD with 1M NaCl, with or without 15 mM glycerol, and collected in any growth phase, all the three strains presented glycerol accumulation against gradient. This is shown in Fig. III3 for cells grown in presence of salt and glycerol. In cells collected in mid-exponential growth phase, according to published results, the uptake V_{max} values were lower than 50 µmoles h^{-1} g^{-1} dry weight and

K_m, consequently, could not be determined (Holst *et al.,* 2000). This was the *criterium* used in previously published work to consider as absence of transport (Holst *et al.,* 2000). During diauxic shift instead, as exemplified in Fig. III4, transport presented much higher V_{max} and for this reason K_m could be determined. The values calculated are presented in Table III1. At first hand, they appear to be identical to the ones previously determined in W303-1A for ethanol-grown cells (Lages and Lucas, 1997; Holst *et al.,* 2000). To sustain this statement, ANOVA test was applied. All the K_m values available from strains with W303-1A background from previous works (Sutherland *et al.,* 1997; Holst *et al.,* 2000) were used together with the ones here obtained. Values were not significantly different: $p>0.05$ in all cases. The presence of 15mM glycerol in media did not change uptake parameters significantly.

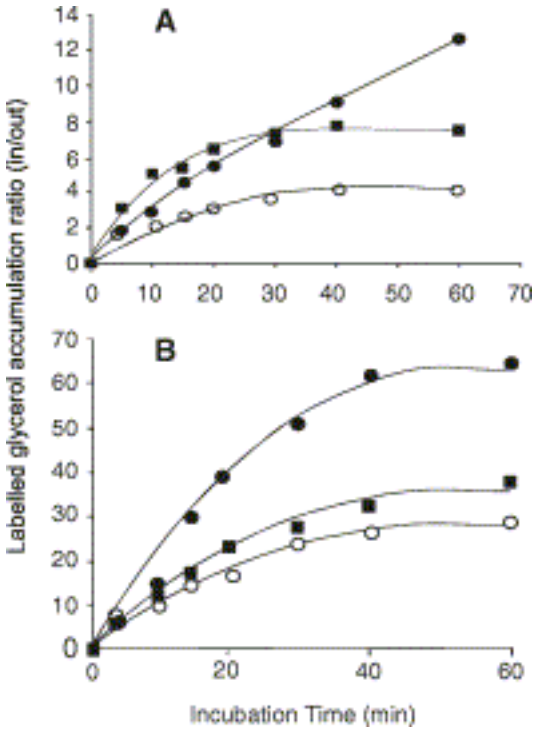


Fig III3. Accumulation ratios of [^{14}C] glycerol at 30ºC of cells cultivated in YPD + 1M NaCl + 15 mM glycerol and harvested during mid-exponential growth phase (A) and diauxic shift (B). W303-1A (●); *gup1gup2* (■); *gup1gup2gut1* (❍).

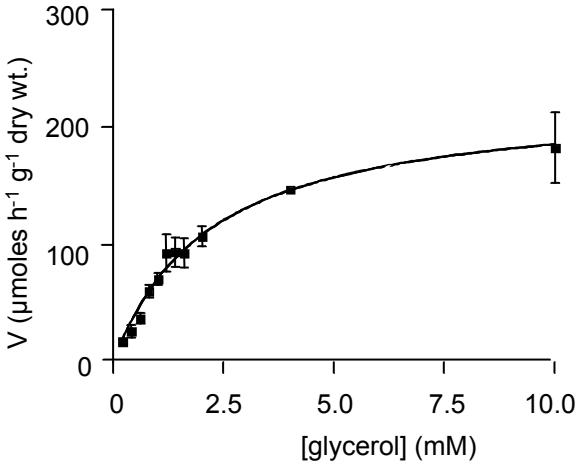


Fig III4. *gup1gup2gut1* [^{14}C] glycerol initial uptake rates at pH5 and 30ºC, in the presence of 1M NaCl. Cells were harvested during diauxic shift on YEPD + 1M NaCl + 15 mM glycerol. Kinetic parameters were determined and evaluated by computer regression analysis (GraphPad PrismR, 1994-97 Copyright GraphPad Software, Inc.).

Table III1. Kinetic parameters of glycerol uptake and accumulation of glycerol against gradient in cells grown on glucose in the presence of salt and 15mM glycerol and collected during diauxic shift.

Strains	[^{14}C] Glycerol uptake		[^{14}C] Glycerol accumulation	External alkalinization upon glycerol addition
	K_m (mM)	V_{max} (µmol h^{-1}g^{-1}dw)		
W303-1A	4.1±1.17 (3)	494±91,5 (3)	+	+
gup1gup2	3,9±2,15 (3)	309±118 (3)	+	+
gup1gup2gut1	2.2±0.31 (3)	226±15.7 (3)	+	+

(n) Number of independent experiments given in parentheses
± Standard error

Glycerol uptake in *gup1gup2gut1* proved to be sensitive to the action of the ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), being V_{max} decreased in about 80%. Incubation in the presence of CCCP prevented glycerol accumulation against gradient, and radiolabelled glycerol efflux was obtained by the addition of either cold glycerol or CCCP, consistently with the fact that this strain cannot metabolise glycerol (Fig. III5). Maximum accumulation ratios did not vary with extracellular pH. This is considered as an indirect consequence of the active transport responsible for accumulation against chemical gradient being dependent on proton-motive force (van der Brock *et al.,* 1982; Lages and Lucas, 1997; Lages *et al.,* 1999). Also, variation of NaCl concentration in assay buffer did not affect accumulation rates significantly. Furthermore, external alkalinization

upon addition of glycerol was observed. Though proton/glycerol stoicheiometry was not determined, these results indicate the presence of a transport system similar to the previously described in *S. cerevisiae*, a H^+/glycerol symport (Lages and Lucas, 1997).

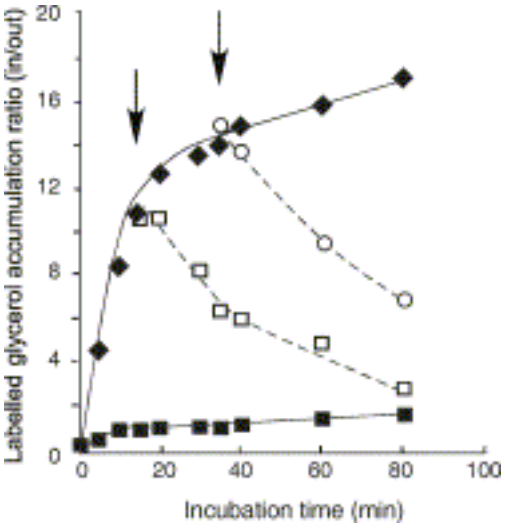


Fig III5. *gup1gup2gut1* [^{14}C] glycerol accumulation ratios at pH5 and 30ºC, in the presence of 1M NaCl (◆). Cells were harvested during diauxic shift on YEPD + 1M NaCl + 15 mM glycerol. Efflux of radioactivity observed after the addition (arrow) of 85mM "cold" glycerol (❍); efflux of radioactivity observed after the addition (arrow) of 50 µM CCCP (❑); accumulation prevented by the addition of 50mM CCCP (■).

Regardless to growth media, cells collected in pre-stationary phase presented the same profile of glycerol uptake and accumulation against gradient as found during diauxic shift, although values obtained were more erratic.

Discussion

Glycerol transport in W303-1A cells, derepressed by growth on non-fermentable carbon sources (Lages and Lucas, 1997), has been previously associated with *GUP1* gene (Holst *et al.,* 2000). On the other hand, transport in cells grown on glucose with salt-stress was measured, but exclusively in a *gpd1gpd2* background. This has been associated with *GUP2* gene (Holst *et al.,* 2000). Previously, Luyten and collaborators (1995) had shown that glycerol could be taken up by *S. cerevisiae* cells in a salt-stress based medium. They also showed that *fps1* mutant strain, which took glycerol very poorly in YPD, increased substantially in glycerol uptake once cultivated under stress. Therefore, glycerol active transport, although

not detected before, was beforehand expected to be present in salt-stress-grown cells (Luyten *et al.,* 1995) in spite of being under glucose repression (Lages and Lucas, 1997).

Results hereby presented show that, regardless of the deletions in *GUP1*, *GUP2* and *GUT1* genes, which abolished active transport in ethanol growing cells (Holst *et al.,* 2000), cells cultured under salt-stress and collected during diauxic shift, *i.e.* under derepression conditions, presented glycerol active uptake. Thus considering, neither of these three genes, though associated with glycerol transport (Holst *et al.,* 2000), cannot, in themselves, be considered as structural gene(s) for the symporter activity described here. This could correspond to a different system from the one previsouly described, functioning in different physiological conditions. Yet, the transport characteristics now observed are identical to the ones published before (Lages and Lucas, 1997; Holst *et al.,* 2000), from which we stress the same K_m value. This points to the possibility of Gup1p and Gup2p not being glycerol carriers, but, instead, regulatory proteins, since they do interfere with transport activity (Holst *et al.,* 2000). This hypothesis is consistent with the *GUP1* and *GUP2* low and constitutive expression (Oliveira and Lucas, 2004) and with the observation that the presence of *GUP1* homologues in several yeasts (Neves *et al.,* 2004) does not correlate to the detection of H^+/glycerol symport activity (Lages *et al.,* 1999; Neves *et al.,* 2004). We thus suggest for Gup1p and Gup2p roles other than glycerol active transport, being the gene encoding for glycerol active transport yet unknown.

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Complementary data

Glycerol transport experiments in other W303-1A mutant strains

Glycerol transport experiments were also done in the following mutant strains, isogenic to W303-1A: Cly5 (*gup2*) (Holst *et al.,* 2000), CLy 7 (*gup1gut1fps1*) (C. Lunde), *gup1gup2fps1* (C. Ferreira). The objective was to complement previous studies on transport regulation according to diverse mutations and to confirm whether *FPS1* deletion has some interaction with gup1p in what relates glycerol uptake.

All the mutant strains grown in glucose with salt stress and collected during diauxic shift presented glycerol accumulation against chemical gradient sensitive to the presence of CCCP, identically to the case of the mutants previously reported (not shown).

The *gup2* strain, that had never been tested as a single mutation, was also tested for initial rates of glycerol uptake, both in cells grown on glucose with salt stress and cells grown on ethanol-based medium. The kinetic parameters determined and evaluated by computer regression analysis (GraphPad PrismR Software) for the first situation were: K_m = 2.5 ± 0.49 mM; V_{max} = 411 ± 36.7 µmol h^{-1} gdw^{-1}. For YPE grown cells the measured kinetic parameters were: K_m = 2.4 ± 0.53 mM; V_{max} = 230 ± 24.9 µmol h^{-1} gdw^{-1}; K_d = 0.003 l h^{-1} gdw^{-1}.

Such results reinforce the previous notions that *GUP1* and *GUP2* genes do not encode the glycerol active transporter and that gup1p and fps1p function independently in relation to glycerol uptake. Besides, we can also state that deleting *FPS1*, in spite of the putative pleitropic nature of this gene, does not affect the previous results on active transport. Accordingly, results obtained with *gup2* strain, namely in cells grown in salt stress media, contradict the suggested existence of a *GUP2*-dependent glycerol uptake for a *gpd1gpd2* strain by Holst and co-workers (2000). On the other hand it is in accordance with the fact that its disruption did not present any phenotype concerning glycerol utilization and osmotic stress (Holst *et al.,* 2000). Actually, if we compare the V_{max} values, the one hereby calculated (411±36.7 µmol h^{-1} gdw^{-1}) with the one published for the wild type cultivated in the same conditions (494±91.5 µmol h^{-1} gdw^{-1}) it becomes clear that they are statistically identical.

Chapter IV

Glycerol transport in *Candida albicans*. Evidence for a constitutive active glycerol uptake system independent of CaGup1p, and a glycerol efflux system independent of Fps1p-like Aqy1p

The work presented in this chapter has been submitted for publication:
Kayingo, G., Neves, L., Martins, A., Lucas, C., and Wong, B. (2004) Glycerol transport in *Candida albicans*. Evidence for a constitutive active glycerol uptake system independent of CaGup1p, and a glycerol efflux system independent of Fps1p-like Aqy1p. (submitted)
Part of the work presented in this manuscript has been done by Gerald Kayingo, at Yale University

Abstract

Candida albicans can utilize glycerol, both as a sole carbon source and as an osmolyte, but the transport systems involved in the uptake, accumulation and release of glycerol across the plasma membrane are unknown. When *C. albicans* was grown in glucose or glycerol medium, the cells revealed the presence of a specific and constitutive proton/glycerol symport. The uptake of [^{14}C] glycerol was accumulative, dependent on proton motive force, and unaffected by osmotic shock. When osmotically stressed cells were exposed to a decrease in external osmolarity, intracellular glycerol was rapidly exported to the external medium. This export process was independent of AQY1, the only known *C. albicans* gene with similiarities to glycerol export channels. We isolated a putative glycerol transporter CaGup1p in a *C. albicans* genomic library, by complementing a *Saccharomyces cerevisiae fps1*Δ*/gup1*Δ mutant and by selection for growth on glycerol medium. Deletion of *CaGUP1* did not abolish active glycerol uptake and accumulation. The *C. albicans gup1* null mutant presents the same kind of glycerol transport of the wild type, with a similar K_m. These results imply that glycerol uptake and accumulation in *C. albicans* could be accomplished via specific transport systems independent of Gup1p. The constitutive nature of glycerol uptake points to a key role of glycerol homeostasis and might be an evolutionary strategy for *C. albicans* to survive in its environment.

Key words

C. albicans, glycerol, transport, proton symport, efflux, channel, *GUP1, FPS1*

Introduction

Glycerol plays a protective role when cells encounter environmental stresses and its formation is essential for redox balancing in yeast (Brown *et al.,* 1978; Yancey *et al.,* 1982; Blomberg and Adler, 1989; Albertyn *et al.,* 1994; Ansell *et al.,* 1997). Yeast and fungal cells have developed adaptive mechanisms to control glycerol flux within limits suitable for growth. These mechanisms include modulation of glycerol production, dissimilation, conservation and increased retention, as well as glycerol transport regulation across the plasma membrane (for review, Kayingo *et al.,* 2001a).

Early studies on glycerol transport assumed that glycerol is a liposoluble molecule that permeates across cell membranes only by simple diffusion (Gancedo *et al.,* 1968). However, evidence has been presented for the existence of specific systems involved in glycerol movements across the cell (Luyten *et al.,* 1995, Lages *et al.,* 1999; Tamás *et al.,* 1999, Oliveira *et al.,* 2003). For example, a glycerol channel protein Fps1p has been described in budding yeast *Saccharomyces cerevisiae* and shown to control glycerol accumulation and export during osmoregulation (Tamás *et al.,* 1999). Similar transport systems have also been demonstrated in other yeasts (Kayingo *et al.,* 2001b; Kayingo *et al.,* 2004) and homologues of Fps1p have been found in many fungal genomes (Kayingo *et al.,* 2001a; Neves *et al.,* 2004b). Besides channel mediated-glycerol transport, active transport systems for glycerol have been reported for several fungi. For example, an active glycerol transport system has been biochemically characterized in *Fusarium oxisporum* and shown to be involved in glycerol utilization and to be repressed by glucose (Castro and Loureiro-Dias, 1991). Active glycerol uptake systems also occur in the osmotolerant yeasts *Debaryomyces hansenii* (Lucas *et al.,* 1990) and *Zygosaccharomyces rouxii* (van Zyl *et al.,* 1990) and have been shown to be salt dependent. Furthermore, H^+/glycerol symport systems, constitutively expressed and neither dependent on salt nor glycerol in the media, were described in *Pichia sorbitophila* (Lages and Lucas, 1995) and in *C. versatilis* (syn *C. halophila*) (Silva-Graça and Lucas, 2003). In *S. cerevisiae*, an active transport system for glycerol was described by Lages and Lucas (1997). It was found to be a proton symport as described before for *P. sorbitophila* (Lages and Lucas, 1995), but differently regulated. It was repressed by glucose and induced by gluconeogenic conditions and apparently not involved in osmotic stress. Later, this transport system was associated with the expression of the genes *GUP1* and *GUP2* whose deletion affects growth on glycerol as a sole carbon source (Holst *et al.,* 2000).

The human pathogen *Candida albicans*, synthesizes and accumulates large amounts of glycerol and D-arabitol in culture and in the tissues of infected animals (Kiehn *et al.,* 1979; Bernard *et al.,* 1981; Wong *et al.,* 1982; San José *et al.,* 1996). Glycerol appears to be the main protective solute with which cells maintain osmotic homeostasis (San José *et al.,* 1996). *C. albicans* mutants that underproduce glycerol are hypersusceptible to environmental stresses and are hypovirulent in mice (San José *et al.,* 1996; Alonso-Monge *et al.,* 1999, 2003). Thus, having efficient mechanisms for regulating glycerol accumulation may be important for pathogenic fungi to colonize and survive in their mammalian host where a plethora

of environmental stresses such as nutrient availability, osmolarity, temperature, oxygen levels and oxidative killing by host phagocytes are frequently encountered. To date, the mechanisms by which glycerol accumulation is regulated in *C. albicans* are not well understood. There have been some studies on glycerol production but the means by which glycerol is transported in and out of *C. albicans* cells or the role of glycerol transport systems in cellular homeostasis have never been reported to our knowledge. The present study was, therefore, carried out to investigate the nature of glycerol transport systems in *C. albicans* and to examine their role in glycerol accumulation.

Materials and methods

Yeast strains, media and growth conditions

Strains used in this work are shown in Table IV1. Batch cultures were grown in YNB (2% glucose or 2% glycerol), in Erlenmeyer flasks with air/culture proportion of 5/1, at 30ºC, 160 r.p.m. and growth was monitored spectrophotometrically. Cells were harvested during exponential growth phase (OD600 of ≈ 1.5).

Table IV1. Yeast strains used in this study

Strain	Genotype	Reference
C. albicans SC5314	Wild-type	Gillum *et al*., 1984
C. albicans CAI4	*ura3::imm434/ura3::imm434*	Fonzi and Irwin, 1993
C. albicans JC0188	ura3::imm434/ura3::imm434 aqy/::hisG-URA3-hisG/aqy/::hisG	Carbrey *et al*., 2001
C. albicans BWP17	*ura3::imm434/ura3::imm434his1::hisG/ his1::hisG arg4::hisG/arg4::hisG*	Wilson *et al*., 1999
C. albicans DAY185	*ura3::imm434 HIS1::his1::hisG ARG4:: URA3 ::arg4::hisGura3::imm434 his1::hisG arg4::hisG*	Davis *et al*., 2000
C. albicans Cagup1	*ura3::imm434/ura3::imm434 his1::hisG/ his1::hisG arg4::hisG/arg4::hisG gup1::URA3-dpl200/gup1::ARG4*	This study
S. cerevisiae W303-1A	*MAT a leu2-3/112 ura3-1 trp1-1 his3-11/15Ade2-1 can1-100 GAL SUC2 mal0*	Thomas and Rothstein, 1989
S. cerevisiae BHY61-1A	*MAT ura3 leu4 trp1 his3 ade2 ygl084c::his5*$^{+}$ fps1*::HIS3*	Holst *et al*., 2000

Glycerol uptake and accumulation studies

To determine initial rates of glycerol uptake, external alkalinisation upon glycerol addition, as well as in/out accumulation ratios, transport studies involving radiolabelled glycerol were carried out following previously described procedures (Lages and Lucas, 1995, 1997; Lages *et al*., 1999). The intracellular volume values used to determine intracellular glycerol molarity have been determined by Lages and Lucas (1997). To estimate the initial uptake rates of radiolabelled glycerol, cells were washed twice and resuspended in ice cold water to a final concentration of about 30 mg/ml dry wt. 10 µl of yeast suspension was mixed with 10 µl (100mM) tris-citrate buffer (pH 5.0) in a conical centrifuge tube and incubated for 2 min at 25 °C in a water bath. The reaction was started by addition of 5 µl of an aqueous solution of [^{14}C] glycerol of varying concentrations (0.98-300mM) with specific activity varying from 200 to 40000dpm/nmol of glycerol. After 10 sec, the reaction was stopped by diluting with 5 ml of ice cold water, the mixtures were immediately filtered through the GF/C filters, washed twice with 5 ml water and immersed in vials containing 5 mls of scintillation fluid (LKB Scintillation products). The radiolabelled glycerol taken up by cells was measured in a Parkard Tri-Carb 2200 CA liquid scintillation counter (Packard Instruments Co., Inc., Rockville, MD, USA). The data for the initial uptake rates as well as the kinetic constants were analysed by a computer-regression analysis program (Graph Pad software, San Diego, CA, U.S.A). To investigate the presence of proton movements upon addition of glycerol, a standard pH meter (PHM82 Radiometer Copenhagen; Denmark) connected to a flat recorder (Kipp and Zonen) was used. The pH electrode was immersed in a water-jacket chamber (10 ml capacity) with a magnetic stirrer at 25 °C. A mixture of 4.5 ml water and 0.5 ml cell suspension was added to the chamber and incubated until the pH stabilized after which the pH was adjusted to 5.0. Proton movements were initiated by adding 100 µl of 1 M glycerol (pH 5.0) to a final concentration of 20 mM and subsequent alkalinization was monitored.

To determine the in/out ratios of [^{14}C] glycerol, the cell suspension (80 µl), and 110 µl of 100 mM tris-citrate (pH 5.0) were transferred in conical glass centrifuge tubes and incubated at 30 °C with magnetic stirring. After 2 minutes of incubation the experiment was started by adding 10 µl (200mM) of [^{14}C] glycerol specific activity (300dpm/nmol). At appropriate time interval, 10 µl samples (aliquots) were taken from the reaction mixture and filtered immediately and directly through Whatman GF/C filters (Whatman, Clifton, NJ, USA). The filters were then washed

twice with 5 ml ice-cold water and transferred to vials containing 5 ml scintillation fluid. The radioactive glycerol accumulated by the cells at different time intervals was then determined. Similar experiments were repeated in presence of 1 M NaCl or with 2% glycerol pre-grown cells. The accumulated glycerol was estimated as a ratio between intracellular and the extracellullar glycerol concentrations following the procedures of Lages and co-workers (1999).

Glycerol efflux assays

To study the patterns and kinetics of glycerol efflux, cells were first grown in medium containing 5% NaCl for 3 hours to accumulate glycerol. Osmotically stressed cells were then harvested and diluted 10X with fresh YNB- medium (hypo-osmotic conditions) to induce glycerol efflux. Control samples were diluted with YNB- medium containing 5% NaCl (iso-osmotic conditions). After a given time interval, triplicate samples (1 ml) were harvested and filtered (Whatman GF/C filter 25 mm) to separate the cell pellets from the supernatants. These were frozen for extracellular glycerol measurements. The cell pellets on filter discs were washed three times with iso-osmolar media and immediately resuspended in 1 ml cold water. Intracellular glycerol was then extracted by boiling for 10 min. After centrifugation, the supernatant containing the extracted intracellular material was retained for further analysis. Glycerol content released into the media and that retained by the cells after a given time interval, were determined with a commercial enzymatic kit (Roche Diagnostics) and D-arabitol content was determined by gas chromatography as previously described (Kiehn *et al*., 1979). For biomass measurements, cells were harvested by filtration and washed with an equal volume of water. The filters containing the cells were oven- dried at 80°C overnight and then weighed.

Isolation and cloning of CaGUP1

The *S. cerevisiae fps1*Δ/*gup1*Δ, which grows poorly on glycerol as a sole carbon and energy source was used for complementation studies to isolate *C. albicans* genes that could correct this defect. It was transformed with a library of genomic DNA fragments from *C. albicans* WO-1 in the shuttle plasmid pEMBLYe23, obtained from P. T. Magee (University of Minnesota). About 40,000 transformants were plated out on selective medium with 0.4% glycerol plus 0.05% peptone and monitored for ten days. Colonies that had grown faster on glycerol

plates and reached the same size as those that grew on glucose plates were isolated for further analysis and plasmid rescue experiments.

The complementing plasmids were isolated and amplified in *Escherichia coli* followed by sequencing. Analysis of the sequences revealed a previously uncharacterized open reading frame with about 53% identity to *S. cerevisia*e Gup1p (Holst *et al*., 2000). The entire *CaGUP1* reading frame and its upstream sequences were amplified by PCR, using the following primers:

*BamH*I-*GUP1*-UP gggatccGGATCTGCTCTTTGGTTAG

*Pst*I-*GUP1*-DO cctgcagAGGGACCAAATTATCTAGTTC.

The PCR product (2.7kb) was purified (QIAquick, QIAGEN Inc. Cat. No. 28704), cloned into pGEMT-easy vector (Promega) and verified by restriction digestion analysis and sequencing. The insert was subsequently subcloned into yeast vector YEp*lac*195 using the *Pst*1 and *BamH*1 linkers introduced into the primers, and transformed into the *S. cerevisiae fps1*Δ/*gup1*Δ mutant strain. Heterologous gene expression was verified by RNA dot blotting using total RNA from *S. cerevisiae fps1*Δ/*gup1*Δ strain transformed with the empty vector as a negative control and total RNA from *C. albicans* SC 5314 as a positive control. To analyze the involvement of *C. albican*s *GUP1* in glycerol uptake and utilization, *S. cerevisiae fps1*Δ/*gup1*Δ strain transformed with the YEp*lac-CaGUP1* plasmid, was monitored for growth on defined medium containing 1% or 0.4% glycerol as a sole carbon and energy source.

Construction of the CaGUP1 deletion mutants

Construction of the homozygous *gup1*Δ*/gup1*Δmutant (strain *GK-Y003*) was performed following the short flanking homology PCR method in the *C. albicans* strain BWP17. The targeting DNA fragments were amplified by PCR from plasmids pRS-Arg4SpeI **(**Wilson *et al*., 1999) and pDDB57, which contains the *URA3-dpl200* cassette (Wilson *et al*., 2000). The following primers were used:

GUP1-DEL5 AGAATTCCCTTCTACTAGATAATGAATTATCTATCAATTAGGG-
AAATTGTTTTCTTTAGAAACATTAGATGTGGAATTGTGAGCGGATA
GUP1-DEL-3 TGATATTCATTCATTAATTGTACCATAATCTCTTTTTAACA
CCTTACATCAATGGCTCTTCTTAATTCTGTTTCCCAGTCACGACGTT.

To delete the first *GUP1* allele, the amplified *URA3* cassette was introduced into the *C. albicans* BWP17 following the lithium acetate protocol (Wilson *et al*., 1999). Uracil$^+$ prototrophs were selected on defined glucose-YNB medium, and tested for proper homologous integration of the *URA3* cassette within the *GUP1*

locus. The resultant *GUP1/gup1*Δ::*URA3-dpl200* strain was transformed by the *ARG4* cassette to replace the second allele and create the homozygous *gup1*Δ*::ARG4/gup1*Δ::*URA3-dpl200* double mutant (strain *GK-Y003*). Proper integrations were confirmed by PCR and by Southern blot hybridization.

Results

Uptake and accumulation of radiolabelled glycerol

Besides increased glycerol production, yeast cells also regulate their intracellular glycerol levels using active transport systems (Van Zyl *et al*., 1990; Lages *et al*., 1999). Therefore, transport experiments were carried out to investigate the existence of an active transport system that might contribute to intracellular glycerol accumulation in *C. albicans*. Glycerol initial uptake and accumulation experiments were performed in cells grown in minimal media with 2% glucose or 2% glycerol as carbon source. *C. albicans* cells grown in presence of glucose, accumulated glycerol intracellularly against a concentration gradient (Fig. IV1). Similar results were obtained in glycerol grown cells (Fig. IV1). Incubation in the presence of CCCP prevented glycerol accumulation against gradient, and radiolabelled glycerol efflux was obtained by the addition of either cold glycerol or CCCP (Fig. IV2). Addition of arabitol, xylitol or ribitol, did not provoke efflux of radiolabelled glycerol (not shown). The effect of salt stress on the accumulation of radiolabelled glycerol was also assayed. Cells were equilibrated in presence of 1 M NaCl before starting the accumulation assay. NaCl shock had no significant effect on accumulative uptake of labelled glycerol. The in/out ratios obtained in the presence or absence of 1 M NaCl were almost identical (not shown). Taken together, the results were consistent with a constitutive and specific active glycerol uptake system that is dependent on proton motive force.

The kinetics of the initial glycerol uptake rates in glucose grown wild type cells is shown in Fig. IV3. It is a Michaelis Menten kinetics, indicative of a saturable transport system. Similar results were obtained for glycerol grown cells. The calculated values of V_{max} and K_m are presented in Table IV2. External alkinization upon addition of glycerol was observed. Although, the proton/glycerol stoicheometry was not determined, these results indicate the presence of an H^+/glycerol symport. A first order kinetics was also measured for higher [^{14}C] glycerol concentrations. The estimated value for the correspondent Kd lies around 0.09 $lh^{-1}g^{-1}$dry wt. Nevertheless, computer regression analysis of uptake raw data

does not corroborate the existence of this component in all the uptake experiments performed.

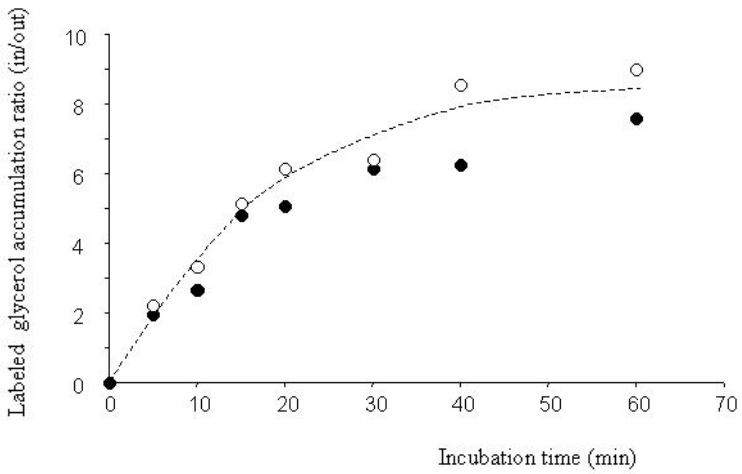


Figure IV1. Accumulation ratios of [^{14}C] glycerol at pH5 and 30ºC, of *C. albicans* cells cultivated in YNB -glucose 2% (❍) or glycerol (●).

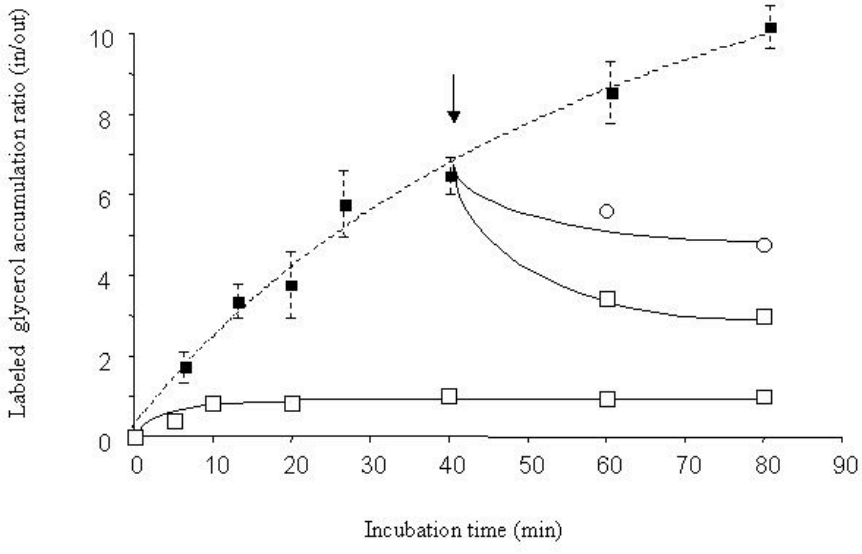


Figure IV2. *C. albicans* [^{14}C] glycerol accumulation ratios at pH5 and 30ºC of cells cultivated in YNB (glucose 2%) and harvested during mid-exponential growth phase (●, dashed lines). Efflux of radioactivity observed after the addition (arrow) of 85mM "cold" glycerol (❍); efflux of radioactivity observed after the addition (arrow) of 50 µM CCCP (❑); accumulation prevented by the addition of 50mM CCCP (■).

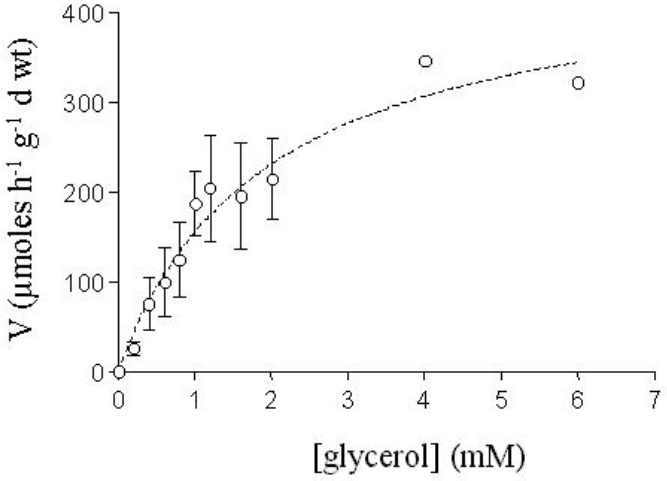


Figure IV3. *C. albicans* wild type [^{14}C] glycerol initial uptake rates at pH5 and 30ºC. Cells were grown in YNB at 30ºC and harvested during exponential growth phase. Kinetic parameters were determined and evaluated by computer regression analysis (GraphPad PrismR, 1994-97 Copyright GraphPad Software, Inc.).

Table IV2. Kinetic parameters of glycerol uptake and accumulation of glycerol against gradient in *C. albicans* cells grown on minimal media with glucose or glycerol and collected during exponential growth phase.

		[^{14}C] Glycerol uptake		[^{14}C] Glycerol accumulation	External alkalinization upon glycerol addition
Strains	Carbon source	K_m (mM)	V_{max} (µmol $h^{-1}g^{-1}$dw)		
wt	glucose	2.1 ± 0.9 (4)	460 ± 110.6 (4)	+	+
	glycerol	2.6 ± 0.7 (3)	268 ± 26.3 (3)	+	nd
gup	glucose	1.0 ± 0.7 (4)	382 ± 87.6 (4)	+	+

(n) Number of independent experiments given in parentheses
± Standard error
nd - not determined

Analysis of glycerol export process in C. albicans

When *C. albicans* cells were transferred from high to low medium osmolarity, approximately 80% of the initial intracellular glycerol was released to the external media in less than 3 min (Fig. IV4A). A similar trend was observed for a D-arabitol, another solute accumulated by *C. albicans* (Fig. IV4A).

Since glycerol export process observed in *C. albicans* was similar to Fps1p-regulated glycerol export in *S. cerevisiae* (Tamás *et al*., 1999), we next determined

if the *C. albicans* MIP channel homologue is also involved in the rapid release of glycerol. We cultivated the *C. albicans aqy1 null* mutant (Carbrey *et al*., 2001) and its isogenic wild type in medium containing 1 M NaCl for 3 hours and then transferred them to media without NaCl. The intracellular polyols (glycerol and D-arabitol) retained at different time points following a hypo-osmotic shock were measured and compared with control where cells were transferred to iso-osmotic media. As shown in Figure 4B, exposing the *C. albicans aqy1* mutant to hypo-osmotic media resulted in a rapid efflux of glycerol and D-arabitol (to a lesser extent). There were no differences between the wild type and the mutant indicating that the sole MIP channel protein CaAqy1 is not the principal mediator of polyol efflux in *C. albicans*.

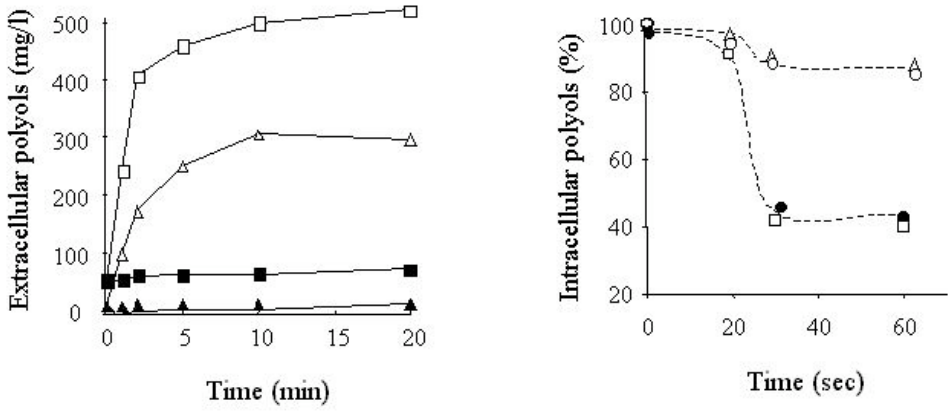


Figure IV4. A. Analysis of glycerol (❑, ■) and D-arabitol (∆,▲) efflux from cells transferred from high osmolarity medium (1 M NaCl) to medium without NaCl (a hypo-osmotic shock) (❑, ∆) and cells transferred to isotonic medium (■ ,▲). **B**. Effect of Ca *aqy1* deletion on the efflux and intracellular retention of polyols. The intracellular content of D-arabitol (∆,O) and glycerol (❑,●) retained by the of the *aqy1* null mutant (●,∆) and the isogenic wild-type CAI4 (❑,O) upon a hypo-osmotic shock were determined and expressed in percentages, taking the iso-osmotic values (transfer to medium containing 1M NaCl), as 100%.

Isolation and functional analysis of CaGUP1

To further investigate the actual protein (s) involved in glycerol transport in *C. albicans*, complementation experiments were undertaken using *S. cerevisiae* mutant lacking Fps1p (glycerol channel) and Gup1p (glycerol uptake), and which grows poorly on glycerol as a sole carbon and energy source. After ten days, some colonies had grown faster on glycerol plates and reached the same size as those that grew on glucose plates. Analysis of complementing plasmids revealed an open reading frame encoding a putative multimembrane spanning protein with 584 amino acids and 53% identity to the *S. cerevisiae* Gup1p.

When the *CaGUP1* was cloned and expressed in the *S. cerevisiae fps1*Δ/*gup1*Δ strain, the cells grew better on glycerol as a sole carbon source than those expressing empty vector (Fig. IV5). We further examined if the *CaGUP1* could also complement the glycerol efflux defect of *S. cerevisiae fps1*Δ/*gup1*Δ mutant. Whereas the wild type *S. cerevisiae* strain released up to 80% of intracellular glycerol in less than five minutes upon a hypo-osmotic shock, the mutant with or without Ca*GUP1* only released about 20% of the intracellular glycerol.

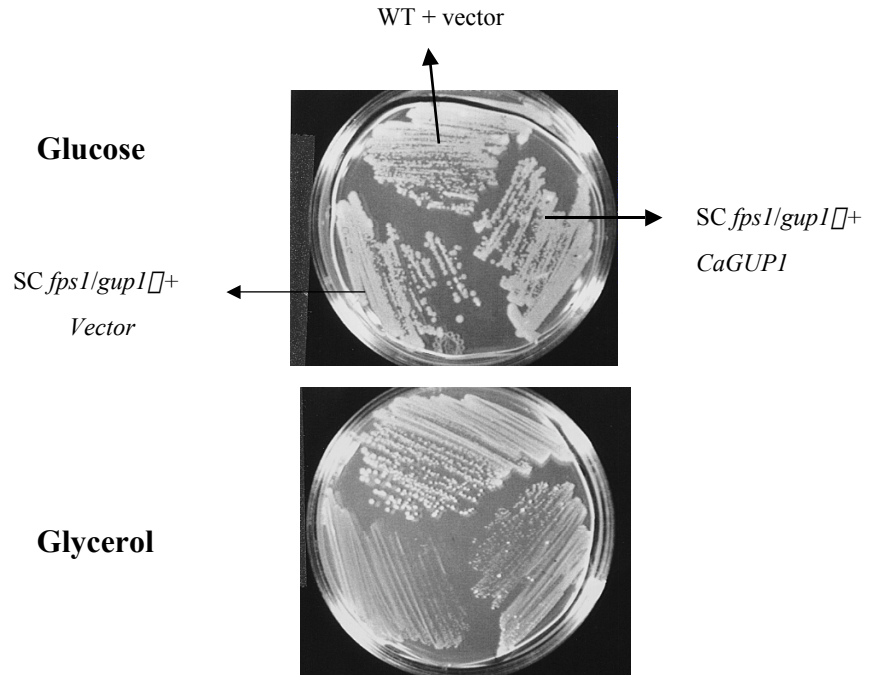


Figure. IV5. Complementation of *S. cerevisiae fps11gup1* mutant. Equal amounts of cells were harvested from overnight cultures, washed three times with water and streaked on minimal medium containing either 2 % glucose or 1% glycerol as a sole carbon and energy source.

To gain additional insights on the transport properties of *CaGUP1* and its roles in polyol accumulation, a deletion mutant was constructed using the *URA3/ARG4* system. Cells lacking the *GUP1* gene grew like the wild type on glucose medium but slightly poorly on medium containing less than 1% glycerol as sole carbon source. We next tested its abilities for the uptake and accumulation of radiolabelled glycerol. The gup1 null mutant presented the same kind of transport system of the wild type (Table IV2).

To investigate if the of *CaGUP1* is required for glycerol accumulation during osmotic regulation, we monitored the intracellular glycerol accumulated when cells

were exposed to hyper or hypo-osmotic stress. Deletion of *CaGUP1* had no effect on the conservation of glycerol during osmo-regulation (Fig. IV6).

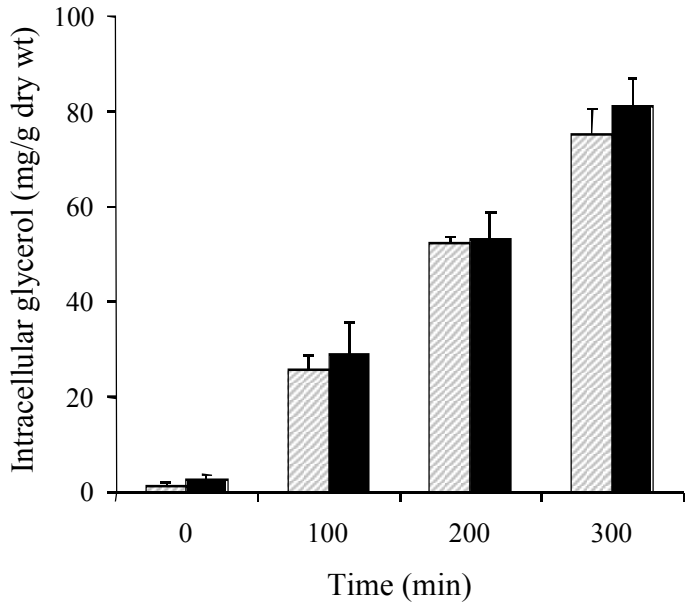


Figure IV6. Effect of *Ca gup1* deletion on intracellular glycerol accumulation during hyper-osmotic stress conditions. Glycerol accumulation by *Ca gup1* null mutant (solid bars) and the wild type DAY185 (hatched bars) was monitored in cells grown at 30ºC in YNB-glucose containing 1 M NaCl

Discussion

An active glycerol transport system in C. albicans

Besides regulating glycerol production (Albertyn *et al.,* 1994), yeast cells have additional control levels at the plasma membrane to conserve the synthesized glycerol intracellularly (Tamás *et al.,* 1999) and even to actively take up glycerol from the medium depending on growth conditions (Lages *et al*., 1999). Results hereby presented show that *C. albicans* cells are able to take up [^{14}C] labelled glycerol by a saturating high affinity transport system. Glycerol uptake was associated with a transient alkalinization of the extracelular media indicating that proton (H^+) movements were involved. The uptake system exhibited accumulative capacity and the accumulation ratios were similar for glucose and glycerol grown cells and were not affected by NaCl. Abolishing the transmembrane proton motive force by the action of an uncoupler (ionophore/protonophore) inhibited glycerol

accumulation. These observations provide evidence for the existence of a constitutive active glycerol uptake system of proton symport type in *C. albicans*, similar to the ones previously reported in the halotolerant yeasts *P. sorbitophila* and *C. halophila* (Lages and Lucas 1995, Silva-Graça and Lucas, 2003). The H^+/glycerol symport observed in *C. albicans* differs from that previously reported in *S. cerevisiae* in which case glycerol is actively transported by proton symport only in cells grown on non-fermentable carbon sources and undetectable in cells grown on glucose (Lages and Lucas, 1997). Furthermore, *C. albicans* differs from *Z. rouxii* and *D. hansenii* in a way that glycerol uptake was not affected by NaCl (Lucas *et al.*, 1990; van Zyl *et al.*, 1990). The fact that the addition of arabitol, xylitol or ribitol did not provoke efflux of radiolabelled glycerol points to a highly specific transport system for glycerol. The physiological implications for having active glycerol uptake system is not yet clear. The constitutive nature of glycerol uptake points to a key role of glycerol homeostasis and might be an evolutionary strategy for *C. albicans* to survive in its environment.

Deletion of CaGUP1 *and* AQY1 *had no effect on glycerol transport*

The accumulation and rapid release of glycerol by *S. cerevisiae* during osmo-regulation is mediated by the plasma membrane export channel Fps1p (Tamás *et al.*, 1999). The kinetics and patterns of glycerol export observed in *C. albicans* resemble that of *S. cerevisiae* and suggest the involvement of channel proteins. Analysis of the *C. albicans* databases (www-sequence.Stanford.edu/group/candida) revealed one putative Fps1p-family channel protein Aqy1p, and prompted us to investigate whether it could be involved in solute transport since many AQY homologues have been shown to transport both water and glycerol (Froger *et al.*; 2001, Ishibashi *et al.*, 2002). However, analysis of the *C. albicans aqy1* null mutant showed no defects in glycerol and arabitol efflux indicating that the MIP channel protein CaAqy1 is not the principal mediator of polyol efflux in *C. albicans*. Fps1p has also been shown to be responsible for the non-saturable glycerol uptake observed with radiolabelled glycerol assays (Oliveira *et al.*, 2003). So, in *C. albicans* the putative first order kinetics of glycerol uptake observed in some of the experimental conditions might be performed by another structurally different protein.

In *S. cerevisiae*, the active glycerol transport system was originally associated with the expression of the genes *GUP1* and *GUP2* as deletion of these genes

affects growth on glycerol as a sole carbon source (Holst *et al.,* 2000). A functional homologue of these genes was isolated from *C. albicans* genomic library by complementation studies. However, analysis of *C. albicans gup1* mutant revealed no significant effects on glycerol uptake, accumulation and efflux, suggesting that glycerol transport in yeast could be accomplished *via* means independent of Gup1p. It has also been recently demonstrated that active glycerol uptake is still present in the *S. cerevisiae gup1/2* mutant under certain conditions (Neves *et al.,* 2004a). Accordingly, it has been proposed that Gup1p and Gup2p might not be glycerol carriers as previous results suggested (Holst *et al*., 2000). Gup1p has also been linked to triglyceride and phospholipid biosynthesis (Oelkers *et al*., 2000). Furthermore, a phylogenetic approach using Gup1p-like sequences allocated these proteins preferentially in the MBOAT family (Neves *et al.,* 2004b). This points to a role in the plasma membrane and eventually wall structure/biogenesis.

In summary, we have demonstrated the presence of a constitutive active glycerol uptake system of proton symport type in *C. albicans*, both in glucose and glycerol grown cells that is independent of NaCl in the growth medium. The kinetics and patterns of glycerol export suggested the involvement of specialized facilitating proteins. However, the *AQY1* and *GUP1* genes, the only known putative homologues of glycerol transporters, are not responsible for the observed glycerol transport. Our future studies will focus on identifying and characterizing the proteins mediating glycerol uptake and efflux in *C. albicans* as well as their role in environmental stress tolerance.

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Chapter V

Strategies for the identification of glycerol transport related genes

Abstract

In order to identify new gene(s) involved in glycerol uptake, two strategies were undertaken. The first corresponded to using a transposon library to mutagenize *S. cerevisiae fps1* mutant strain. This strain was chosen for enabling to avoid artifacts generated by the Fps1p channel activity. Due to unexpected difficulties encountered in *fps1* manipulation a second approach was chosen. A screen for glycerol defective growth was done using a subset of the EUROSCARF single mutant collection. Several genes putatively involved in glycerol consumption were isolated, but neither proved to be affected in glycerol uptake, presenting levels of glycerol accumulation identical to the wild type. The unveiling of the molecular phenomena underlying the glycerol phenotype here presented will require further studies.

Key words

Glycerol transport; *FPS1;* mTn-lacZ/LEU2 genomic library; EUROSCARF mutant collection

Introduction

Saccharomyces cerevisiae is able to utilize glycerol as a sole carbon and energy source. Its catabolism involves transmembrane transport and two further enzymatic steps: phosphorylation catalysed by cytosolic glycerol kinase, encoded by *GUT1* (Pavlik *et al.,* 1993) and dehydrogenation catalysed by mitochondrial glycerol 3-phosphate dehydrogenase, encoded by *GUT2* (Sprague and Cronan, 1977; Rønnow and Kielland-Brandt, 1993).

Physiological characterization of glycerol uptake in *S. cerevisiae* has shown that glycerol enters the cell following a Michaelis-Menten kinetics due to the presence of an active proton symporter (Lages and Lucas, 1997; Lages *et al.,* 1999). Furthermore, it also enter the cell through a first order kinetics, previously considered as passive diffusion (Lages and Lucas, 1997), and later associated with Fps1p (Oliveira *et al.,* 2003), representing the open channel-mediated diffusion. This, operates independent and simultaneously to active H^+/glycerol symport system (Sutherland *et al.,* 1997).

Fps1p belongs to the MIP (Major Intrinsic Protein) family of channel proteins, that transport water, small molecules like glycerol, urea, NH_3, CO_2 or ions, through an energy independent mechanism (van Aelst *et al.,* 1991, Park and Saier, 1996). Depending on substrate specificity, three subgroups of the MIP family have been proposed: aquaporins (water specific channels), aquaglycerolporins (water and other solutes) and glycerol facilitators (Froger *et al.,* 1998; Hohmann *et al.,* 2000). Most microbial genomes tend to have at least two MIP channels (one for water transport and another for glycerol and/or other solute transport) (Kayingo *et al.,* 2001). The MIP proteins exhibit a typical structure with six transmembrane spanning domains, an internal sequence repeat, highly conserved motifs and about 200-300 amino acids (Park and Saier, 1996). *S. cerevisiae* has four homologues, two aquaporins and two glycerol facilitators, Fps1p and its close homologue *YFL054*c (Luyten *et al.,* 1995). Fps1p has been considered an atypical member of the glycerol facilitator group, due to the long amino- and carboxyl-terminal extensions and the lack of the characteristic NPA motifs (Park and Saier 1996, Hohmann *et al.,* 2000). These long terminal ends have been associated with the regulation of channel opening/closure by osmotic stress (Bill *et al.,* 2001; Tamás *et al.,* 2003; Hedfalk *et al.,* 2004). It was shown to encode a channel-type protein of the MIP family (Luyten *et al.,* 1995) responsible for regulating glycerol export under sudden osmotic down-shock (Tamás *et al.,* 1999), or in reaction to a cell turgor change (Tamás *et al.,* 2000).

The proton/glycerol symport system has been associated with *GUP1* and *GUP2* genes (Holst *et al.,* 2000), as it was demonstrated that *GUP1* was essential for growth on glycerol as sole carbon and energy source, as well as for a glycerol mediated salt phenotype recovery in a *gpd1gpd2* background (Holst *et al.,* 2000). *GUP2* disruption in a wild-type background did not present any phenotype. In further studies, it has been shown that *GUP1* is active under derepression conditions, while *GUP2* seemed to be active under salt stress, provided glycerol production was impaired. Nevertheless, active transport could still be measured on ethanol-grown cells deleted in both *GUP1* and *GUP2*. This was attributed to transport measurements artefacts, created by glycerol kinase (Gut1p), as no glycerol active uptake could be measured in the triple mutant *gup1gup2gut1* grown on ethanol based medium (Holst *et al.,* 2000). Recently, new studies on *S. cerevisiae* glycerol transport showed that the above mentioned mutant strain, *gup1gup2gut1*, was able to transport actively glycerol if grown on salt-stressed media provided cells were collected during diauxic shift (Neves *et al.,* 2004a). Furthermore, both *GUP1* and *GUP2* genes have been shown to be constitutively

expressed and no relation was found between the intracellular levels of glycerol and transport results (Oliveira and Lucas, 2004). Also, and in spite that these genes were attributed an exclusive family in the Transport Protein database (TC-DB2.A.50.1.1), they were preferentially included in a superfamily of membrane-bound O-acyl transferases (MBOAT) (Hofmann, 2000; Neves *et al.,* 2004b). All this new data raised a problem concerning the affiliation of H^+/glycerol symport to *GUP1* and *GUP2*, suggesting for Gup1p and Gup2p roles other than transport. In fact, presently, it is our belief that *GUP1* and *GUP2* despite interfering in some way with glycerol active transport (Holst *et al.,* 2000), are not the genes responsible for encoding the glycerol transporter(s) (Neves *et al.,* 2004a,b). Results obtained in *C. albicans* transport reinforced this idea.

Taking all these different data into consideration, it was considered necessary to make further efforts to find genes involved in glycerol uptake and try to clarify their roles in relation to that same function. The strategies followed were two. One corresponded to the use of a transposon library to mutagenize *fps1* mutant and the other corresponded to a screen in the EUROSCARF collection for glycerol-related phenotypes.

Materials and methods

Yeast strains

S. cerevisiae strains used in this study include two different genetic backgrounds: W303-1A (Thomas and Rothstein, 1989) and BY4742, derived from S288C, the strain used for sequencing of the *S. cerevisiae* genome (Brachmann *et al.,* 1998). The collection of single mutants in BY4742 was obtained from EUROSCARF (European *Saccharomyces cerevisiae* archive for functional analysis). The mutant strains isogenic to W303-1A used were YSH642 (*gpd1gpd2*) and YSH294 (fps1::LEU2) (S. Hohmann).

Media and growth conditions

Yeast cells were grown in YP medium [1% (w/v) yeast extract; 2% (w/v) peptone] with 2% (w/v) glucose (YPD), 2% glycerol (YPG) or 2%ethanol (YPE) at 30ºC, 160 rpm, and growth was monitored by spectrophotometry at 640 nm. Cultures were maintained in YPDA. For plates 2% agar was added to the medium to be used.

Transformants selection was performed in synthetic complete media SC (1% (w/v) succinic acid, 0.6% (w/v) NaOH, 0.67%(w/v), YNB w/o amino acids (DIFCO)), with 2% (w/v) glucose and 2% (w/v) agar, supplemented for auxotrophic requirements.

Bacterial cells were grown in LB liquid medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 1 N NaOH to adjust to pH 7.5) or solid agar plates with additional 2% (w/v) agar. Cultivations were done at 37ºC with aeration at 200 r.p.m. for liquid media. For plasmid maintenance and amplification, growth was performed in LB medium supplemented with 100 µg/ml ampicillin (Lbamp). All biological material was kept at -80ºC for long-term storage in 50% (v/v) glycerol.

Genomic library

A mTn-lacZ/LEU2 genomic library was used to transform *fps1* strain to create, by random mutagenesis, a library of mutants in which each mutant would contain, a further unique disruption in the chromosome. In parallel, and as a control, the same was done in a *gpd1gpd2* strain. Library propagation and transformation were carried out as described by Ross-MacDonald and co-workers*,* (1995) and in the URL: http//ygac.med.yale.edu/mtn/.

Escherichia coli strain DH5 [F- 80d*lac*ZM15 (*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17(r_k^- m_k^+) *deo*R *thi*-1 *pho*A *sup*E44 -*gyr*A96 *rel*A1] was used for maintenance and amplification of genomic library and it was transformed with a $CaCl_2$-based protocol (Sambrook *et al*. 1989). Competent cells were prepared according to Inoue and co-workers (1990).

Glycerol transport studies

For glycerol uptake experiments ethanol grown cells were harvested at exponential growth phase by centrifugation at 7400 *g*, 4ºC, 2 min and washed twice with ice-cold distilled water. After washing, cells were resuspended in ice-cold distilled water to a final concentration of about 300 mg dry weight ml^{-1} and kept on ice.

Glycerol accumulation ratios, were determined as previously described (Lages and Lucas, 1995, 1997; Lages *et al*, 1999). The intracellular volume values used to determine intracellular glycerol molarity have been determined by Lages and Lucas (1997).

Results

Transposon-based mutagenesis

In an attempt to identify genes that encode, or at least are involved, in glycerol uptake, we set up a screen, which would allow us to identify mutants with a loss-of-function phenotype. Knowing that glycerol could enter the cell both through Fps1p (Oliveira *et al.,* 2003) and by a H^+/glycerol symport system (Lages and Lucas, 1997), we decided to use *fps1* strain as the receptor for the mTn-lacZ/LEU2 (Ross-MacDonald *et al.,* 1995), in order to create, by random mutagenesis, a library of mutants. The reason underlying this choice resides in the fact that *fps1* mutants can grow on glycerol-based media due to active transport induction. This strategy should allow the identification of clones putatively involved in glycerol uptake.

For presently unknown reasons*, fps1* strain resisted transformation procedures of diverse type. We begun by using the method recommended in the protocols available for mTn-lacZ/LEU2-mutagenized library, which is a modification of the classical lithium acetate method (Ausubel *et al.*, 1996). Furthermore, the base method of chemical transformation was also tried, varying the quantities of the different components used, as well as electroporation (Ausubel *et al.*, 1996; Becker and Guarente, 1991). The efficiency of transformation was in either case very low, not enough to enable screening (<100 transformants/µg DNA).

To test whether this problem related to the recipient strain and not in the methods used, we applied the same methodology to a *gpd1gpd2* strain for control, in identical circumstances as previously used (Holst *et al.*, 2000). In this case the efficiency of transformation was high: > 20 000 transformants/µ DNA. This strategy was not further followed.

Screen on EUROSCARF collection for mutants impaired in glycerol utilization

We proceeded doing a screen for glycerol utilization on glycerol-based plates using the EUROSCARF mutant collection. As a control, the growth capacity of the same mutants was also tested in YPD plates. To avoid mutants defective in respiration, they were also analysed for proper growth on ethanol-based plates. According to results, strains were grouped as: (1) negative phenotypes in both ethanol and glycerol (313 ORFs); (2) positive growth on glycerol and negative growth on ethanol (154 ORFs) and (3) considerable growth on YPE and non-

growth on YPG (209 ORFs) (not shown). Strains of this last group were then analysed in relation to the number of transmembrane domains. The ones comprising ORFs with more than ten predicted transmembrane domains were: YBL089w, YBR132c, YDL054c, YGL104c, YGL114w, YGL139w, YGL140c, YLR081w, YMR011w and YPL 189w. This last ORF corresponds to the gene *GUP2*, which has previously been described as being involved in glycerol uptake (Holst *et al.,* 2000), and for this reason it was not further analysed.

Glycerol accumulation studies in EUROSCARF strains

To establish the possible relation between the defective growth on glycerol-based media and glycerol uptake, the mentioned strains, as well as the parental strain, were tested for [^{14}C] glycerol accumulation. As it was shown before that active glycerol uptake is done *via* a proton symport inducible by growth on non-fermentable carbon sources (Lages and Lucas, 1997; Sutherland *et al.,* 1997) the experiments were done in cells growing exponentially on ethanol.

All the strains presented glycerol accumulation against gradient, prevented by the presence of the protonophore/ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) as expected in the case of a proton motive force-dependent active uptake mechanism (Fig. V1). Maximum accumulation rates of the mutant strains defective in the ORFs above mentioned, were not significantly different from the wild type strain, except for YLR081w, which present a maximum accumulation ratio more than two times the wild type (Fig. V2).

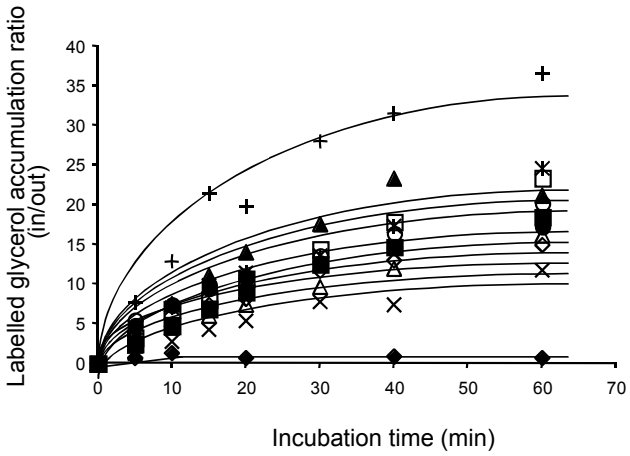


Fig. V1. [^{14}C] glycerol accumulation ratios at pH5 and 30ºC of: BY4741(●); YBL089W(▲); YBR132c(■); YDL054(❍); YGL104(❒); YGL114W(□); YGL139(□); YGL140c(✶); YLR081W(+); YMR011(x). Accumulation is prevented by the presence of 50mM CCCP in the assay buffer (◆).

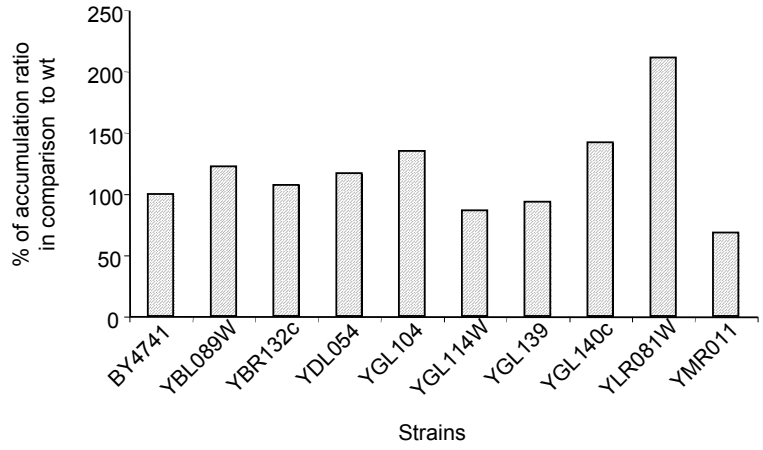


Fig. V2. Percentage of accumulation ratios of EUROSCARF mutants strains in relation to the correspondent wt strain.

Discussion

The strategy for using mTn-*lacZ/LEU2*-mutagenized plasmid library to create mutants by integration in homologous regions of the genome in a *fps1* strain was based on the assumption that lack of growth on glycerol would enable the identification of the gene corresponding to glycerol active transport or other glycerol transport-related genes in *S. cerevisiae*. The difficulties we faced in the transformation of that strain can presumably be related to differences in the plasma membrane composition in relation to wild type (Sutherland *et al.*, 1997).

The EUROSCARF library screen yielded several putatively interesting clones, with non-growth phenotype on glycerol and supposedly not respiratory deficient due to the normal growth on ethanol they presented. From these a sub-set of nine ORFs were used for transport assays. All displayed accumulation ratios above equilibrium indicative of the activity of glycerol transporter.

The non-growth phenotypes on glycerol from these mutants are apparently not corroborated by data available in literature. The strains deleted in the ORFs YBR132c, YDL054c, YGL114w, YLR081w and YMR011w, in opposition to the results here obtained, have been described as being able to grow normally on glycerol-based media (van Roermund *et al.,* 1999; Makuc *et al.,* 2001 Pearson and Schweizer, 2002; Wieczorke *et al.,* 1999), while a strain deleted in the ORF YBL089w was shown to exhibit a growth defect on non-fermentable carbon sources as ethanol (Russnak *et al.,* 2001). Different results could be explained by different genetic backgrounds of the strains used, as glycerol utilization by *S. cerevisiae* has been described as ~~a~~ strain dependent (Barnett *et al.*, 1990).

The ORF YBL089w, corresponding to *AVT5* (Amino acid Vacuolar Transport) has been considered based on sequence similarity, as a putative transporter, member of a family of seven *S. cerevisiae* genes (*AVT1-7*) related to vesicular GABA-glycine transporters. Yet, no amino acid transport function could be attributed to the correspondent protein (Russnak *et al.,* 2001). The lack of growth on glycerol medium observed by us could be due to the growth defect on a non-fermentable (respiratory) carbon source referred for this mutant deleted in this ORF but in a different genetic background (Russnak *et al.,* 2001). Yet, a mutant deleted in this ORF showed a good growth on ethanol, which is not in accordance with a respiratory growth defect.

The ORF YBR132c, named *AGP2* (high-Affinity Glutamine Permease) was included in the family of aminoacid permeases by sequence similarity (André, 1995; Nelissen *et al.,* 1997). It encodes a plasma membrane carnitine carrier, down-regulated by osmotic stress through the Hog1 pathway and it seems to be linked to fatty acid oxidation (van Roermund *et al.,* 1999; Lee *et al.,* 2002). Agp2p also functions as a low-affinity amino acid permease on poor nitrogen sources (Schreve and Garrett, 2004). It is more highly expressed in cells in which the major permeases are deleted and present a broad substrate range (Schreve and Garrett, 2004). This data could point to a broad range in this gene functionality and could explain somehow its relation to glycerol uptake, namely through its relation to the Hog1 pathway. Also, apparently, it functions as a carnitine-H^+ symporter repressed by glucose and derepressed by glycerol (van Roermund *et al.,* 1999), according with what has been described for the *S. cerevisiae* glycerol/H^+ symporter (Lages and Lucas, 1997). Nevertheless, the mutant strain in *S. cerevisiae* BJ1991 showed no growth defect on glycerol (van Roermund *et al.,* 1999), what contradicts the presented results.

YDL054c, *MCH1*, encodes for a putative monocarboxilate permease homologue (André, 1995), but does not show any involvement in the uptake or secretion of monocarboxilates such as lactate, pyruvate or acetate (Makuc *et al.,* 2001). It showed no growth defects on glycerol or ethanol batch cultures compared to wild type (CEN.PK113-13D) and its transcription is repressed by lactate but not by glucose (Makuc *et al.,* 2001). Once again our results seem to be contradicted. Nevertheless, growth in aerobic glucose-limited chemostat cultures show a reduction in biomass yield in the *mch1-5* mutant strain (Makuc *et al.,* 2001) and so its involvement in glycerol utilization can not be discarded.

YGL104c, *VPS73* (Vacuolar Protein Sorting) encodes a protein involved only in the carboxypeptidase Y pathway (CPY). Null mutant secretes CPY. This is

dependent on the genetic background of the parental strains (Bonangelino *et al.,* 2002). A function in cell wall biosynthesis was also predicted (Samanta and Liang, 2003).

In what concerns YGL114w, an ORF postulated to be a member of the oligopeptide transporter (OPT) family of membrane transporters, on the basis of sequence similarity (Paulsen *et al.,* 1998), the disruptants are viable and show no growth defects on glucose or glycerol at 15, 30 and 37ºC compared to wt strain (CENPK2) (Pearson and Schweizer, 2002). So, we have no clues for its involvement in glycerol utilization. The same is true for YGL139w and YGL140c, which have no significant homology to any protein sequence available in the public databases (Voet *et al.,* 1997).

YMR011w, *HXT2*, encodes for a high affinity glucose transporter (Kruckeberg *et al.,* 1999; Maier *et al.,* 2002). Hxt2p is induced by low glucose concentrations and it is removed from the membrane by endocytosis in the presence of high glucose concentrations (Wieczorke *et al.,* 1999). This is in accordance with what probably happens to the active glycerol transporter, but this is in fact not the case since the mutant deleted in this ORF presented glycerol accumulation capacity similar to the wild type strain.

The ORF YLR081w corresponds to the *GAL2* gene, which product, a galactose permease, belongs to the HXT transporters family. This protein is required for utilization of galactose, but is also able to transport glucose. Previous studies point to an involvement of *GAL2* transporter in glucose permeation mechanisms, as it was observed that in the *hxt1-7* null mutant there was no significant permeation of glucose, but after *GAL2* induction there was, apart from the existence of facilitated-diffusion transport of glucose, a significant first order kinetics glucose entry (Maier *et al.,* 2002). This points directly to an involvement of Gal2p in both permeation mechanisms. It could also be involved in glycerol permeation mechanisms, acting as a negative regulator, and that could explain the high glycerol accumulation capacity presented by the correspondent deleted strain.

Summarizing, differences found in glycerol accumulation capacity of some of these strains may be related to an indirect involvement of the correspondent genes in the uptake, in a similar way to what happens with *GUP1* and *GUP2* (Neves *et al.,* 2004a,b, Oliveira and Lucas, 2004). Yet, the definition of the roles of those genes, in particular the most promising case of *GAL2*, in glycerol consumption/transport, if any, requires further studies.

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Final remarks and future perspectives

Until the present work a model system for glycerol transport was established for *S. cerevisiae*, which mainly involved a glycerol channel, encoded by *FPS1* (Luyten *et al*; 1995; Sutherland *et al.*, 1999), which is responsible for glycerol retention/release in osmotic stressed conditions (Tamás *et al*., 1999) and for the so-called passive diffusion of glycerol (Oliveira *et al.,* 2003), and a glycerol/H^+ symport system responsible for active glycerol uptake (Lages and Lucas, 1997). This system was described as being repressed by glucose and was related with the expression of the genes *GUP1* and *GUP2* (Holst *et al.,* 2000). In other yeasts active glycerol uptake systems had also been described (Lages and Lucas, 1995; Lages *et al.,* 1999; Silva-Graça and Lucas, 2003), but the genes involved are unknown.

GUP1/2 and *FPS1* orthologues cloned in this work were used to enlarge the correspondent gene families (Bateman *et al.,* 2002; Park and Saier, 1996) and were able to complement *S. cerevisiae* mutant phenotypes. This does not demonstrate that these genes, once in their native genetic environment correspond to identical molecular/physiological response to the same stimulus, but suggests a function close to the one described for their homologues in *S. cerevisiae*. Search for Fps1-like yeast proteins in databases show that some species like *C. albicans* and *Schz. pombe* do not present *FPS1* homologues. Yet, they are still able to rapidly release glycerol and recover from hypoosmotic shock (Kayingo *et al.,* 2004, Chapter IV). So, it is likely that in some yeasts, the roles of the Fps1p are performed by a structurally different protein. Phylogenetic analysis of Gup-like proteins belonging to different organisms clearly indicated their preferential allocation in the MBOAT-family (Hofmann, 2000; Chapter II, Neves *et al.,* 2004b), as well as its presence in yeasts, which do not present glycerol active uptake, like *Schz. pombe* (Lages *et al.,* 1999).

Glycerol uptake experiments in *S. cerevisiae,* in physiological conditions different from the ones previously described (Lages and Lucas, 1997; Holst *et al.,* 2000), indicate that the active glycerol/proton uptake system besides being induced by non-fermentable carbon sources (Lages and Lucas, 1997) is also induced by salt stress and is independent of Gup1 and Gup2 proteins (Chapter III, Neves *et al.,* 2004a).

C. albicans is able to utilize glycerol as a sole carbon source but nothing was known about the way glycerol is transported. Furthermore, glycerol plays a yet not fully understood but important role on this yeast pathogenesis. In this work characterization of glycerol transport in a wild type strain was done. The presence of a specific constitutively expressed proton/glycerol symport was evidenced. As *C.*

albicans genome presents only a *GUP1* homologue, a deleted strain in this gene was constructed and tested for glycerol active uptake. This mutant strain presented the same kind of glycerol transport of the wild type. So, Gup1p is not responsible for it (Chapter IV).

All the results mentioned so far suggest that, despite the involvement of Gup1p and Gup2p in glycerol uptake, these are not the actual glycerol transporters. Consequently, the glycerol active transport observed in yeasts should be performed by other(s) protein(s).

With this in mind, strategies for the identification of new glycerol transport related genes were delineated. These led to the identification of several glycerol-related ORFs, none of which, though, corresponded to a glycerol transporter protein. The disclosure of that gene will be an essential step to further study the relation between glycerol transport and metabolism, as well as its regulation, both at molecular and physiological levels. As soon as the gene encoding the active uptake system for glycerol is cloned and available the correspondent yeast orthologues will be searched in databases and cloned. On the other hand, the search for yeast orthologues of *GUP1, GUP2* and *FPS1* involved in glycerol uptake, has become easier with the work developed and phylogenetic analysis can proceed. We cannot disregard the pertinence of including in the future work the identification and characterization of the nature and roles of *GUP1/2*, Gup1/2p.

Besides, the regulation of these genes can be assessed in order to keep characterizing molecularly in *S. cerevisiae* the regulation processes underlying glycerol metabolism and transport/retention in connection with the vital cell responses it relates to.

We are specially interested in cloning and analyse genes, as well as their promoter regions, from yeasts much more resistant to salt stress than *S. cerevisiae*, and in which glycerol transport systems were physiologically characterized like *C. albicans* (Chapter IV), *D. hansenii* (Lucas *et al.,* 1990), *P. sorbitophila* (Lages and Lucas, 1995), *Z. rouxii* (van Zyl *et al.,* 1990) and *C. halophila* (Silva-Graça and Lucas, 2003). All these yeasts present constitutive glycerol active transport systems differently regulated. It is expected that similarly expressed genes display identical regulatory sequences at the promoter regions. Hence, genes constitutively expressed, repressible by glucose and inducible by osmotic stress will be identified or, at least, an indication of the mechanisms of transcriptional regulation will be available.

This will contribute to uncover the regulation of expression of those genes and help to understand how genes involved in glycerol transport have evolved and the

relation of this evolution with the adaptation to particular environments, namely, to hyper-osmotic habitats. In the case of the human pathogenic yeast *C. albicans* the understanding of the way glycerol transport and metabolism is regulated could be of great help in diagnostic and therapeutic processes since glycerol is involved in its infectious capacity (San José *et al*., 1996, Alonso-Monge *et al*., 1999, 2003). Also, with this knowledge at hand, construction of transgenic yeasts strains with improved capacity of glycerol uptake and able to grow on hyperosmotic media will be possible, and that could be an impact in several biotechnological processes.

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