Yeast orthologues associated with glycerol transport and metabolism

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

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. All the genes hereby cloned presented a high degree of similarity to *S. cerevisiae*'s and were able to complement the correspondent mutant phenotypes. A phylogenetic analysis is presented. The allocation of *GUP* genes in the MBOAT family is suggested as more consistent than the inclusion in the TC-DB/Glycerol Uptake family.

Introduction

Glycerol plays a central role in yeast metabolism. Besides acting as compatible solute under low a_w stress environments [1,2,3], glycerol is involved in different metabolic pathways. Its production relates with glycolytic flux [4,5,6,7,8,9,10] and is involved in lipid metabolism [11,12,13] as well as in the regulation of inorganic phosphate turnover [14,15]. 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* [16] and *FPS1* [15,17,18]. 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 [19,20,21,22]. But, despite all the similarities in what concerns glycerol uptake and metabolism in yeasts [22], it is also known that different yeasts deal differently in what concerns their needs for glycerol [3,23].

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 from glycerol production pathway in *S. cerevisiae* [24,25,26] and *GUT1,* encoding for glycerol kinase, the first enzyme from glycerol assimilation pathway also from *S. cerevisiae* [27,28]. Another metabolic pathway has been described before in *Schizosaccharomyces pombe* [29], from which genes encoding for similar enzymatic steps have been shown to exist also in *S. cerevisiae* [30]. Nevertheless, the metabolic role and importance of this pathway is still not understood [31].

We began by using the partial sequencing data available in the Génolévures Program [32], for a series of hemiascomycetous yeast species with biomedical or industrial interest [33], *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* presenting full genome sequence available.

Genomic sequences retrieved showed a high degree of similarity to *S. cerevisiae*'s 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* IGC3886, *K. thermotolerans* CBS137, *P. angusta* IGC 4129, *P. farinosa* (syn. *P. sorbitophila*) CBS 7064, *Z. rouxii* CBS 732, *C. versatilis (*syn. *C. halophila*) CBS 4019 and *Debaryomyces hansenii* CCMI 1941 [34]. IGC is synonym of PYCC (Portuguese Yeast Culture Collection), a CBS associated microorganism collection. CCMI stands for Collection of Industrial Microrganisms from the INETI (Instituto Nacional de Engenharia, Tecnologia e Inovação (Portugal)).

P. sorbitophila was recently renamed as *P. farinosa* [35,36]. Nevertheless, it is known from previous work [22] that the type strain of *P. farinosa* (IGC 2459) presents different phenotypes 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, *MAT*a *leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100* [37], 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* [16].

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*) [16]; 1,4 M NaCl (for *GPD1*/*2*) [25] or replacing glucose for glycerol as carbon and energy source in a *gut*-diagnostic media (for *GUT1*) [38]. For *FPS1* phenotypic analysis, cells were grown on YPD with1M sorbitol and then transferred to YPD plates (hypoosmotic shock) [17]. 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 [39]. Plasmids were amplified from one single colony of *Escherichia coli* transformants, by growth in LB supplemented with 100 μg/ml ampicillin (LBamp) and isolated by the alkaline lysis method [39,40]. The concentration and purity of the acid nucleic 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 DH5α [F- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17(r_k^- m_k^+) *deo*R *thi*-1 *pho*A *sup*E44 λ-*gyr*A96 *rel*A1] was used for plasmid maintenance and amplification of isolated clones. Bacteria 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 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 [40]. Each transformation was done with 100 ng of plasmid DNA and 100 μl of competent cells prepared according to Inoue and co-workers [41]. Selection of transformants was done by plating cells on agar plates containing LBamp with X-gal/IPTG.

S. cerevisiae cells were transformed with the lithium acetate method [39]. 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 [42] 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) [43,44], using FirstChoicet™ RLM-RACE (Ambion) according to the manufacturer's instructions. Sequencing was done by Genómica STAB Vida, Lda (Portugal).

Following, PCR reactions were designed to retrieve the genomic complete 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 94ºC, 35 cycles: 30 sec at 94ºC, 30 sec at the corresponding annealing temperature (55 to 62ºC), 30 sec at 72ºC, and finally 7 min at 72ºC. The specific primers used are listed in Table 1. 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 [45]. 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).

Southern-blot analysis

Southern blot was done by standard procedures [39] using *S. cerevisiae, C. halophila, P. sorbitophila, D. hansenii, Z. rouxii, C. albicans, C. tropicalis, K. marxianus, P. angusta, K. lactis and K. thermotolerans* 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™AlKPhos Direct™ 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: *GPD*f - 5'CAAAACGTGAAATACTTGCC3'; *GPD*r - 5'AGCAACAACGTTCTTCAAAGCAC3'; *GUT1*f - 5'CCTACAGTCTTGCCCTCCAC3'; *GUT1*r - 5´AACATTCCCGCAACACTTTC3´; *GUP1*f - 5'CGTGGTTCCCTTGATGTTCT3'; *GUP1*r - 5'TCGTTCCTTTTCCTCCAGAA3'; *FPS1*f - 5'CCTACAGTCTTGCCCTCCAC3'; *FPS1*r - 5'AACATTCCCGCAACACTTTC3'.

Genomic sequences 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 [46,47] and Multalin [48] 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 [49,50] and TMPRED [51] transmembrane helices prediction programs.

Results

Sequences retrieval

The complete sequences of *K. thermotolerans* and *P. angusta GPD*-like ORF*s*, *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 [52]. 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. 1) 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 [25,53]. 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 [24,54], while Gpd2p is under redox control [5,55]. 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 [5,55, 56].

Complementation analysis for *GUT1*-like sequence was done using a *gut*-diagnostic medium [38], 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. 2).

As *S. cerevisiae GUP1* presented a salt-stress associated phenotype [16], 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. 3). 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.

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 [17]. All the putative orthologues could restore partially the wild type capability to recover from hypoosmotic shock (Fig. 4).

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. 1). 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).

Southern blot analysis

In order to complete this study, including all the chosen genes (*GPD1/2, GUT1, GUP1/2* and *FPS1*) from all the yeasts mentioned above, we proceeded with a different methodology to find the sequences which were not available, not even partially, in the data-

bases. Furthermore, we included in the series of yeasts to use, *D. hansenii* and *C. halophila*. These are very halo/osmotolerant yeasts with known specific features in what concerns glycerol metabolism and uptake [23,57,58,59]. *C. halophila* has never been subjected to a genetic approach or sequencing program and *D. hansenii* genome database from Génolévures Program is not yet completely available.

Probes were designed for all the genes mentioned. In the case of the *GPD1/2* it was chosen a highly conserved region in what concerns the alignment of eight sequences encoding glycerol 3-phosphate dehydrogenases from very different organisms (not shown). In what concerns the other genes probes, identical procedure was not possible due to the inexistence in the literature or in databases, at the time this work begun, of enough sequences from similar genes.

Southern Blot was made using two sets of genomic DNA, one partially digested with *Sau*3A and another digested with *Eco*RI. Controls were made using *S. cerevisiae* gDNA. All probes gave a good signal in *S. cerevisiae* as expected (not shown). *GPD1* probe was positive in all strains. *FPS1* probe gave a positive signal in *Z. rouxii, K. marxianus* and *K. lactis*, as expected by the results above presented, 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 any detectable signal in any strain, at any of the hybridization temperatures used, maybe 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.

From the positive results, we chose *C. halophila GPD* putative sequence to follow. The reason for this choice lays in that glycerol 3-phosphate dehydrogenase from *C. halophila* has been reported as being able to utilize both NADH and NADPH as co-factors [59]. Two parallel gels were run, one of which was used for Southern hybridization. Fragments of digested gDNA correspondent to the hybridization results 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 phenotype, nevertheless the attempts to sequence these were unfruitful,

apparently due to plasmid instability in *E. coli*. This has been reported before for *P. sorbitophila,* another halotolerant yeast also used in this work [60].

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 (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 (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 *Saccharomyces 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 *ScGUPp2*. *CaGUP1p* is more similar to *ScGUP1* (65.1%) than to *ScGUP2* (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 [61,62,63,64,65]. 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 to amino acid sequence [65,66]. Despite the different number of predicted TMM, *PsGUP*1 complemented the *gup1* mutant phenotype of *S. cerevisiae* (Fig. 3). 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 [14,15,17,67]. 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 [68] through the analysis method of one thousand replicates, gave the confidence values for the groups included in the consensus tree thus obtained (Fig. 5). The trees obtained for the deduced amino acid sequences of those genes confirm the phylogenetic positions of those yeasts determined by 18S and 25S rRNA sequence comparison [33] and reflect the identity/similarity relationships above mentioned.

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 [26,69,70,71] 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* [72,73]. 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 [74]. Also, in spite of the importance of Fps1p in yeast physiology and metabolism [14,15,17,18,75,76,77], 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* [78] 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 [79] and that *Schz. pombe* takes up glycerol precisely in such a fashion [22], 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 [14,71]. 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 [80,81]. 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 [67,71,82,83,84]. 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 - MSF - Family number 7 of proteins of unknown function [85]. Later, due to the homology these proteins present to several acyltransferases, they were included in the MBOAT superfamily (Membrane-Bound *O*-Acyl Transferases) [70]. 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 *gup1* strain [86]. *GUP1* and *GUP2* were further shown to present sequence similarity to DGAT (diacylglycerol *O*-acyltransferase), a mammalian acyltransferase, which belongs to the ACAT (acyl-coA cholesterol acyltransferase) gene family, which also includes yeast *ARE1* and *ARE2 (*ACAT Related Enzymes). These two isoenzymes catalyze intracellular sterol esterification [86]. More recently, in the COG (Cluster of Orthologous Groups of Proteins) database at NCBI, which includes genomes of prokaryotes and unicellular eukaryotes, *GUP1* and *GUP2* were included in a cluster of bacterial predicted membrane proteins, COG1696, involved in the incorporation of D-alanin into lipoteichoic acids (DltB) [87], which are also members of the MBOAT superfamily. According to the recently released KOG (Eukaryotic Orthologous Groups) database [88], *GUP1* and *GUP2* 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 [16], 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 [16,21,22,89]. 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 [22], corroborating the extremely poor growth this yeast presents on glycerol-based media.

GUP1 and *GUP2* inclusion in MSF family was duly done according to the unquestionable transmembrane nature of the correspondent proteins [85]. On the other hand, the inclusion of these two genes 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 [70], in relation to an extensively represented family of proteins with well established function, corroborated by the lipid and *ts* phenotypes [86]. In opposition, the inclusion of *GUP1* and *GUP2* in the Glycerol Uptake (GUP) family is based exclusively on data concerning glycerol uptake related phenotypes [16]. Nevertheless, recently it has been published that *GUP1* and *GUP2* expression does not match glycerol transport regulation [53]. 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 [86] could mean that the function of *GUP1* and *GUP2* could be connected to cell membrane and/or wall

modifications and thus influence glycerol transport indirectly. 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. 6). Gup1p/Gup2p-like sequences are clustered together, but not distant from other clusters. In spite of the high degree of similarity between *GUP1* and *GUP2* [16], Gup2p-like sequences group together and so do Gup1p-like sequences (Fig. 6). Clustering may reflect some functional differences between proteins, but it can also be due to the fact that phylogenetic closeness of proteins is largely determined according to the *phylum* of the organisms of origin [33]. Thus, since all the Gupp included in this tree belong to yeasts, it increases the probability of them clustering together. 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 function must be undertaken.

Acknowledgements

We thank the laboratories of C. Gaillardin, S. Casarégola and J-L Souciet for providing the clones of yeast gDNA which were partially sequenced by the Génolevures Project. We also thank our colleague A. Correia for helping in the design of the phylogenetic trees, as well as G. Kayingo, from Yale University, for kindly revising the part of the text referring to *FPS1*.

Supplementary data available in the journal page: http://www…….

References

[1] Blomberg, A. and Adler, L. (1989) Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD^+) in acquired osmotolerance of *Saccharomyces cerevisiae*. J. Bacteriol. 171, 1087-92

[2] Tokuoka, K. and Ishitani, T. (1991) Minimum water activities for the growth of yeasts isolated from high-sugar foods. J. Gen. Appl. Microbiol. 37, 111-9

[3] van Eck, J.H., Prior, B.A. and Brandt, E.V. (1993) The water relations of growth and polyhydroxyalcohol production by ascomicatous yeasts. J. Gen. Microbiol. 139, 1047-54

[4] van Dijken, J.P. and Scheffers, W.A. (1986) Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol. Rev. 32, 199-224

[5] Björkqvist, S., Ansell, R., Adler, L. and Lidén, G. (1997) Physiological response to anaerobicity of glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 63, 128-32

[6] Larsson, C., Påhlman, I.-L., Ansell, R., Rigoulet, M., Adler, A. and Gustafsson, L. (1998) The importance of the glycerol 3-phosphate shuttle during aerobic growth of *Saccharomyces cerevisiae*. Yeast 14, 347-57

[7] Overkamp, K.M., Bakker, B.M., Kotter, P., van Tuijl, A., de Vries, S., van Dijken, J.P. and Pronk, J.T. (2000) *In vivo* analysis of the mechanisms for oxidation of cytosolic NADH by *Saccharomyces cerevisiae* mitochondria. J. Bacteriol. 182, 2823-30

[8] Blomberg, A. (2000) Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. FEMS Microbiol. Lett. 182, 1-8

[9] Påhlman, A.K., Granath, K., Ansell, R., Hohmann, S., Adler, L. (2001) The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress. J. Biol. Chem. 276, 3555-63

[10] Påhlman, I.L., Larsson, C., Averét, N., Bunoust, O., Boubekeur, S., Gustafsson, L. and Rigoulet, M. (2002) Kinetic regulation of the mitochondrial glycerol-3-phosphate

dehydrogenase by the external NADH dehydrogenase in *Saccharomyces cerevisiae*. J. Biol. Chem. 277, 27991-5

[11] Christiansen, K. (1978) Triacylglycerol synthesis in lipid particles from baker's yeast (*Saccharomyces cerevisiae*). Biochim. Biophys. Acta 530, 78-90

[12] Racenis, P.V., Lai, J.L., Das, A.K., Mullick, P.C., Hajra, A.K. and Greenberg, M.L. (1992) The acyl dihydroxyacetone phosphate pathway enzymes for glycerolipid biosynthesis are present in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 174, 5702-10

[13] Grauslund, M. and Rønnow, B. (2000) Carbon source-dependent transcriptional regulation of the mitochondrial glycerol-3-phosphate dehydrogenase, *GUT2*, from *Saccharomyces cerevisiae*. Can. J. Microbiol. 46, 1096-100

[14] van Aelst, L., Hohmann, S., Zimmermann, F.K., Jans, A.W.H. and Thevelein, J.M. (1991) A yeast homologue of the bovine lens fibre MIP gene family complements the growth defect of a *Saccharomyces cerevisiae* mutant on fermentable sugars but not its defect in glucose-induced RAS-mediated cAMP signalling. EMBO J. 10, 2095-104

[15] Luyten, K., Albertyn, J., Skibbe, W.F., Prior, B.A., Ramos, J., Thevelein, J.M. and Hohmann, S. (1995) Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. EMBO J. 14, 1360-71

[16] Holst, B., Lunde, C., Lages, F., Oliveira, R., Lucas, C. and Kielland-Brandt, M. (2000) *GUP1* and its close homologue *GUP2*, encoding multimembrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisiae*. Mol. Microbiol. 37, 108-124

[17] Tamás, M.J., Luyten, K., Sutherland, F.C.W., Hernandez, A., Albertyn, J., Valadi, H., Li, H., Prior, B.A., Kilian, S.G., Ramos, J., Gustafsson, L., Thevelein, J.M. and Hohmann, S. (1999) Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. Mol. Microbiol. 31, 1087-104

[18] Tamás, M.J., Rep, M., Thevelein, J.M. and Hohmann, S. (2000) Stimulation of the yeast high osmolarity glycerol (HOG) pathway: evidence for a signal generated by a change in turgor rather than by water stress. FEBS Lett. 472, 159-65

[19] Reed, R.H., Chudek, J.A., Foster, R., Gadd, G.M. (1987) Osmotic significance of glycerol accumulation in exponentially growing yeasts. Appl. Environ. Microbiol. 53, 2119-23

[20] Yagi, T. (1992) Regulation of intracellular osmotic pressure during the initial stages of salt stress in a salt-tolerant yeast *Zygosaccharomyces rouxii*. Microbios. 70(283), 93-102

[21] Lages, F. and Lucas, C. (1997) Physiological characterization of mediated glycerol uptake in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. 1322, 8-18

[22] Lages, F., Silva-Graça, M. and Lucas, C. (1999) Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species. Microbiol. 145, 2577-85

[23] Silva-Graça, M. and Lucas, C. (2003) Physiological studies on long-term adaptation to salt stress in the extremely halotolerant yeast *Candida versatilis* CBS 4019 (syn. *C. halophila*) FEMS Yeast Res. 3, 247-60

[24] Larsson, K., Ansell, R., Eriksson, P. and Adler, L. (1993) A gene encoding *sn*-glycerol 3-phosphate dehydrogenase (NAD^+) complements an osmosensitive mutant of *Saccharomyces cerevisiae*. Mol. Microbiol. 10, 1101-11

[25] Albertyn, J., Hohmann, S., Thevelein, J.M. and Prior, B.A. (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol. 14, 4135-44

[26] Eriksson, P., André, L., Ansell, R., Blomberg, A. and Adler, L. (1995) Cloning and characterization of *GPD2*, a second gene encoding *sn*-glycerol 3-phosphate dehydrogenase (NAD^+) in *Saccharomyces cerevisiae* and its comparison with *GPD1*. Mol. Microbiol. 17, 95-107

[27] Sprague, G.F. and Cronan, G,R, (1977) Isolation and characterisation of *Saccharomyces cerevisiae* mutants defective in glycerol catabolism. J. Bacteriol. 129, 1335-42

[28] Pavlik, P., Simon, M., Schuster, T. and Ruis, H. (1993) The glycerol kinase (*GUT1*) gene of *Saccharomyces cerevisiae*: cloning and characterization. Curr. Genet. 24, 21-5

[29] May, J.W., Marshall, J.H. and Sloan, J. (1982) Glycerol utilization by *Schizosaccharomyces pombe*: phosphorylation of dihydroxyacetone by a specific kinase as the second step. J. Gen. Microbiol. 128, 1763-1766

[30] Norbeck, J. and Blomberg, A. (1997) Metabolic and regulatory changes associated with growth of *Saccharomyces cerervisiae* in 1.4 M NaCl. Evidence for osmotic induction of glycerol dissimilation via dihydroxyacetone pathway. J. Biol. Chem. 272, 5544-5554

[31] Molin, M., Norbeck. J. and Blomberg, A. (2003) Dihydroxyacetone kinases in *Saccharomyces cerevisiae* are involved in detoxification of dihydroxyacetone. J. Biol. Chem. 278, 1415-23

[32] Feldmann, H. (2000) Génolevures – a novel approach to ‘evolutionary genomics’. FEBS Lett. 487, 1-2

[33] Souciet, J., Aigle, M., Artiguenave, F., Blandin, G., Bolotin-Fukuhara, M., Bon, E., Brottier, P., Casarégola, S., Montigny, J., Dujon, B., Durrens, P., Gaillardin, C., Lépingle, A., Llorente, B., Malpertuy, A., Neuvéglise, C., Ozier-Kalogeropoulos, O., Potier, S., Saurin, W., Tekaia, F., Toffano-Nioche, C., Wésolowski-Louvel., M., Winker, P. and Weissenbach, J. (2000) Genomic exploration of the hemiascomycetous Yeasts: 1. A set of yeast species for molecular evolution studies. FEBS Lett. 487, 3-12

[34] Amaral-Collaço, M.T., Gírio, F.M. and Peito, M.A. (1989) Utilization of a hemicellulosic fraction of agroindustrial residues by yeasts. In: Coughland MP (Ed) Enzyme systems for lignocellulosic degradation. Elsevier Applied Science, London, pp 221-230

[35] Barnett, J.A., Payne, R.W. and Yarrow, D. (Eds) (2000) Yeasts: Characteristics and Identification, 3th edn. Cambridge University Press, UK

[36] Kurtzman, C.P. and Fell, J.W. (Eds) (1998) The Yeasts, A Taxonomic Study, 4th ed. Elsevier, Amsterdam

[37] Thomas, B.J and Rothstein, R. (1989) Elevated recombination rates in transcriptionally active DNA. Cell 56, 619-30

[38] Rønnow, B. and Kielland-Brandt, M.C. (1993) *GUT2*, a gene for mitochondrial glycerol 3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. Yeast 9, 1121-30

[39] Ausubel, F.M., Brent, R., Kingston, R.E., Moor, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds) (1996) Current Protocols in Molecular Biology, Vols. 1-3, John Wiley & Sons, Inc., New York

[40] Sambrook, J., Fritsch, E.F. and Maniatis, T. (Eds) (1989) Molecular Cloning: A Laboratory Manual, 2nd ed, Vols. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

[41] Inoue, H., Nogima, H. and Okayama, H. (1990) High efficiency transformation of *Escherichia coli* with plasmids. Gene 96, 23-28

[42] Altschull, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search program. Nucleic Acids. Res. 25, 3389-402

[43] Maruyama, K., Sugano, S. (1994) Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNA with oligo ribonucleotides. Gene 138, 171-4

[44] Schaefer, B.C. (1995) Revolutions in rapid amplification of cDNA ends: new strategies for polymerase chain reaction cloning of full-lenght cDNA ends. Anal. Biochem. 227, 255-73

[45] Mumberg, D., Müller, R. and Funk, M, (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156, 119-122

[46] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673-4680

[47] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The clustalX-windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25, 4876-4882

[48] Corpet, F. (1998) Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res. 16, 10881-90

[49] Sonnhammer, E.L.L., von Heijne, G. and Krogh, A. (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. In: J. Glasgow, T. Littlejohn,

F. Major, R. Lathrop, D. Sankoff, and C. Sensen (eds) Proceedings of the Sixth International Conference on intelligent Systems for Molecular Biology, Menlo Park CA. AAAI Press, pp 175-182

[50] Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E.L.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305, 567-580

[51] Hofmann, K. and Stoffel, W. (1993) TMbase - A database of membrane spanning proteins segments. Biol. Chem. Hoppe-Seyler 374, 166

[52] LangkjÆr, R.B., Cliften, P.F., Johnston, M. and Piskur, J. (2003) Yeast genome duplication was followed by asynchronous differentiation of duplicated genes. Nature 421, 848-52

[53] Oliveira, R. (2003) Molecular and physiological approaches towards the characterization of glycerol transport in *Saccharomyces cerevisiae*. PhD Thesis, Minho Univ., Portugal

[54] André, L., Hemming, A. and Adler, L. (1991) Osmoregulation in *Saccharomyces cerevisiae*. Studies on the osmotic induction of glycerol production and glycerol-3-phosphate dehydrogenase (NAD^+). FEBS Lett. 286, 13-17

[55] Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M. and Adler, L. (1997) The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J. 16, 2179-87

[56] Remize, F., Barnavon, L. and Dequin, S. (2001) Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in *Saccharomyces cerevisiae*. Metabol. Eng. 3, 301-12

[57] Lucas, C., da Costa, M. and van Uden, N. (1990) Osmoregulatory active sodium-glycerol co-transport in the halotolerant yeast *Debaryomyces hansenii*. Yeast 6, 187-91

[58] Neves, L., Oliveira, R. and Lucas, C. (1997) Metabolic flux response to salt-induced stress in the halotolerant yeast *Debaryomyces hansenii*. Microbiol. 143, 1133-1139

[59] Silva-Graça, M., Neves, L. and Lucas, C. (2003) Outlines for the definition of halotolerance/halophily in yeasts: yeast *Candida versatilis* (*halophila*) CBS 4019 as the archetype? FEMS Yeast Res. 3, 347-62

[60] Montigny, J., Spehner, C., Souciet, J., Tekaia, F., Dujon, B., Wincker, P., Artiguenave, F. and Potier, S. (2000) Genomic exploration of the hemiascomycetous yeasts: 15. *Pichia sorbitophila*. FEBS Lett. 487, 52-55

[61] Ohama, T., Suzuki, T., Mori, M., Osawa, S., Ueda, T., Watanabe, K. and Nakase, T. (1993) Non-universal decoding of the leucine codon CUG in several *Candida* species. Nucleic Acids Res. 21, 4039-45

[62] Santos, M. and Tuite, M. (1995) The CUG codon is decoded in vivo as serine and not leucine in *Candida albicans*. Nucleic Acids Res. 23, 1481-6

[63] Sugita, T. and Nakase, T. (1999) Non-universal usage of the leucine CUG codon and the molecular phylogeny of the genus *Candida*. Syst. Appl. Microbiol. 22, 79-86

[64] Suzuki, C., Kashiwagi, T. and Hirayama, K. (2002) Alternative CUG codon usage (Ser for Leu) in *Pichia farinosa* and the effect of a mutated killer gene in *Saccharomyces cerevisiae*. Protein Eng. 15, 251-5

[65] Tekaia, F., Blandin, G., Malpertuy, A., Llorente, B., Durrens, P., Toffano-Nioche, C., Ozier-Kalogeropoulos, O., Bon, E., Gaillardin, C., Aigle, M., Bolotin-Fukuhara, M., Casarégola, S., Montigny, J., Lépingle, A., Neuvéglise, C., Potier, S., Souciet, J., Wésolowski-Louvel, M. and Dujon, B. (2000) Genomic exploration of the hemiascomycetous yeasts: 3. Methods and strategies used for sequence analysis and annotation. FEBS Lett. 487, 17-30

[66] Blandin, G., Llorente, B., Malpertuy, A., Wincker, P., Artiguenave, F. and Dujon, B. (2000) Genomic exploration of the hemyascomycetous. Yeasts: 13. *Pichia angusta*. FEBS Lett. 487, 76-81

[67] Bill, R.M., Hedfalk, K., Karlgren, S,, Mullins, J.G.L., Rydström, J. and Hohmann, S. (2001) Analysis of the pore of the unusual major intrinsic protein channel, yeast Fps1p. J. Biol. Chem. 276, 36543-49

[68] Felsenstein, J. (1985) Confidence limits of phylogenies: an approach using the bootstrap. Evol. 39, 783-91

[69] Bateman, A., Birney, E., Cerruti, L., Durbon, R., Etwiller, L., Eddy, S.R., Griffiths-Jones, S., Howe, K.L., Marshall, M. and Sonnhammer, E.L.L. (2002) The Pfam Protein Families Database. Nucl. Acids Res. 30, 276-80

[70] Hofmann, K. (2000) A superfamily of membrane-bound *O*-acyltransferases with implications for Wnt signalling. Trends Biochem. Sci. 25, 111-12

[71] Park, J.H. and Saier, M.H.Jr. (1996) Phylogenetic characterization of the MIP family of transmembrane channel proteins. J. Membr. Biol. 153, 171-80

[72] Saier, M.H.Jr. (1994) Computer-Aided Analyses of Transport Protein Sequences: Gleaning Evidence concerning Function, Structure, Biogenesis, and Evolution. Microbiol. Rev. 58, 71-93

[73] Gaillardin, C., Duchateau-Nguyen, G., Tekaia, F., Llorente, B., Casarégola, S., Toffano-Nioche, C., Aigle, M., Artiguenave, F., Blandin, G., Bolotin-Fukuhara, M., Bon, E., Brottier, P., Montigny, J., Dujon, B., Durrens, P., Lépingle, A., Malpertuy, A., Neuvéglise, C., Ozier-Kalogeropoulos, O., Potier, S., Saurin, W., Termier, M., Wésolowski-Louvel, M., Wincker, P., Souciet, J. and Weissenbach, J. (2000) Genomic exploration of the hemiascomycetous yeast: 21. Comparative functional classification of genes. FEBS Lett. 487, 134-149

[74] Oshiro, K. and Yagi, T. (1996) Regulation of intracellular osmotic pressure andd some factors that influence the promotion of glycerol synthesis in a respiration-deficient mutant of the salt-tolerant yeast *Zygosaccharomyces rouxii* during salt stress. J. Gen. Appl. Microbiol. 42, 201-212

[75] Philips, J. and Herskowitz, I. (1997) Osmotic balance regulates cell fusion during mating in *Saccharomyces cerevisiae*. J. Cell Biol. 138, 961-74

[76] Toh. T.H., Kayingo, G., van der Merwe, M.J., Kilian, S.G., Hallsworth, J.E., Hohmann, S. and Prior, B.A. (2001) Implications of *FPS1* deletion and membrane ergosterol content for glycerol efflux from *Saccharomyces cerevisiae*. FEMS Yeast Res. 1, 205-11

[77] Henry, M.F., Mandel, D., Routson, V., Henry, P.A. (2003) The yeast hn-RNP-like protein Hrp/Nab4 accumulates in the cytoplasm after hyperosmotic stress: A novel Fps1-dependent response. Mol. Biol. Cell 14, 3929-41

[78] Kayingo, G., Sirotkin, V., Hohmann, S. and Prior, B.A. (2004) Accumulation and release of the osmolyte glycerol is independent of the putative MIP channel Spac977.17p in *Schizosaccharomyces pombe* Antonie van Leeuwenhoek 85, 85-92

[79] Oliveira, R., Lages, F., Silva-Graça, M. and Lucas, C. (2003) Fps1p channel is a mediator of the major part of glycerol passive diffusion in *Saccharomyces cerevisiae*. Artefacts and re-definitions, Biochim. Biophys. Acta 1613, 57-71

[80] Froger, A., Tallur, B., Thomas, D. and Delamarche, C. (1998) Prediction of functional residues in water channels and related proteins. Prot. Sci. 7, 1458-68

[81] Hohmann, S., Nielsen, S. and Agre, P. (2001) Aquaporins. Aacdemic Press, USA

[82] Hohmann, S., Bill, R.M., Kayingo, I. and Prior, B.A. (2000) Microbial MIP channels. Trends Microbiol. 8, 33-38

[83] Tamás, M.J., Karlgren, S., Bill, R.M., Hedfalk, K., Allegri, L., Ferreira, M., Thevelein, J.M., Rydstrom, J., Mullins, J.G. and Hohmann, S. (2003) A short regulatory domain restricts glycerol transport through yeast Fps1p. J. Biol. Chem. 278, 6337-45

[84] Hedfalk, K., Bill, R.N., Mullins, J.G., Karlgren, S., Philipsson, C., Bergstron, J., Tamás, M.J., Rydstron, J. and Hohmann, S. (2004) A regulatory domain in the C-terminal extension of the yeast glycerol channel Fps1p. J. Biol. Chem. 279, 14954-60

[85] Nelissen, B., de Watcher, R. and Goffeau, A. (1997) Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 21, 113-4

[86] Oelkers, P., Tinkelenberg, A., Erdeniz, N., Cromley, D., Billheimer, J.T. and Sturley, S.L. (2000) A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast. J. Biol. Chem. 275, 15609-12

[87] Perego, M., Glaser, P., Minutello, A., Strauch, M.A., Leopold, K. and Fischer, W. (1995) Incorporation of D-alanin into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. Identification of genes and regulation. J. Biol. Chem. 270, 15598-606

[88] Tatusov, R.L., Fedorova, N.D., Jackson, J.D., Jacobs, A.R., Kiryutin, B., Koonin, E.V., Krylov, D.M., Mazumder, R., Mekhedov, S.L., Nikolskaya, A.N., Rao, B.S.,

Smirnov, S., Sverdlov, A.V., Vasudevan, S., Wolf, Y., Yin, J.J. and Natale, D.A. (2003) The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4, 41

[89] Lages, F. and Lucas, C. (1995). Characterization of a glycerol/H^+ symport in the halotolerant yeast *Pichia sorbitophila*. Yeast 11, 111-9

Legends

Fig.1. 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*.

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

Fig 3. Complementation of *S. cerevisiae gup1* mutant strain complemented with multicopy vector p426GPD. 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*.

Fig. 4. Complementation of *S. cerevisiae fps1* mutant strain complemented with multicopy vector p426GPD. 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.

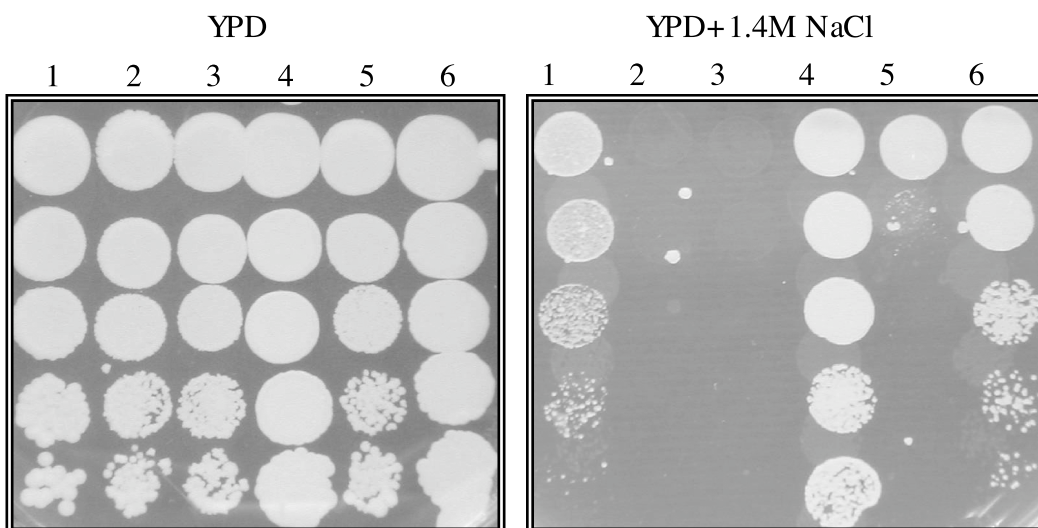
Fig 5. Phylograms for the predicted amino acid sequences of the genes: A - *GPD1/2*; B - *GUT1*; C - *GUP1/2*; D - *FPS1*. *Genes cloned and tested for phenotypic complementation in this work. Accession numbers are presented between brackets. Strains nomenclature: *Ca - C. albicans, Ct - C. tropicalis, Dh – D. hansenii, Kd – K. delphinensis, Kl – K. lactis, Kt –*

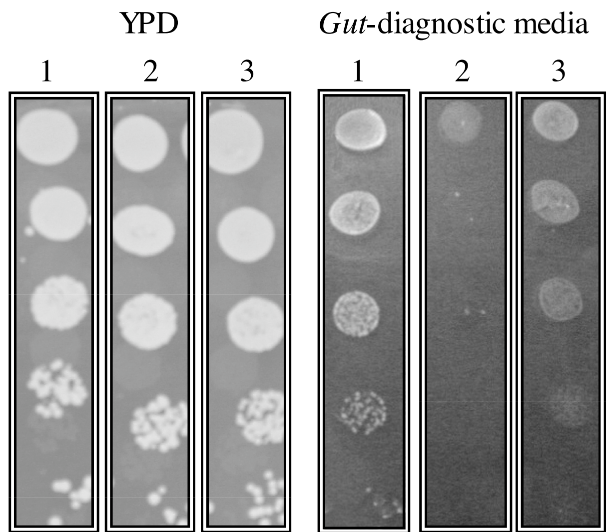
K. thermoltolerans, Pa – P. angusta, Ps - P. sorbitophila, Sb - S. bayanus, Sca – S. castelli, Sc - S. cerevisiae, Sk – S. kluyvery, Sku - S. kudriavzevii, *Sm - S. mikatae, Sp - S. paradoxus*, *Schp – Sch. pombe, Yl – Y. lipolitica* and *Zr – Z. rouxii*.

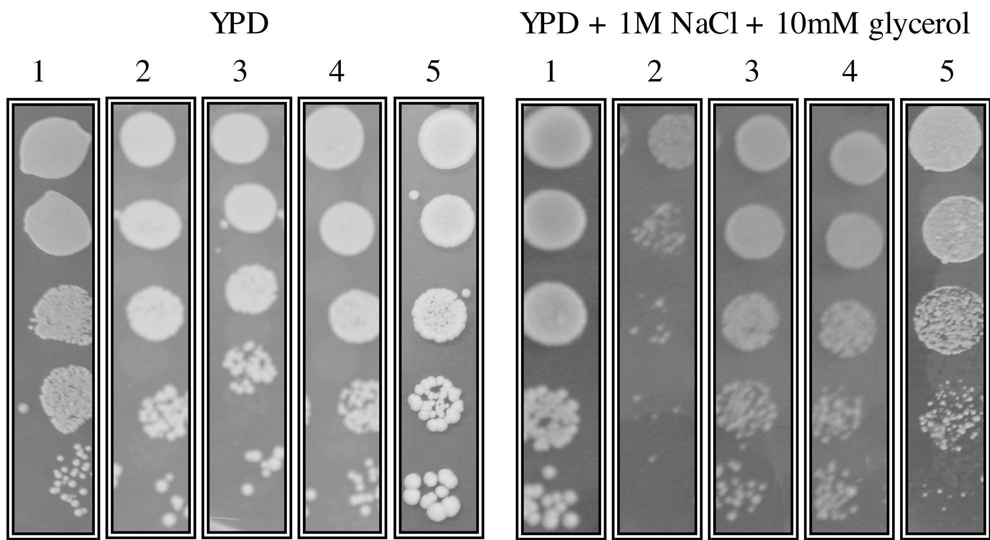
Fig. 6. Phylogram of Gup-related predicted aminoacid sequences. A – Gup-like sequences; B – proteins from MBOAT/DltB cluster; C – proteins from MBOAT/KOG3860 cluster. Accession numbers are presented between brackets. Only complete ORF sequences were used. Organisms nomenclature, other than yeasts: *At – 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 palmytolation 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).

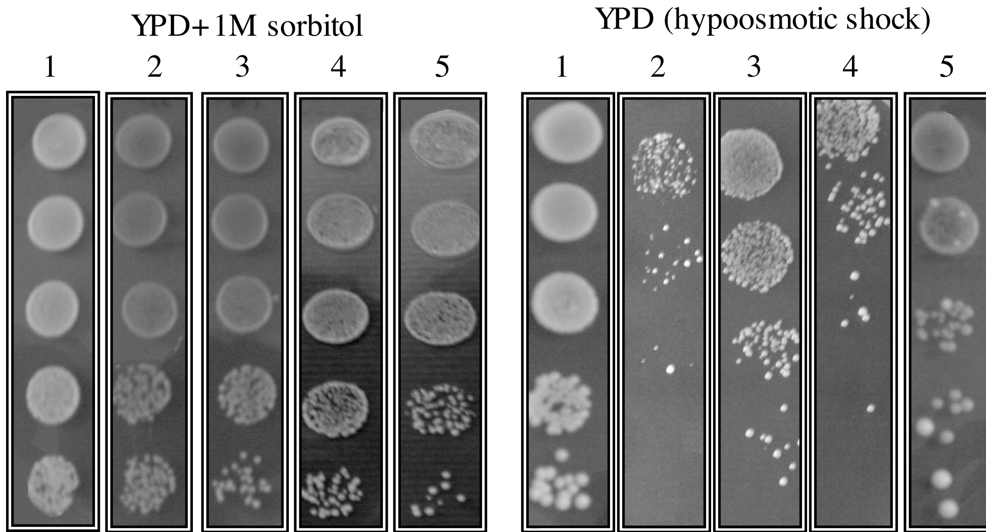
Table 1. Primers used in PCR reactions. Yeast strains nomenclature: Ca - *C. albicans, Ct - C. tropicalis, Km - K. marxianus, Kt - K. thermotolerans, Pa - P. angusta, Ps - P. sorbihophila, Kl - K. lactis, Zr - Z. rouxii.*

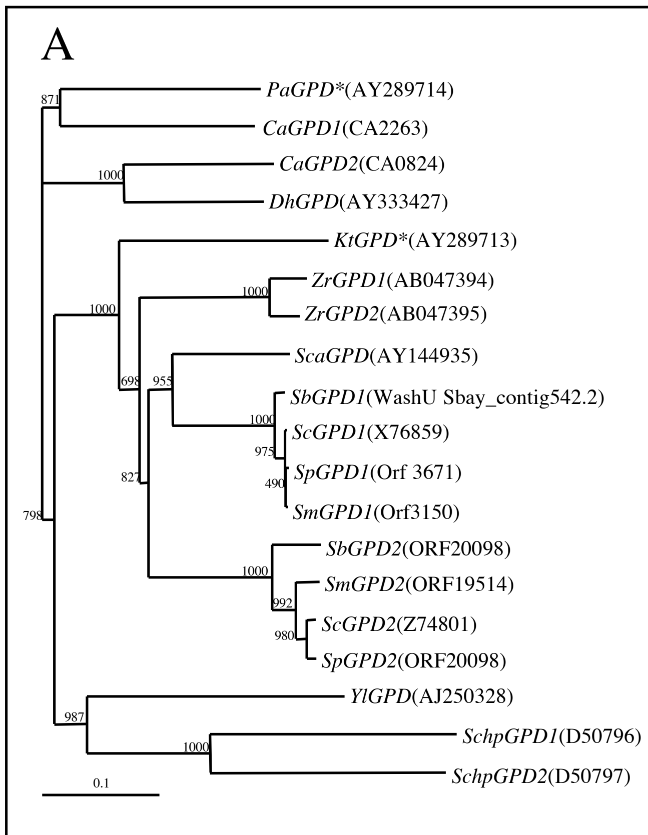
KlGPD-f	5’- GACAGCTGCTCCAACAGAAA - 3’
KlGPD-r	5’- CCTTCCGCTGTTGGTTTATG - 3’
PaGPD-f	5’- TAGTCCTCAGATGCCTCAAG - 3’
PaGPD-r	5’ - GCAGGTAAACGTCATACTGC - 3’
CtGUP-f	5’ - CGTCAGATGGTTGATTATCG - 3’
CtGUP-r	5’ - GTTGTACTGAAGGGTTTGGG - 3’
PsGUP-f	5’ - GTGGAATACTTGGGAATTTC - 3’
PsGUP-r	5’ - CACTGATGTGCACTCATGCT - 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’


YPD
YPD+1.4M NaCl
1
2
3
4
5
6
1
2
3
4
5
6

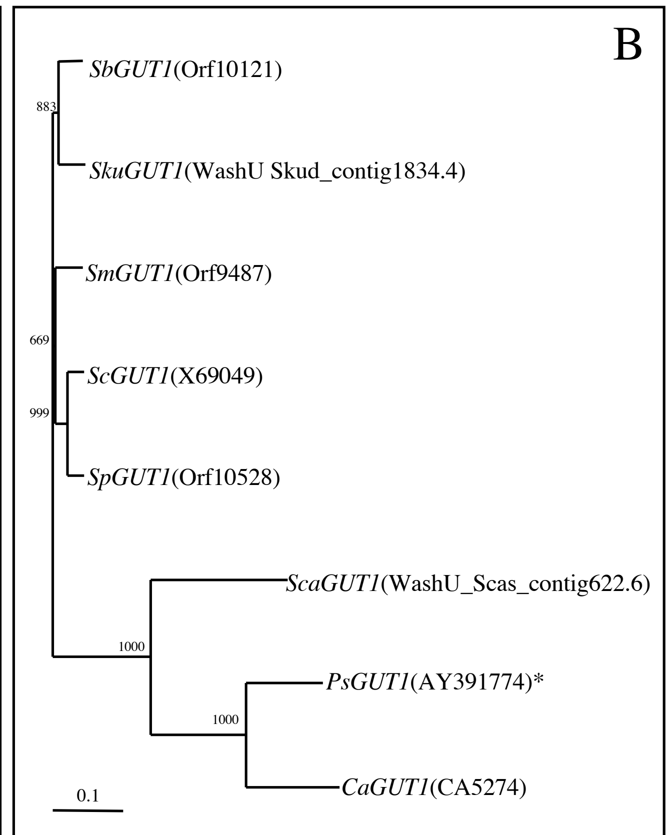

YPD
Gut-diagnostic media
1
2
3
1
2
3


YPD
YPD + 1M NaCl + 10mM glycerol
1
2
3
4
5
1
2
3
4
5

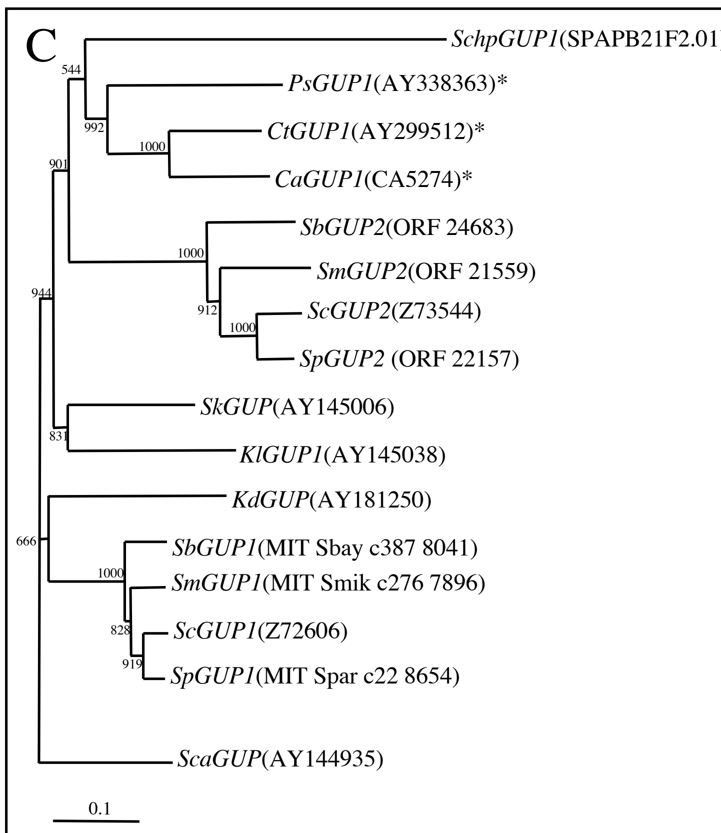

YPD+1M sorbitol
1
2
3
4
5
YPD (hypoosmotic shock)
1
2
3
4
5



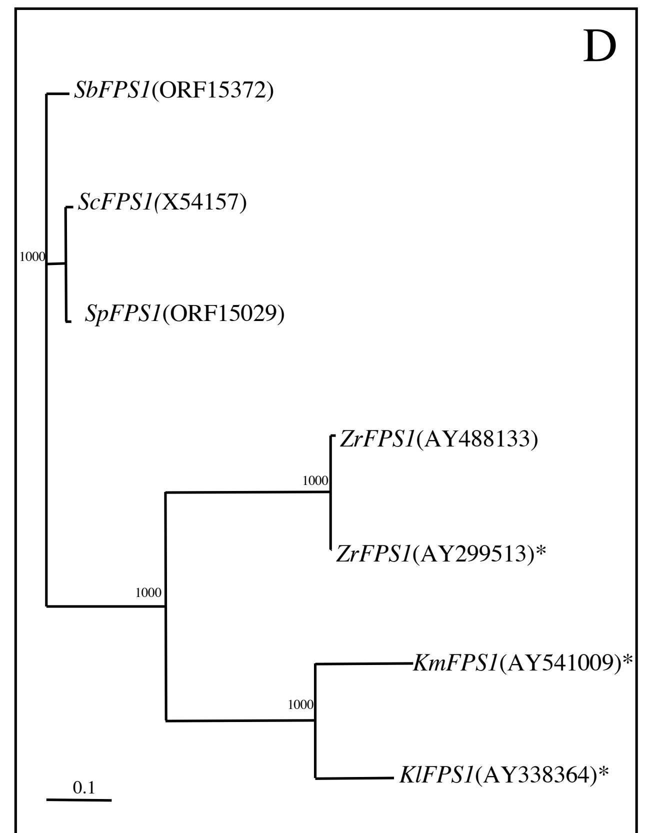
A
PaGPD*(AY289714)
CaGPD1(CA2263)
CaGPD2(CA0824)
DhGPD(AY333427)
KtGPD*(AY289713)
ZrGPD1(AB047394)
ZrGPD2(AB047395)
ScaGPD(AY144935)
SbGPD1(WashU Sbay_contig542.2)
ScGPD1(X76859)
SpGPD1(Orf 3671)
SmGPD1(Orf3150)
SbGPD2(ORF20098)
SmGPD2(ORF19514)
ScGPD2(Z74801)
SpGPD2(ORF20098)
YlGPD(AJ250328)
SchpGPD1(D50796)
SchpGPD2(D50797)
0.1



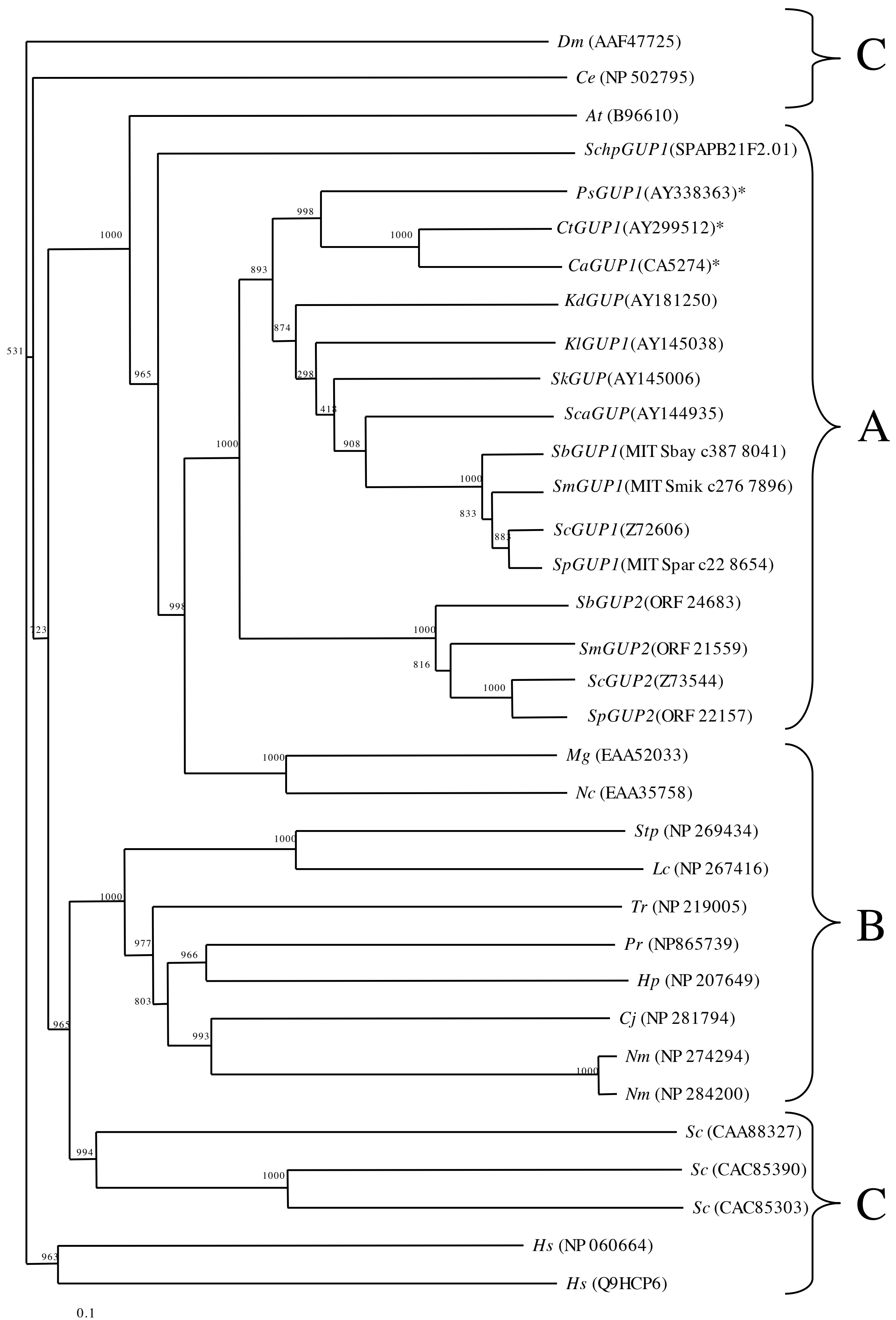
B
SbGUT1(Orf10121)
SkuGUT1(WashU Skud_contig1834.4)
SmGUT1(Orf9487)
ScGUT1(X69049)
SpGUT1(Orf10528)
ScaGUT1(WashU_Scas_contig622.6)
PsGUT1(AY391774)*
CaGUT1(CA5274)
0.1



C
SchpGUP1(SPAPB21F2.01)
PsGUP1(AY338363)*
CtGUP1(AY299512)*
CaGUP1(CA5274)*
SbGUP2(ORF 24683)
SmGUP2(ORF 21559)
ScGUP2(Z73544)
SpGUP2 (ORF 22157)
SkGUP(AY145006)
KlGUP1(AY145038)
KdGUP(AY181250)
SbGUP1(MIT Sbay c387 8041)
SmGUP1(MIT Smik c276 7896)
ScGUP1(Z72606)
SpGUP1(MIT Spar c22 8654)
ScaGUP(AY144935)
0.1



D
SbFPS1(ORF15372)
ScFPS1(X54157)
SpFPS1(ORF15029)
ZrFPS1(AY488133)
ZrFPS1(AY299513)*
KmFPS1(AY541009)*
KlFPS1(AY338364)*
0.1



Dm (AAF47725)
Ce (NP 502795)
C
At (B96610)
SchpGUP1(SPAPB21F2.01)
PsGUP1(AY338363)*
CtGUP1(AY299512)*
CaGUP1(CA5274)*
KdGUP(AY181250)
KlGUP1(AY145038)
SkGUP(AY145006)
ScaGUP(AY144935)
SbGUP1(MIT Sbay c387 8041)
SmGUP1(MIT Smik c276 7896)
ScGUP1(Z72606)
SpGUP1(MIT Spar c22 8654)
SbGUP2(ORF 24683)
SmGUP2(ORF 21559)
ScGUP2(Z73544)
SpGUP2(ORF 22157)
A
Mg (EAA52033)
Nc (EAA35758)
Stp (NP 269434)
Lc (NP 267416)
Tr (NP 219005)
Pr (NP865739)
Hp (NP 207649)
Cj (NP 281794)
Nm (NP 274294)
Nm (NP 284200)
B
Sc (CAA88327)
Sc (CAC85390)
Sc (CAC85303)
Hs (NP 060664)
Hs (Q9HCP6)
C
531
1000
965
998
893
874
298
418
908
1000
833
883
1000
998
1000
816
1000
723
1000
1000
1000
977
966
803
993
1000
965
994
1000
963
0.1