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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

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The *Saccharomyces cerevisiae FPS1* gene, which encodes a channel protein belonging to the MIP family, has been isolated previously as a multicopy suppressor of the growth defect of the *fdp1* mutant (allelic to *GGS1/TPS1*) on fermentable sugars. Here we show that overexpression of *FPS1* enhances glycerol production. Enhanced glycerol production caused by overexpression of *GPD1* encoding glycerol-3-phosphate dehydrogenase also suppressed the growth defect of *ggs1/tps1*Δ mutants, suggesting a novel role for glycerol production in the control of glycolysis. The suppression of *ggs1/tps1*Δ mutants by *GPD1* depends on the presence of Fps1. Mutants lacking Fps1 accumulate a greater part of the glycerol intracellularly, indicating that Fps1 is involved in glycerol efflux. Glycerol-uptake experiments showed that the permeability of the yeast plasma membrane for glycerol consists of an Fps1-independent component probably due to simple diffusion and of an Fps1-dependent component representing facilitated diffusion. The *Escherichia coli* glycerol facilitator expressed in a yeast *fps1*Δ mutant can restore the characteristics of glycerol uptake, production and distribution fully, but restores only partially growth of a *ggs1/tps1*Δ*fps1*Δ double mutant on glucose. Fps1 appears to be closed under hyperosmotic stress when survival depends on intracellular accumulation of glycerol and apparently opens rapidly when osmostress is lifted. The osmostress-induced High Osmolarity Glycerol (HOG) response pathway is not required for inactivation of Fps1. We conclude that Fps1 is a regulated yeast glycerol facilitator controlling glycerol production and cytosolic concentration, and might have additional functions.

Key words: glycerol facilitator/glycolysis/MIP family/osmoregulation/*Saccharomyces cerevisiae*

Introduction

We have previously isolated Fps1, a yeast member of the MIP family of channel proteins, as a suppressor of the yeast *fdp1* mutant which has a defect in the control of glycolysis. Using a combination of genetic and biochemical analysis, we demonstrate in the present paper that Fps1 is a glycerol facilitator which appears to suppress the *fdp1* mutant because of its stimulating effect on glycerol production. The existence of a channel mediating glycerol permeation through the yeast plasma membrane suggests a role for this protein in osmoadaptation, which we have studied as well.

The MIP family is a rapidly growing group of currently ~20 channel proteins that have been identified in organisms ranging from bacteria to man [recently reviewed by Reizer *et al.* (1993)]. Most of them are ~250–280 amino acids long and consist of six putative membrane-spanning domains. Recently, specific functions have been attributed to some of these proteins (Reizer *et al.*, 1993). MIP itself, the major intrinsic protein of lens fibre gap junctions in mammals, provides voltage gated channels between adjacent cells (Zamphigi *et al.*, 1985). CHIP28 has been identified in erythrocytes and kidney proximal tubes where it regulates water flux through the membrane (Preston *et al.*, 1992). Similarily, WCH-CD functions as a water channel in kidney collecting tubule cells (Fushimi *et al.*, 1993). Isoforms of tonoplast intrinsic protein (TIP) which have been identified in different plant species also form water channels, in this case between the cytosol and the vacuole (Maurel *et al.*, 1993; Chrispeels and Agre, 1994). Nodulin-26, which is located in the peribacteroid membrane of root nodules in soybean and is produced by the plant during symbiosis, may serve as a channel for dicarboxylic acids (Ouyang *et al.*, 1991). Finally, glycerol facilitators belonging to the MIP family have been described in bacteria and a function in glycerol transport and utilization has been confirmed for the *Escherichia coli* protein (Heller *et al.*, 1980; Sweet *et al.*, 1990; Maurel *et al.*, 1994). Fps1 differs from most other members of the MIP family by having long hydrophilic N- and C-terminal extensions (total open reading frame 669 amino acids). The only other known protein of the MIP family that has been reported to have such extensions is the *Drosophila* BIB (big brain) protein (Rao *et al.*, 1990). However, the extensions of Fps1 and BIB neither show any sequence similarity to each other nor to any other known protein. The part of Fps1 containing the putative membrane-spanning domains shows the highest degree of overall sequence similarity to the glycerol facilitator of *E.coli* (30.5% identical over 220 amino acids; Muramatsu and Mizuno, 1989) and of *Bacillus subtilis* (33.0% identical over 233 amino acids; Holmberg *et al.*, 1990).

The *FPS1* gene has been isolated as a multicopy suppressor of the growth defect on fermentable sugars of the yeast *fdp1* mutant (Van Aelst *et al.*, 1991). We have recently cloned the actual *FDP1* gene and it turned out to be allelic to *byp1-3*, *cif1* and *glc6* (González *et al.*,

1992; Hohmann *et al.*, 1992; Van Aelst *et al.*, 1993; Cannon *et al.*, 1994) and we have renamed this gene *GGS1* (for component of the proposed *G*eneral *G*lucose *S*ensor; Thevelein, 1992; Van Aelst *et al.*, 1993). Mutants defective in *GGS1* cannot grow in the presence of a fermentable carbon source like glucose or fructose, but they grow normally on non- or slowly fermentable substrates like ethanol, glycerol or galactose (Van Aelst *et al.*, 1991, 1993; Hohmann *et al.*, 1992, 1993, 1994; Neves *et al.*, 1995). The inability of such mutants to grow on fermentable sugars is due to an imbalance in glycolysis. When glucose is added to *ggs1* mutants, they accumulate large amounts of sugar phosphates and the levels of ATP and inorganic phosphate (P_i) rapidly decrease. The glyceraldehyde-3-phosphate dehydrogenase reaction, which requires P_i as a substrate, apparently limits glycolytic flux. This phenotype, together with the finding that deletion of the gene for hexokinase PII suppressed the growth defect of the *ggs1/tps1*Δ mutant, suggested that Ggs1 has a role in controlling sugar influx into glycolysis. In addition, *ggs1* mutants show pleiotropic defects: they lack glucose-induced signalling (Van Aelst *et al.*, 1991, 1993; Hohmann *et al.*, 1992), homozygous diploids do not sporulate (Van Aelst *et al.*, 1993; Neves *et al.*, 1995) and most of the mutants are unable to accumulate trehalose (Charlab *et al.*, 1985; Van Aelst *et al.*, 1993; Neves *et al.*, 1995). The basis for the latter effect was explained in a simple way after the *GGS1* gene was also cloned as encoding the small subunit of the trehalose synthase complex under the names *TPS1* (Bell *et al.*, 1992) and *TSS1* (Vuorio *et al.*, 1993). Ggs1/Tps1 is most likely responsible for the trehalose-6-phosphate synthase activity in this complex (Bell *et al.*, 1992; McDougall *et al.*, 1993; W.Bell, S.Hohmann and J.M.Thevelein, unpublished results). However, it is still unclear whether the pleiotropic effects of mutants lacking Ggs1/Tps1 can all be attributed solely to a role of Ggs1/Tps1 in trehalose synthesis or whether this protein has additional regulatory roles, hence the use of the dual name (Thevelein, 1992; Thevelein and Hohmann, 1995).

Glycerol synthesis resembles that of trehalose in various aspects. First of all, the production of both compounds is enhanced under certain stress conditions. Trehalose is thought to have a protective role under heat and desiccation stress, and its synthesis is induced at high temperatures (Thevelein, 1984; Hottiger *et al.*, 1987, 1994; Van Laere, 1989; Wiemken, 1990; de Virgilio *et al.*, 1994). Glycerol serves as a compatible solute to adjust the intracellular osmotic pressure relative to that of the medium and its production is induced under osmotic stress (André *et al.*, 1991; Blomberg and Adler, 1992; Albertyn *et al.*, 1994a,b). Secondly, the precursors for both, trehalose and glycerol, derive from the upper part of glycolysis, i.e. from metabolites that accumulate in *ggs1/tps1* mutants. Finally, production of both trehalose and glycerol releases P_i, which is believed to be limiting for glycolytic flux in *ggs1/tps1* mutants (Hohmann *et al.*, 1993; Van Aelst *et al.*, 1993; Thevelein and Hohmann, 1995). Therefore we tested whether enhanced glycerol production could suppress the growth defect of *ggs1/tps1* mutants. We show that this is indeed the case and we characterize *GPD1* encoding NAD-dependent glycerol-3-phosphate dehydrogenase (Larsson *et al.*, 1993; Albertyn *et al.*, 1994b) as a novel multicopy suppressor of *ggs1/tps1* mutants.

NAD-dependent glycerol-3-phosphate dehydrogenase catalyses the first step of glycerol production by reducing the glycolytic intermediate dihydroxyacetone phosphate to glycerol-3-phosphate which is subsequently converted to glycerol by glycerol-3-phosphatase (Gancedo *et al.*, 1968). Glycerol-3-phosphate dehydrogenase is encoded by at least two homologous genes, *GPD1* and *GPD2* (Larsson *et al.*, 1993; Albertyn *et al.*, 1994b; P.Ericksson, L.André, R.Ansell, A.Blomberg and L.Adler, submitted for publication). Mutants lacking *GPD1* still have low enzyme activity and they produce up to 20% of the amount of glycerol compared with the wild-type. *GPD1* expression is osmoregulated (Albertyn *et al.*, 1994b). A gene for glycerol-3-phosphatase has not yet been described.

Results

Overexpression of GPD1 suppresses the growth defect of ggs1/tps1Δ mutants in an FPS1-dependent way

We have investigated in detail the genetic relationship between *GGS1/TPS1*, *FPS1* and *GPD1*. For this purpose, *FPS1* or *GPD1* were either deleted in a *ggs1/tps1*Δ background or overexpressed by transformation with a multicopy plasmid carrying the respective gene. To minimize genetic background effects, we used in parallel two wild-type strains for mutant and transformant constructions: YSH 6.36.-3B (so-called M5 background; Hohmann *et al.*, 1993) and W303-1A (Thomas and Rothstein, 1989). Both backgrounds gave consistent results, except that W303-1A is generally more affected by *ggs1/tps1* mutations: W303-1A *ggs1/tps1*Δ mutants cannot grow on glucose or fructose, while M5 *ggs1/tps1*Δ mutants grow on glucose but not on fructose. Fructose causes a more stringent growth inhibition of *ggs1/tps1* mutants in general (Hohmann *et al.*, 1992, 1993; Neves *et al.*, 1995).

Overexpression of *GPD1* suppressed the growth defect of *ggs1/tps1*Δ mutants both on glucose (in W303-1A, not shown) and on fructose medium (Figure 1). Deletion of *GPD1*, which alone does not confer a phenotype on fermentable sugars (Albertyn *et al.*, 1994b), resulted in a growth defect of the M5 *ggs1/tps1*Δ mutant even on glucose. Thus, the growth inhibition of *ggs1/tps1*Δ mutants on fermentable sugars is less pronounced with a high capacity of glycerol production and more pronounced when only little glycerol can be produced.

Unexpectedly, overexpression of *FPS1* did not suppress the growth defect of the *ggs1/tps1*Δ mutant, in contrast to our earlier findings with the allelic *fdp1* mutant. This might be ascribed to a different genetic background used in our previous study (Van Aelst *et al.*, 1991) which was unsuitable for further genetic analysis. On the other hand, additional deletion of *FPS1* in the M5 *ggs1/tps1*Δ mutant, like deletion of *GPD1*, resulted in a growth defect on glucose (Figure 1). The *fps1*Δ alone does not confer any obvious growth phenotype (Van Aelst *et al.*, 1991, and results not shown).

Overexpression of *GPD1*, which caused a very strong suppression of the growth defect of the *ggs1/tps1*Δ mutant, did not restore growth of the *ggs1/tps1*Δ*fps1*Δ double

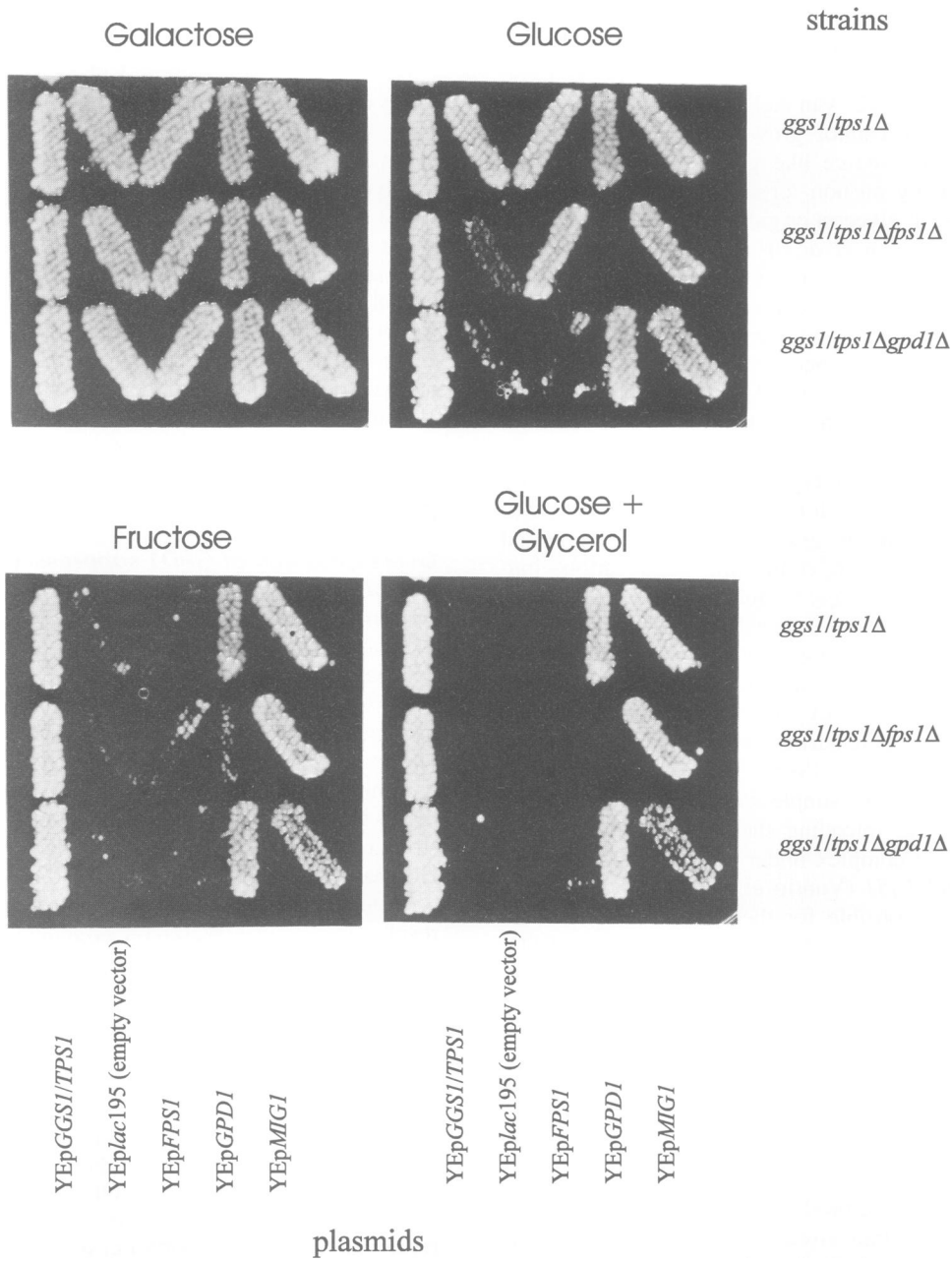

Fig. 1. Growth of different yeast strains after replica plating from synthetic medium containing 2% galactose onto yeast extract/peptone plates containing 2% galactose, 2% glucose, 2% fructose and 2% glucose plus 5% glycerol. All strains are isogenic to M5 (YSH 6.36.-3B). Each row represents a different strain transformed with the plasmids as indicated from left to right.

mutant on glucose or fructose. Thus, suppression by *GPD1* depends on the presence of the Fps1 channel protein, pointing to a role of Fps1 in glycerol metabolism. This result, together with the sequence similarity of Fps1 to channel proteins, suggested that Fps1 has a role in glycerol efflux.

To corroborate this idea further, we inhibited glycerol efflux by the addition of high levels of glycerol to the medium. The addition of 5% glycerol led to a growth defect of the M5 *ggs1/tps1*Δ mutant on glucose (Figure 1). Glycerol at 3% was equally effective (not shown). On such media, the growth defect of the *ggs1/tps1*Δ mutant was indistinguishable from that of the *ggs1/tps1*Δ*fps1*Δ double mutant, regardless of the presence in either strain of the multicopy plasmid carrying *FPS1*, suggesting that the high external glycerol concentration blocked the effect conferred by Fps1. Addition of 5% sorbitol instead had no such inhibitory effect (not shown), indicating that it is specific for glycerol. The addition of 5% glycerol did not abolish suppression of the *ggs1/tps1*Δ mutant by overexpression of *GPD1*, but it was less effective in the W303-1A background (not shown). Probably, the higher levels of glycerol produced could still be at least partially excreted against the high external level.

MIG1 overexpression has been shown previously to suppress the growth defect of the *byp1-3* mutant which is allelic to *GGS1/TPS1* (Hohmann *et al.*, 1992). *MIG1* encodes a transcriptional repressor protein involved in

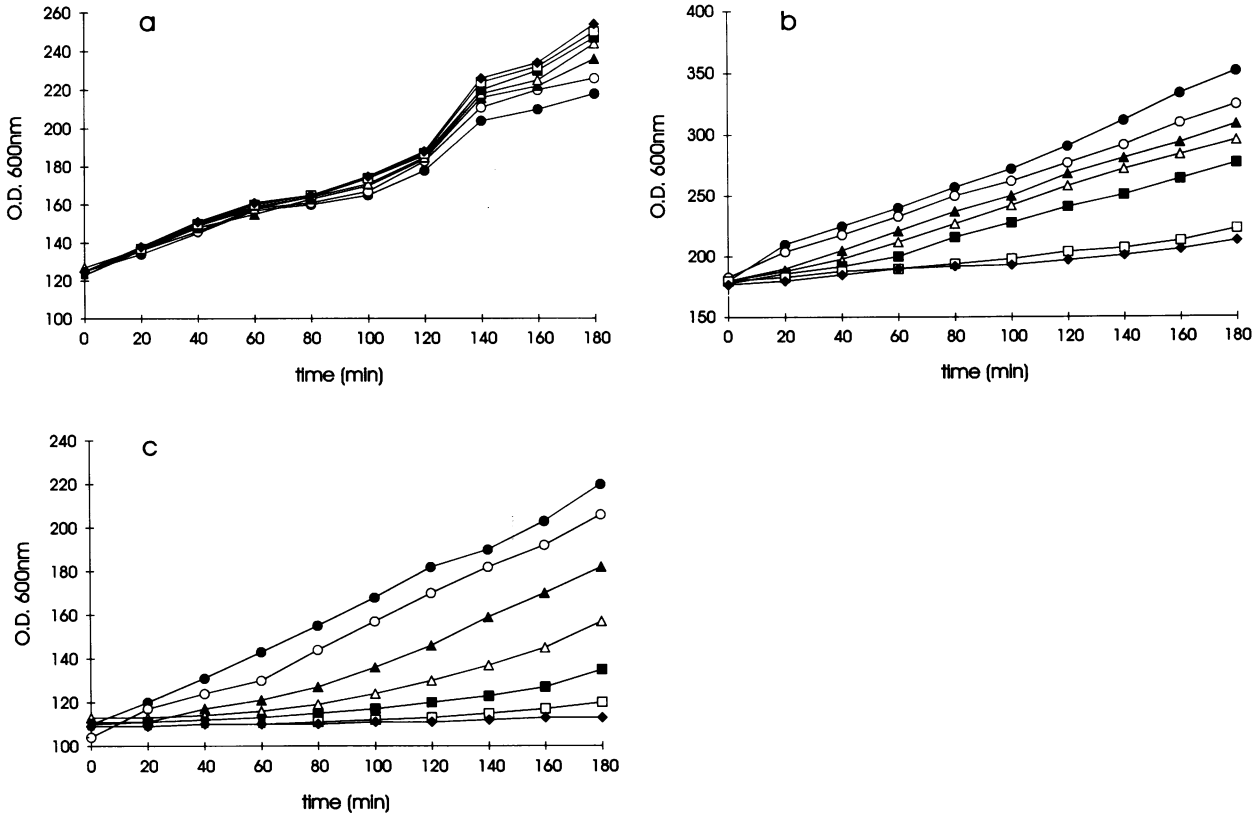

Fig. 2. Growth of different strains (isogenic to YSH 6.36.-3B) in synthetic medium containing 2% (100 mM) galactose after the addition of different concentrations of glucose at time zero. (**a**) Wild-type. (**b**) *ggs1/tps1*Δ. (**c**) *ggs1/tps1*Δ transformed with the multicopy plasmid YEp*lac*195 containing *GPD1*. Glucose concentrations: no glucose (●), 0.5 mM glucose (○), 1 mM glucose (▲), 2 mM glucose (△), 5 mM glucose (■), 10 mM glucose (□) and 20 mM glucose (◆).

glucose repression (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991), but the way it suppresses the *byp1-3* mutant is unknown. *MIG1* overexpression also suppressed the *ggs1/tps1*Δ mutation in both genetic backgrounds used (Figure 1 and not shown) and deletion of *MIG1* in a *ggs1/tps1*Δ mutant led to a more severe growth defect (not shown). Overexpression of *MIG1* also suppressed, in both backgrounds, the double mutants *ggs1/tps1*Δ*fps1*Δ and, to a lesser extent, *ggs1/tps1*Δ*gpd1*Δ, showing that *MIG1* does not suppress via stimulation of glycerol production or efflux. Five per cent glycerol in the medium did not reduce the suppressive effect of *MIG1* overexpression, confirming this conclusion. The suppression of *ggs1/tps1*Δ*fps1*Δ by *MIG1* overexpression also shows that the inability of *GPD1* to suppress this double mutant is not simply due to the stronger growth defect of this strain, but that it is specific for the mechanism by which *GPD1* suppresses.

We have shown recently that *ggs1/tps1*Δ mutants are highly sensitive to low glucose concentrations in liquid medium with even 0.5 mM of glucose already causing a transient growth inhibition in the presence of 100 mM of galactose as carbon source (Neves *et al.*, 1995). Glucose at 20 mM prevents growth of the *ggs1/tps1* mutant in liquid medium completely. This difference to growth on plates is probably due to the cells having better access to the medium in liquid culture. *GPD1* overexpression in *ggs1/tps1*Δ mutants did not significantly alter the initial sensitivity to glucose in the early exponential growth phase in liquid medium (Figure 2). In contrast, deletion of *HXK2* (encoding the hexokinase isoenzyme PII) suppressed the growth defect of *ggs1/tps1*Δ mutants on glucose plates (Hohmann *et al.*, 1993) and also the initial glucose sensitivity (Neves *et al.*, 1995). Thus, deletion of *HXK2* apparently prevents the metabolic disorder in glycolysis caused by the *ggs1/tps1*Δ mutation. Overexpression of *GPD1*, on the other hand, does not appear to prevent the metabolic disturbance, but causes subsequent restoration of a normal glycolytic flux, probably by degradation of the accumulated sugar phosphates to glycerol. Growth of the *ggs1/tps1*Δ mutant transformed with the *GPD1* multicopy plasmid resumed ~6–8 h after the addition of 20 mM of glucose.

Fps1 affects glycerol production and the ratio of intra- to extracellular glycerol

We have investigated the effects of *FPS1* on the intra- and extracellular glycerol concentrations after the addition of glucose to cells pre-grown on ethanol (gluconeogenic growth). This was performed with the wild-type strain and the *ggs1/tps1*Δ mutant in the presence or absence of *GPD1* overexpression. When glucose is added to a *ggs1/tps1*Δ mutant culture, the cells accumulate large amounts of sugar phosphates, which are metabolites of the upper part of glycolysis. Since the precursor for glycerol production, dihydroxyacetone phosphate, also accumulates (Hohmann *et al.*, 1993), it is not surprising that glycerol production is also enhanced in *ggs1/tps1*Δ mutants upon glucose addition. Indeed, the *ggs1/tps1*Δ mutant produced

much more intra- and extracellular glycerol upon glucose addition than the wild type (Figure 3a and b). Interestingly, the *ggs1/tps1*Δ mutant already had higher glycerol levels when growing in ethanol medium, suggesting that the inability to produce trehalose as one end product of gluconeogenesis results in an accumulation of precursors which are then deviated to glycerol. Deletion of *GPD1* largely prevented glycerol production in the *ggs1/tps1*Δ mutants with the residual glycerol produced being probably due to the activity of the *GPD2* gene product. Overexpression of *GPD1* increased the glycerol production further compared with the untransformed *ggs1/tps1*Δ strain, very apparent by the continuous increase of the external level. Thus, the suppressive effect of *GPD1* is most probably due to enhanced degradation of the accumulating metabolites of the upper part of glycolysis to glycerol and concomitant recovery of free phosphate. Deletion of *GPD1* accentuates the growth problem of *ggs1/tps1*Δ mutants (Figure 1) most probably because it eliminates the glycerol rescue pathway.

The presence or absence of the Fps1 channel protein in a *ggs1/tps1*Δ background affected the ratio of intra- to extracellular glycerol very much (Figure 3c and d). When *FPS1* was deleted in a *ggs1/tps1*Δ mutant, more glycerol accumulated inside the cell and much less glycerol appeared in the extracellular growth medium. When Fps1 was overproduced, much more glycerol was extruded from the cell. However, the level of intracellular glycerol was also consistently higher in *ggs1/tps1*Δ mutants transformed with *FPS1* on a multicopy plasmid than in untransformed cells. Thus, not only was the intra/extracellular distribution of glycerol affected by the level of Fps1, but also the total amount of glycerol produced.

The same effect of Fps1 on glycerol distribution was also observed when *GPD1* was overexpressed in a *ggs1/tps1*Δ background (Figure 3e and f). In the absence of *FPS1*, such a strain initially accumulated much more glycerol inside the cell and much less glycerol appeared in the medium. Considering that the external medium has a much larger volume than the cells, the total amount of glycerol and the production rate were also diminished. This effect may explain the inability of *GPD1* overexpression to suppress the growth defect of the *ggs1/tps1*Δ*fps1*Δ double mutant (Figure 1). Overexpression of *GPD1* in the *ggs1/tps1*Δ*fps1*Δ double mutant diminished further the already low viability of the mutant upon storage at 4°C. This indicates that a high intracellular glycerol level might be deleterious to the cells by possibly affecting the osmotic equilibrium of the cytosol.

Fps1 also affected the glycerol distribution in a *GGS1/TPS1* wild-type strain both during normal or enhanced glycerol production (Figure 3g and h). Again, deletion of *FPS1* resulted in higher glycerol levels inside the cell and in lower levels in the medium. As was already observed in the *ggs1/tps1*Δ background, overexpression of *FPS1* increased both intra- and extracellular glycerol levels.

In summary, these results strongly argue for a role of Fps1 in glycerol efflux and, in addition, Fps1 also appears to affect total glycerol production. Since higher glycerol levels were found both inside and outside of cells overexpressing *FPS1* compared with normal cells, it is unlikely that this observation can be explained simply by faster glycerol efflux.

Fps1 is required for glycerol uptake by facilitated diffusion

Next we investigated whether the presence or absence of Fps1 affects glycerol uptake into the cell. It has been shown previously that the permeability of the *Saccharomyces cerevisiae* plasma membrane for glycerol is relatively low compared with that of another yeast, *Candida utilis* (Gancedo *et al.*, 1968). Thus, we have measured glycerol uptake as the accumulation of radioactivity derived from glycerol over a longer time period than is used for the study of transport characteristics. Thus, glycerol may be metabolized to a certain extent during the course of the experiment. Because of the low membrane permeability for glycerol, we used a high glycerol concentration, 100 mM, containing 20 μM radioactive glycerol.

The amount of glycerol taken up by the cells at 28°C is reduced to about one-third in the *fps1*Δ mutant compared with the wild-type (Figure 4a and b). When the same experiment was performed at 0°C instead of 28°C, glycerol uptake by the *fps1*Δ mutant was virtually absent, whereas the wild-type strain showed reduced but still substantial glycerol influx (Figure 4a). Simple diffusion is affected more than facilitated diffusion by lower temperature since it requires a higher Arrhenius activation energy (e.g. Chrispeels and Agre, 1994). Hence, the plasma membrane permeability for glycerol appears to consist of two components. The first component, independent of Fps1, is undetectable at low temperature and probably represents simple (or free) diffusion through the phospholipid bilayer. The second component, dependent on Fps1, appears to be much less affected by low temperature and probably constitutes facilitated diffusion. At 28°C, the Fps1-dependent process accounts for about two-thirds of the total glycerol influx. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which allows protons to equilibrate across the membrane and therefore inhibits active transport processes, did not affect glycerol uptake in similar experiments as those described above (data not shown). This result confirms that glycerol uptake occurs by diffusion.

In cells pre-grown in glycerol instead of glucose medium, the Fps1-dependent component represented ~80% of glycerol influx at 28°C and glycerol uptake occurred much faster (Figure 4b; note the different time scale of Figure 4a and b). Thus, synthesis or activity of the Fps1-mediated glycerol uptake is apparently stimulated by the presence of glycerol. Since these results point to a possible role of Fps1 in glycerol utilization, we tested whether growth of the *fps1*Δ mutant on glycerol as sole carbon source was affected. No such effect was observed under standard growth conditions (30°C) on synthetic medium containing different glycerol concentrations (not shown). Also, at lower growth temperature (15°C), where free diffusion might have been limiting, the *fps1*Δ mutant grew like the wild-type strain. Thus, the free diffusion component of glycerol uptake also appears to be sufficient for glycerol assimilation at the lower temperature.

Our results show that Fps1 affects the permeability of the plasma membrane for glycerol in both directions, i.e. uptake and efflux (Figures 3 and 4). Another metabolite that is both produced by yeast cells as well as utilized as carbon source is ethanol and it is also believed to permeate the membrane passively (Kotyk and Alonso, 1985). Deletion of Fps1, however, did not effect the rate of ethanol

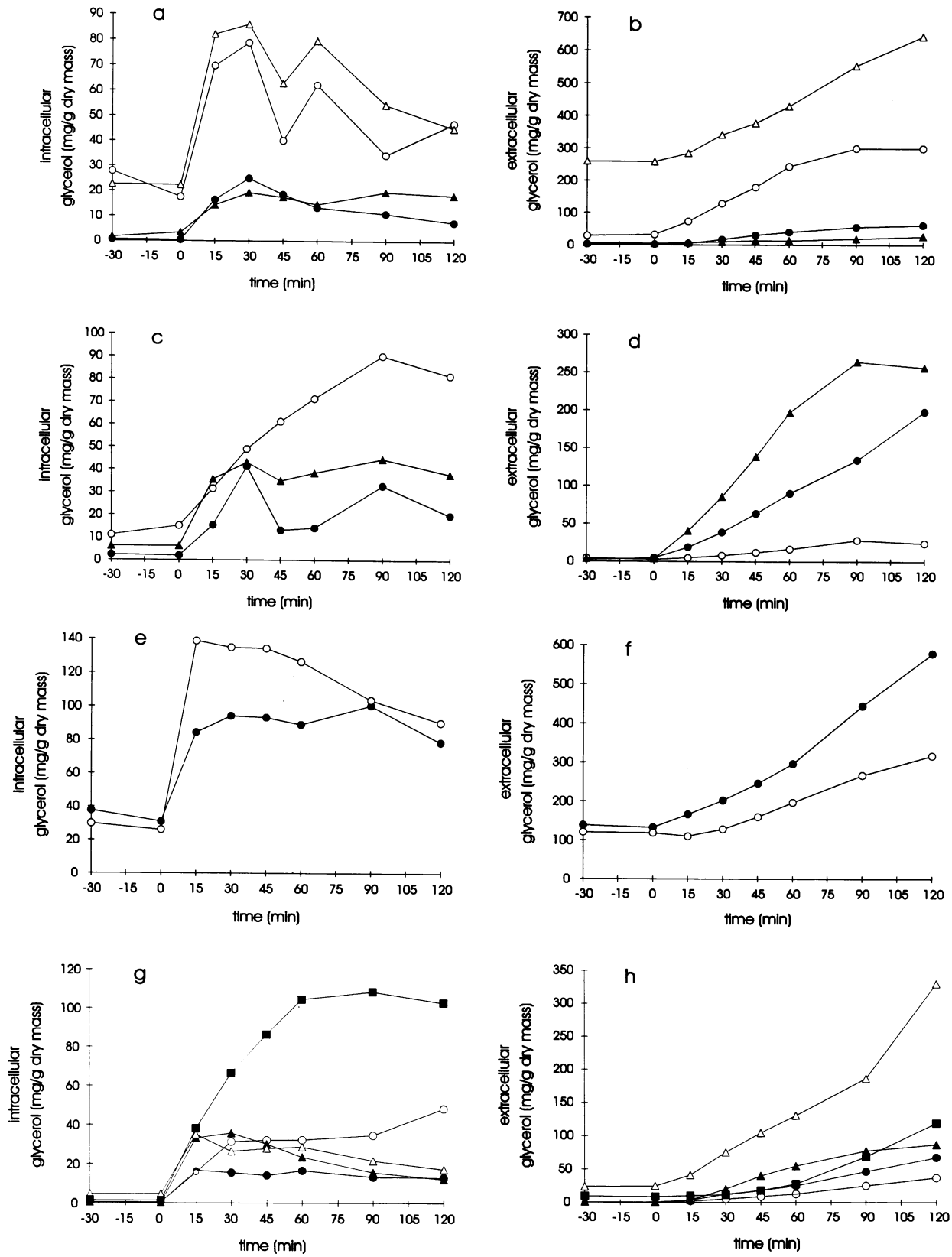

Fig. 3. Intra- and extracellular glycerol in different yeast strains pre-grown in synthetic medium containing 3% ethanol after the addition of 100 mM glucose at time zero. (**a**) Intra- and (**b**) extracellular glycerol in the wild-type (●), the *ggs1/tps1*Δ mutant (○), the *ggs1/tps1*Δ*gpd1*Δ double mutant (▲) and the *ggs1/tps1*Δ mutant transformed with the multicopy plasmid YEp*lac*195 containing *GPD1* (△). (**c**) Intra- and (**d**) extracellular glycerol in the *ggs1/tps1*Δ mutant (●), the *ggs1/tps1*Δ*fps1*Δ double mutant (○) and the *ggs1/tps1*Δ mutant transformed with the multicopy plasmid YEp*lac*195 carrying *FPS1* (▲). (**e**) Intra- and (**f**) extracellular glycerol in the *ggs1/tps1*Δ mutant transformed with the multicopy plasmid YEp*lac*195 containing *GPD1* (●) and the *ggs1/tps1*Δ*fps1*Δ double mutant transformed with the same plasmid (○). (**g**) Intra- and (**h**) extracellular glycerol in the wild-type (●), the *fps1*Δ mutant (○), the wild-type transformed with the multicopy plasmid YEp*lac*195 carrying *FPS1* (▲) or *GPD1* (△), and the *fps1*Δ mutant transformed with the same plasmid carrying *GPD1* (■).

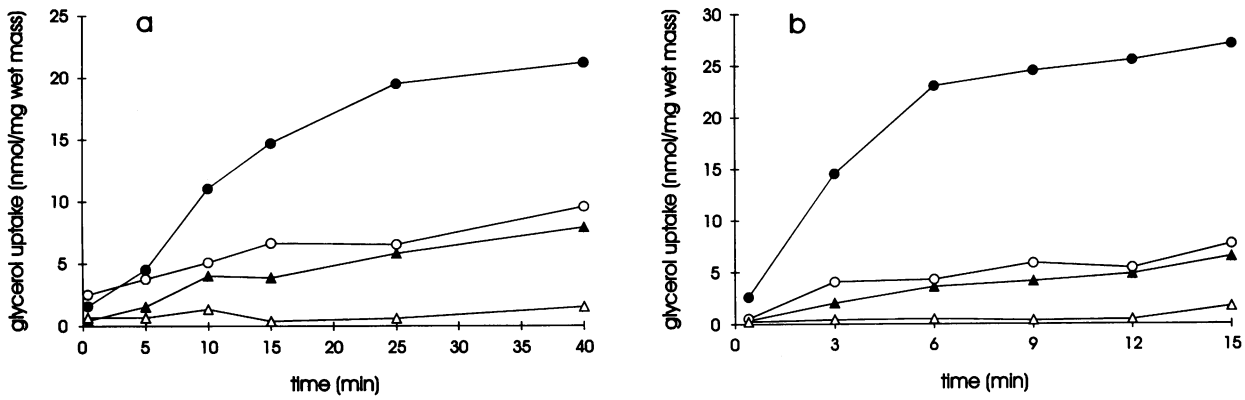

Fig. 4. Uptake of glycerol measured as the accumulation of radioactivity derived from labelled glycerol as a function of time after addition of glycerol to a final concentration of 100 mM (containing 20 μM radioactive glycerol). (**a**) Cells pre-grown in yeast extract/peptone medium containing 2% glucose. (**b**) Cells pre-grown in yeast extract/peptone medium, but containing 3% glycerol. Strains and conditions: wild-type incubated at 28°C (●) and at 0°C (○), the *fps1*Δ mutant incubated at 28°C (▲) and at 0°C (△).

influx into yeast cells in experiments similar to those described for glycerol. This indicates that either Fps1 does not transport ethanol or that free diffusion of ethanol occurs at a rate too rapid to detect a possible role for Fps1 (results not shown).

The E.coli glycerol facilitator partially substitutes for yeast Fps1

If Fps1 functions as a glycerol facilitator in yeast, the *E.coli* counterpart might be able to substitute for Fps1 in an *fps1*Δ strain. Using the yeast *PGK1* promoter and a multicopy plasmid, we have expressed the *E.coli* glycerol facilitator (Muramatsu and Mizuno, 1989) in the *ggs1/tps1*Δ*fps1*Δ double mutant which cannot grow on glucose. Transformation with a multicopy plasmid carrying the *FPS1* wild-type gene restores growth on glucose (Figures 1 and 5). The plasmid carrying the *E.coli* glycerol facilitator (GlpF) expression construct conferred growth on glucose, but much less than the *FPS1* plasmid (Figure 5). Since addition of 5% glycerol, but not of 5% sorbitol (not shown), completely prevented growth on glucose, suppression by GlpF is likely to be due to enhanced glycerol export (Figure 5). Expression of GlpF had very much the same effect on glycerol production and efflux as the yeast facilitator protein, in that the intracellular glycerol concentration was reduced and the extracellular glycerol level enhanced (Figure 6a and b). In addition, GlpF restored glycerol uptake in an *fps1*Δ mutant at 28°C, as did the wild-type Fps1 protein, and it even enhanced glycerol uptake at 0°C (Figure 7). We conclude that yeast Fps1 and the *E.coli* GlpF are interchangeable in yeast with respect to their effects on glycerol production and transport, but only partially for the control of growth on fermentable sugars in the *ggs1/tps1*Δ mutant strain.

The function of Fps1 is affected by osmotic stress

Glycerol plays an essential role as compatible solute for growth of yeast cells under reduced water availability, a condition generally described as hyperosmotic stress (for a review, see Blomberg and Adler, 1992). *Gpd1*Δ mutants lacking glycerol-3-phosphate dehydrogenase are unable to accumulate large amounts of glycerol and hence they are osmosensitive (Albertyn *et al.*, 1994b). Expression of *GPD1* is induced by osmotic stress and this induction depends on the *H*igh *O*smolarity *G*lycerol (HOG) response

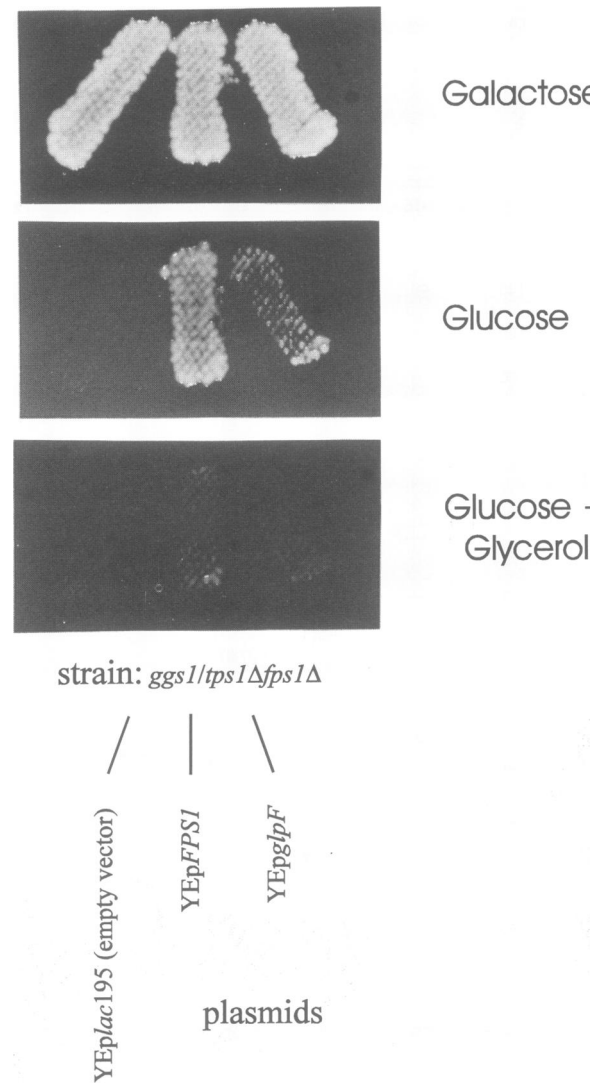

Fig. 5. Partial suppression of the growth defect on glucose of the *ggs1/tps1*Δ*fps1*Δ double mutant by overexpression of the gene for the *E.coli* glycerol facilitator (using the yeast *PGK1* promoter and a multicopy plasmid). Cells were pre-grown on synthetic medium containing 2% galactose and replicated onto yeast extract/peptone medium containg 2% galactose, 2% glucose or 2% glucose plus 5% glycerol. The strain *ggs1/tps1*Δ*fps1*Δ was transformed with the indicated plasmids.

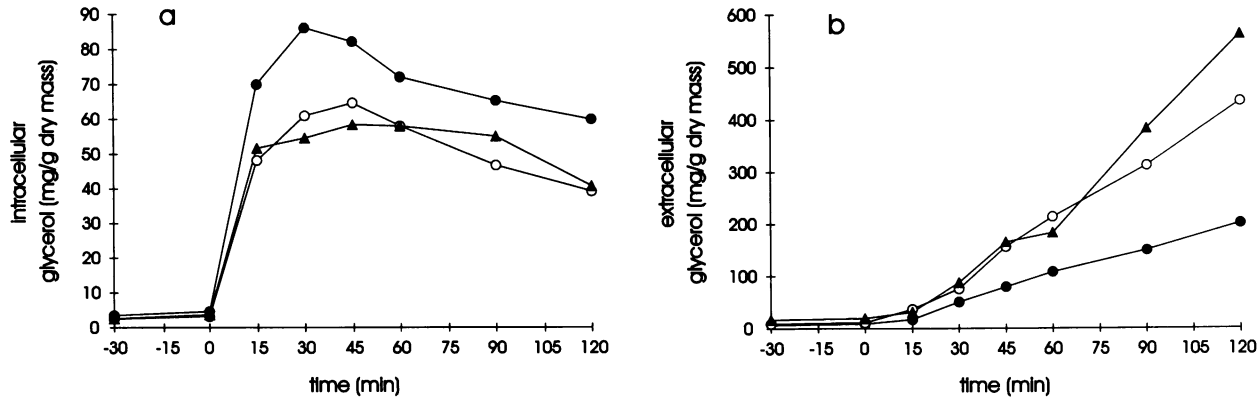

Fig. 6. Glycerol levels in yeast cells expressing the *E.coli* glycerol facilitator. (**a**) Intra- and (**b**) extracellular glycerol after the addition of glucose at time zero to ethanol-grown cells of the *ggs1/tps1Δfps1Δ* double mutant (●) and the same mutant transformed with a multicopy plasmid containing either *FPS1* (○) or the gene for the *E.coli* glycerol facilitator (▲).

pathway, of which Hog1 is the most terminal protein kinase known. *Hog1Δ* mutants are also osmosensitive (Brewster *et al.*, 1993; Albertyn *et al.*, 1994b). Since glycerol must both be retained in the cytosol under osmotic stress and released when hyperosmotic stress is lifted, a possible function of Fps1 in controlling membrane permeability for glycerol under such conditions appeared plausible.

Under hyperosmotic stress (5% salt), cells of an *fps1Δ* mutant accumulated the same amount of glycerol as those of the wild-type strain, consistent with the idea that Fps1 might be inactive under such conditions (Figure 8, time zero). When the cells were transferred into salt-free medium (i.e. a hypo-osmotic shock was given), the wild-type cells lost ~50% of the accumulated glycerol within 3 min. The cells of the *fps1Δ* mutant, on the other hand, required ~2 h to lose 50% of their glycerol, eventually again reaching the same intracellular glycerol level as the wild-type (Figure 8). This indicates that under hyperosmotic stress the Fps1 channel remains present, but is apparently closed, and that it can be opened quickly upon a hypo-osmotic shock. Survival of the *fps1Δ* mutant cells fell to <5% of that of the wild-type after a hypo-osmotic shock from 8% NaCl into water, suggesting that the quick release of the accumulated glycerol is an essential step in the adaptation to lower external osmolarity.

Since the Fps1 glycerol channel functions in both directions, we also investigated its control under osmotic stress using glycerol-uptake experiments. We included the *hog1Δ* mutant in these experiments in order to check whether the HOG pathway is involved in the control of membrane permeability for glycerol. When glycerol uptake by osmotically stressed cells was measured at 0°C, where free diffusion is extremely low, none of the three strains tested—the wild-type, the *fps1Δ* and the *hog1Δ* mutant—showed detectable glycerol uptake (Figure 9a). The same results were obtained with a *gpd1Δ* mutant which was used as an osmosensitive control strain (not shown). The *fps1Δ* mutant also did not show glycerol uptake when grown under normal conditions, as had been described above (Figure 4). When glycerol transport under conditions of osmotic stress was measured at 28°C, where simple diffusion also contributes significantly to glycerol influx, the wild-type strain, the *fps1Δ* mutant and the *hog1Δ*

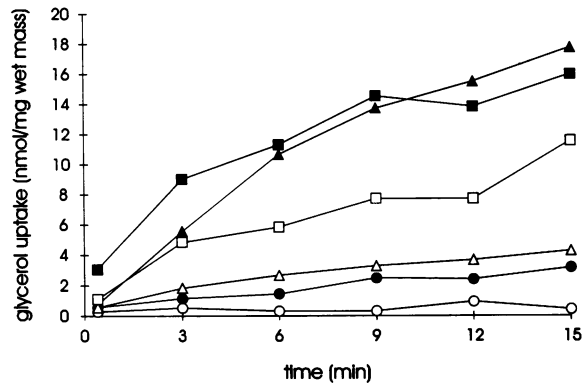

Fig. 7. Glycerol uptake in yeast cells expressing the *E.coli* glycerol facilitator. At 28°C: *Fps1Δ* mutant transformed with the empty vector YEp*lac*195 (●), the multicopy plasmid carrying the wild-type *FPS1* gene (▲) or the *glpF* gene for the *E.coli* glycerol facilitator (■). At 0°C: *Fps1Δ* mutant transformed with the empty vector YEp*lac*195 (○) and the *fps1Δ* mutant transformed with the multicopy plasmid carrying the wild-type *FPS1* gene (△) or the *glpF* gene for the *E.coli* glycerol facilitator (□).

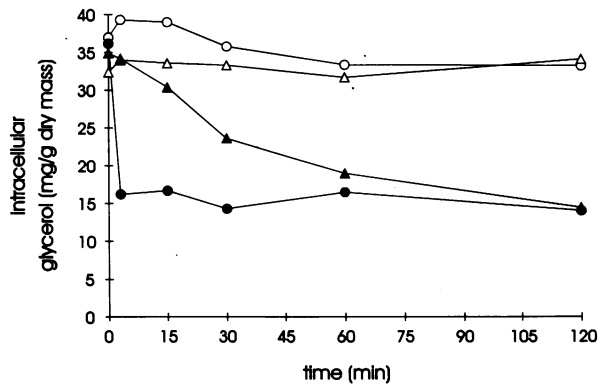

Fig. 8. Intracellular glycerol concentrations before and after release from hyperosmotic stress (hypo-osmotic shock). Strains and conditions: wild-type (●) and the *fps1Δ* mutant (▲) shifted from medium with 5% NaCl at time zero to medium containing no salt and the wild-type (○) and the *fps1Δ* mutant (△) shifted again to medium with 5% NaCl as control.

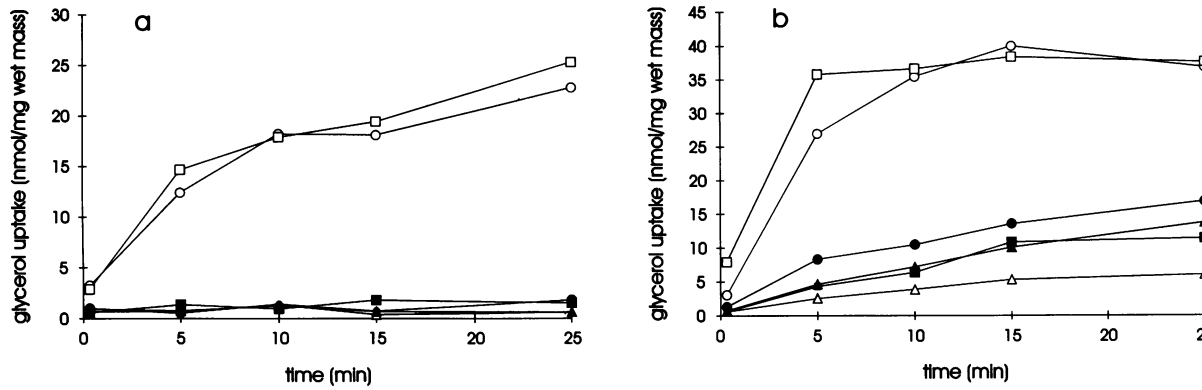

Fig. 9. Glycerol uptake of yeast cells under osmotic stress. (**a**) Incubation at 0°C to reduce simple diffusion. (**b**) Incubation at 28°C where both simple diffusion and facilitated diffusion by Fps1 are present. Strains: wild-type under osmotic stress after a 2 h incubation in the presence (●) or absence (○) of 7% NaCl, the *fps1*Δ mutant under osmotic stress (▲) and grown without salt (△) and the *hog1*Δ under osmotic stress (■) and grown without salt (□).

mutant showed about the same glycerol uptake rate (Figure 9b). Since under such conditions no uptake occurred at 0°C, the influx observed at 28°C must be mainly or exclusively due to free diffusion. Thus, free diffusion of glycerol can still occur under osmotic stress, but facilitated diffusion through the Fps1 channel is inactive. This inactivation appears to be independent of the Hog1 protein kinase since the *hog1*Δ mutant showed the same reduction in glycerol uptake as the wild-type. Interestingly, glycerol uptake at 28°C was consistently about two times higher in an *fps1*Δ mutant incubated under osmotic stress than in the same strain grown under normal conditions (Figure 9b), indicating that additional uptake mechanisms might be operating under osmotic stress to sequester glycerol from the medium.

Discussion

Fps1 is a glycerol facilitator

The yeast member of the MIP family of channel proteins, Fps1, is most closely related to the bacterial glycerol facilitators at the amino acid sequence level (Reizer *et al.*, 1993). Consistent with this sequence similarity, Fps1 also functions as a glycerol facilitator and it does so for glycerol influx and efflux. Genetic evidence for a role of Fps1 in glycerol metabolism was obtained through its relationship to *GPD1*, encoding glycerol-3-phosphate dehydrogenase, in the suppression of *ggs1/tps1*Δ mutants for growth on fermentable carbon sources. This evidence was corroborated by the partial complementation of an *fps1*Δ mutation in a *ggs1/tps1*Δ strain by the *E.coli* glycerol facilitator. Biochemical experiments showed that the absence of Fps1 enhances the ratio of intra- to extracellular glycerol and this was particularly clear in strains where glycerol is overproduced, for instance in *ggs1/tps1*Δ mutants or in strains overexpressing *GPD1*.

Glycerol-uptake experiments showed that the permeability of the yeast plasma membrane for glycerol consists of two components: one independent of Fps1 and probably due to free diffusion through the phospholipid bilayer, and a second component of higher capacity apparently due to facilitated diffusion by the Fps1 channel protein. The existence of a channel protein for glycerol allows the yeast cell to control and to adjust rapidly the permeability of the membrane for glycerol depending on the growth conditions. A high permeability seems advantageous when glycerol is utilized as a carbon source. Consistent with this, we found the Fps1-dependent component of glycerol influx to be higher in cells growing with glycerol than with glucose as carbon source. On the other hand, the cell must reduce efflux of glycerol through the plasma membrane when intracellularly produced glycerol has to be accumulated. Consistent with this, we found that under osmotic stress the Fps1 channel is apparently closed (see below).

We have established the role of Fps1 in glycerol transport both for glycerol influx and efflux. While the role in glycerol efflux was demonstrated in biochemical and genetic experiments, a possible role in glycerol influx was inferred from glycerol-uptake experiments. No phenotype has been observed in *fps1*Δ mutants related to an inability to take up glycerol efficiently, e.g. in utilization of glycerol as carbon source. Thus, simple diffusion of glycerol through the phospholipid bilayer appears to be sufficient for the capacity of the glycerol catabolic pathway. We suggest that Fps1 may be more important for the control of cytosolic glycerol concentration in relation to internally produced glycerol rather than for glycerol uptake and utilization. However, we cannot exclude such a role for the latter under specific conditions.

Glycerol transport has been studied in other fungi much more extensively than in *S.cerevisiae* (Gancedo *et al.*, 1968) and two apparently different types of glycerol transporters have been reported. Genetic evidence suggests the existence of glycerol transporters involved in glycerol utilization in *Aspergillus nidulans* (Visser *et al.*, 1988) and in *Neurospora crassa* (Denor and Courtright, 1982), and such an uptake system has been characterized biochemically in *Fusarium oxysporum* (Castro and Loureiro-Dias, 1991), but none of the structural genes have been reported. Osmotolerant yeasts like *Zygosaccharomyces rouxii* (van Zyl *et al.*, 1990) and *Debaryomyces hansenii* (Lucas *et al.*, 1990) possess active glycerol-uptake systems which are induced under osmotic stress to accumulate glycerol. Also, in these instances, the isolation of a gene has not yet been reported. Since yeast Fps1 is neither

involved in glycerol utilization nor does it appear to constitute an active uptake system, it is possible that the glycerol transporters of other fungi are unrelated to Fps1. However, the *E.coli* glycerol facilitator, one of the closest homologues of Fps1, appears to be involved in glycerol catabolism since mutants lacking it grow more slowly on glycerol and the structural gene is part of the *glp* regulon for glycerol utilization (Sweet *et al.*, 1990). It remains to be established whether Fps1 homologues are involved in some way in glycerol utilization in fungi or whether they solely function as regulators of glycerol production and distribution.

Two independent observations appear to point to additional roles of Fps1 besides its function as a glycerol channel. First, the bacterial glycerol facilitator GlpF could fully complement for Fps1 function with respect to glycerol production, distribution of internally produced glycerol and glycerol uptake, but only partially for growth on fermentable sugars. A simple explanation for this effect could be that the expression of bacterial *glpF* from the strong yeast *PGK1* promoter causes some deleterious, unspecific effect. On the other hand, evidence has been reported that GlpF interacts specifically with glycerol kinase in glycerol catabolism (Voegele *et al.*, 1993). Thus, Fps1 could also interact with certain proteins, but GlpF might be unable to recognize these yeast proteins. This would then suggest that Fps1 has a specific role during growth on glucose, possibly by sensing the intracellular glycerol level and then controlling glycerol metabolism and probably its own activity as well. Although we do not know how such a control function might look, the second observation points in a similar direction. Overexpression of Fps1 leads to higher intra- and extracellular glycerol levels, and thus to a stimulation of total glycerol production, while deletion of *FPS1* has the opposite effect. The effect on total glycerol production could be explained simply by the channel function, through a faster efflux of the glycerol produced drawing the equilibrium towards glycerol production. However, the higher intracellular glycerol levels in cells overexpressing *FPS1* indicate a more direct control function.

Role of Fps1 in osmoregulation

Several members of the MIP family of channel proteins have been implicated in osmoregulation, i.e. in the control of cell water and turgor. CHIP28 (Preston *et al.*, 1992) and WCH-CD (Fushimi *et al.*, 1993) in mammalian cells, and γ-TIP (Maurel *et al.*, 1993) in plant vacuoles, are water channels. For the latter two proteins, it has been shown that their activity is regulated. WCH-CD is involved in urine concentration in kidney collecting tubules and the water permeability of the plasma membrane of these cells is regulated by vasopressin which might exert its effect through protein kinase A (Fushimi *et al.*, 1993). γ-TIP is strongly expressed in root tips of plants and the appearance of certain isoforms of TIP is induced by water stress (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992; Ma, 1993; Maurel *et al.*, 1993). Calcium-dependent protein phosphorylation might be involved in this control, as has been shown for nodulin-26, a member of the MIP family that transports malate (Ouyang *et al.*, 1991). Water channels have not been found in micro-organisms and the *E.coli* glycerol facilitator was unable to transport water when expressed in *Xenopus* oocytes (Maurel *et al.*, 1993, 1994). Osmoregulation in microorganisms might be exerted mainly through the production of compatible solutes rather then transport of water, an obvious strategy considering their unicellular nature (Yancey *et al.*, 1982; Blomberg and Adler, 1992).

Glycerol is the sole compatible solute in *S.cerevisiae* (Blomberg and Adler, 1992). As such, it has to be accumulated under hyperosmotic conditions and it has to be released again when conditions return to normal or lower osmolarity. The membrane permability for glycerol is indeed controlled by medium osmolarity and this control appears to be exerted mainly or solely through regulation of the Fps1 channel. Thus, Fps1 is involved in osmoregulation by controlling the cytoplasmic concentration of the compatible solute. Since membrane permeability for glycerol is very rapidly restored in wild-type but not in *fps1*Δ cells when osmotic stress is lifted, the inactivation of the Fps1 channel must be reversible, pointing to a protein modification like phosphorylation. We have tested the most obvious candidate protein kinase, the product of the *HOG1* gene, which is known to be essential for osmoadaptation and for osmostress-induced expression of *GPD1* (Brewster *et al.*, 1993; Albertyn *et al.*, 1994b; Brewster and Gustin, 1994). However, *hog1*Δ mutants are still able to reduce glycerol influx under osmostress like the wild-type. Thus, the Fps1-dependent component of membrane permeability for glycerol is controlled by other mechanisms than the HOG pathway. It has been shown previously that although *hog1*Δ mutants do not increase glycerol-3-phosphate dehydrogenase activity under osmotic stress, they still accumulate up to 40% of the wild-type glycerol level (Brewster *et al.*, 1993; Albertyn *et al.*, 1994b). This further confirms that *hog1*Δ cells can still reduce membrane permeability for glycerol under osmostress and also points to flux into the glycerol biosynthetic pathway being controlled in a Hog1-independent manner.

It has been suggested that an active glycerol-uptake system might be induced under osmotic stress to accumulate glycerol from the environment, as in the distantly related, osmotolerant yeasts *Z.rouxii* and *D.hansenii* (Lucas *et al.*, 1990; Van Zyl *et al.*, 1990). No direct evidence has been obtained for such an uptake mechanism in *S.cerevisiae*. However, three independent observations indicate that such an uptake system might exist. First, *gpd1*Δ mutants which produce only little glycerol increase their intracellular glycerol level under osmotic stress at the expense of external glycerol (Albertyn *et al.*, 1994b). Physiological evidence for the existence of such an active uptake system comes from the observation that *gpd1*Δ mutants partially recover from osmotic stress when as little as 5 mM glycerol is added to the medium. Fps1 is probably not involved in this process since the *gpd1*Δ*fps1*Δ double mutant showed the same recovery phenomenon with 5 mM glycerol on high-salt medium (R.Ansell and S.Hohmann, unpublished results). The third indication comes from the glycerol-uptake experiments in the present paper, carried out with osmotically stressed *fps1*Δ cells which took up more glycerol than the cells grown under normal conditions (Figure 9). Probably, an osmostress-inducible uptake system has escaped attention up to now because of the large amount of glycerol produced by

S.cerevisiae. Hence, a *gpd1Δfps1Δ* mutant appears the right strain to look for such a transport system in more detail since it produces only little glycerol and facilitated diffusion is absent.

Glycerol production and the control of yeast glycolysis by the inorganic phosphate level

Ggs1/tps1 mutants have been studied for many years under different names (*fdp1*, *cif1*, *byp1*). The role of the *GGS1/TPS1* gene product in the regulation of glycolysis, as predicted from the phenotype of the mutants lacking it, appears to be in restricting glucose influx into the pathway. We have demonstrated previously that upon glucose addition, *ggs/tps1Δ* mutants consume their entire intracellular inorganic phosphate (P_i) pools for the production and accumulation of sugar phosphates (Van Aelst *et al.*, 1991). Also, external P_i was shown by *in vivo* ^{31}P-NMR experiments to be utilized for the same purpose, but at a much slower rate. Addition of up to 50 mM of P_i to the growth medium and/or overexpression of a P_i transporter did not rescue a *ggs1/tps1Δ* mutant on glucose medium (S.Hohmann and J.M.Thevelein, unpublished results). Probably, the P_i uptake capacity cannot cope with the high metabolic flux through glycolysis. *ggs1/tps1* mutants appear to suffer from a bottleneck in glycolysis at the level of glyceraldehyde-3-phosphate dehydrogenase, which requires NAD, ADP and P_i as substrates. Since the NAD/NADH ratio is unaffected in these mutants and the ADP level even increases substantially at the expense of ATP (Van Aelst *et al.*, 1991; Hohmann *et al.*, 1993), the fall in the P_i level seems to be a major consequence of the lack in sugar influx control and the real cause of the inhibition of glycolysis.

Suppression by enhanced glycerol production of the growth defect of *ggs1/tps1Δ* mutants on glucose seems to confirm this conclusion. Since glycerol synthesis liberates P_i, it has the potential to secure continued availability of P_i. It has been appreciated for a long time that glycerol production is important for maintaining an NAD^+/NADH ratio favourable for glycolytic flux. NAD^+ consumed by the glyceraldehyde-3-phosphate dehydrogenase reaction is normally re-oxidized by alcohol dehydrogenase in the terminal step of alcoholic fermentation (Van Dijken and Scheffers, 1986; Gancedo and Serrano, 1989). If this activiy is for some reason reduced, NADH can be oxidized alternatively by glycerol-3-phosphate dehydrogenase. Our results suggest that glycerol production might also play a role in balancing the ratio of free to bound phosphate in the cytosol.

The actual role of Ggs1/Tps1 in the control of sugar influx into glycolysis is still not clear. In a first model, it has been proposed that the *GGS1/TPS1* gene product, which is responsible for trehalose-6-phosphate synthase activity, has an additional function controlling directly sugar transport and/or phosphorylation (Thevelein, 1992; Van Aelst *et al.*, 1993). In the second model, trehalose synthesis was proposed to act as a metabolic buffer for P_i homeostasis during transition from respiratory to fermentative metabolism. Under this condition, glucose influx was presumed to occur too rapidly, necessitating a mechanism to rescue the cells from P_i depletion due to hyperaccumulation of sugar phosphates (Hohmann *et al.*, 1993). This model predicted that stimulation of glycerol production might have the same effect since it also liberates P_i. This idea led to the finding reported in the present paper that overexpression of *GPD1* efficiently suppresses the growth defect of the *ggs1/tps1Δ* mutant. Therefore the data presented here appear to support this second model without providing definite proof. Recently, a third model for the mechanism by which Ggs1/Tps1 restricts glucose influx into glycolysis has been proposed. It is based on the finding that trehalose-6-phosphate inhibits hexokinase *in vitro*, but *in vivo* evidence has not been reported (Blázquez *et al.*, 1993). A detailed discussion of the possible role of Ggs1/Tps1 in the control of glycolysis has been published elsewhere (Thevelein and Hohmann, 1995).

Materials and methods

Yeast genetics

The yeast strains used in this study are either isogenic to YSH 6.36.-3B *MATα leu2-3/112 ura3-52 trp1-92 SUC GAL mal0* (ATCC 90756; Schaaff *et al.*, 1989; Hohmann *et al.*, 1993), the so-called M5-background, or to W303-1A *MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2* (Thomas and Rothstein, 1989). Consistent results were obtained in experiments where these two backgrounds were compared (suppression by *GPD1* and *FPS1*, and glycerol production and distribution dependence on Fps1). The construction of deletion mutants for the various genes has been described previously: *ggs1/tps1Δ::TRP1* (Hohmann *et al.*, 1993); *fps1Δ::LEU2* (Van Aelst *et al.*, 1991); *gpd1Δ::TRP1* or *gpd1Δ::LEU2* (Albertyn *et al.*, 1994b).

Plasmid construction

For overexpression of specific genes, the multicopy vector YEp*lac*195 carrying *URA3* as selective marker was used throughout (Gietz and Sugino, 1988). *GGS1/TPS1* was cloned into this vector on a 4.6 kb *Hin*dIII/*Bam*HI fragment (Van Aelst *et al.*, 1993), *FPS1* on a 3.8 kb *Sal*I/*Hin*dIII fragment (Van Aelst *et al.*, 1991), *GPD1* on an 5.2 kb *Eco*RI fragment (Albertyn *et al.*, 1994b) and *MIG1* on a 3.6 kb *Xba*I/*Hin*dIII fragment (Nehlin and Ronne, 1990; Hohmann *et al.*, 1992).

For expression of the *E.coli* glycerol facilitator, a 1.8 kb *Hin*dIII fragment from plasmid pMA91 carrying the strong yeast promoter of the *PGK1* gene encoding phosphoglycerate kinase (Mellor *et al.*, 1983) was subcloned into YEp*lac*195. The *glpF* gene was cloned into the *Bgl*II site just downstream of the *PGK1* promoter on a 1.3 kb *Bam*HI fragment derived from plasmid pBENT6$^+$ which contains parts of the *E.coli glp* operon including the entire *glpF* gene and a fragment of *glpK* (Mizuno, 1987; Muramatsu and Mizuno, 1989). Expression in yeast was confirmed by Northern blot analysis.

Growth conditions

Yeast cells were grown in medium containing 2% peptone and 1% yeast extract (YEP) supplemented with carbon sources as indicated. Selection and growth of transformants carrying a replicating plasmid was done on synthetic medium as described by Sherman *et al.* (1986). For glucose-sensitivity assays, cells were pre-grown overnight in synthetic medium, transferred to the same medium containing 2% galactose and grown for one generation before glucose was added to different final concentrations. Tolerance to hyperosmotic stress was measured in YEP medium containing 2% glucose and NaCl to a final concentration of 5 or 7%, as indicated.

For experiments in which hyperosmotic stress was removed (hypo-osmotic shock), cells were pre-grown in YEP supplemented with 2% glucose up to an $OD_{600\ nm}$ of ~1.0. Then cells were stressed by the addition of NaCl to a final concentration of 5% and incubated for another 2 h with vigorous shaking. Cells were then sedimented and either resuspended in YEP glucose without salt or with 5% NaCl as control, and a first sample was taken after 3 min. All media were constantly kept at 30°C.

Glycerol determination

Intra- and extracellular glycerol was determined enzymatically using a commercial glycerol determination kit (Boehringer Mannheim) as described previously (Albertyn *et al.*, 1994a). For glycerol influx experiments, cells were pre-grown in YEP medium containing 5%

glucose or 3% glycerol as carbon source. For the preparation of osmotically stressed cells, the yeast was pre-grown in YEP medium with 2% glucose, sedimented and resuspended in the same medium containing 7% NaCl, and further incubated under shaking for 2 h. Cells were harvested by centrifugation, washed in water (or 7% NaCl) and resuspended in 25 mM 2-(*N*-morpholino) ethanesulphonic acid/$Ca(OH)_2$ pH 5.5–6.0, 0.1 mM $MgSO_4$ (containing 7% NaCl for osmotic stress experiments). Glycerol uptake was measured with an external concentration of 100 mM glycerol plus 20 μM ^{14}C-labelled glycerol (160 mCi/mmol). Samples were withdrawn by filtration, washed with ice-cold water (or 7% NaCl when osmotically stressed cells were investigated) and the radioactivity on the filter was determined as a measure for the glycerol taken up by the cells.

Data presented show the results of one typical experiment out of at least three independent experiments giving consistent results.

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