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Fps1p channel is the mediator of the major part of glycerol passive diffusion in *Saccharomyces cerevisiae*: artefacts and re-definitions

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Abstract

Glycerol has been shown to cross the plasma membrane of *Saccharomyces cerevisiae* through (1) a H^+/symport detected in cells grown on non-fermentable carbon sources, (2) the constitutively expressed Fps1p channel and (3) by passive diffusion. The Fps1p channel has been named a *facilitator* for mediating glycerol low affinity transport of the facilitated diffusion type. We present experimental evidence that this kinetic is an artefact created by glycerol kinase activity. Instead, the channel is shown to mediate the major part of glycerol's passive diffusion. This is not incompatible with Fps1p's major role in vivo, which has been previously shown to be the control of glycerol export under osmotic stress or in reaction to turgor changes. We also verified that *FPS1* overexpression caused an increase in H^+/symport V_{max}. Furthermore, *yfl054c* and *fps1* mutants were equally affected by exogenously added ethanol, being the correspondent passive diffusion stimulated. For the first time, to our knowledge, a phenotype attributed to the functioning of *YFL054c* gene is presented. Glycerol passive diffusion is thus apparently channel-mediated. This is discussed according to glycerol's chemical properties, which contradict the widely spread concept of glycerol's *liposoluble nature*. The discussion considers the multiple roles that the intracellular levels of glycerol and its pathway regulation might play as a central key to metabolism control.

Keywords: *S. cerevisiae*; Glycerol transport/permeability artefact; *FPS1*; *YFL 054c*; *GUT1*; *GUT2*

1. Introduction

In the yeast *Saccharomyces cerevisiae*, glycerol plays important roles in metabolism, from which the most studied is the one of compatible solute, accumulated under osmotic stress conditions. A lot of information is available on how the genes from glycerol pathways respond to osmotic stress signalling [1–5], regulating the production of this substrate in accordance to the degree of stress the cells are subjected to. Despite this, a retention problem remains to be completely clarified, essentially due to the so-called *liposoluble nature* of this compound [6,7]. Accordingly, glycerol should leak through the plasma membrane, regardless of the environmental conditions. This has been accepted as common knowledge among the yeast scientific community, somehow hampering the straightforward accepted of the existence of proteins able to permeate and control glycerol movements across the yeast plasma membrane.

According to previous results [8,9], glycerol is actively transported by a proton symport in cells cultivated in non-fermentable carbon sources, like ethanol, glycerol or acetate. It was shown to be subjected to glucose regulation, being undetectable in cells repressed by growth on glucose [8]. These, instead, take up glycerol through Fps1p, described as a low affinity transport of the facilitated diffusion type [10]. The basis for this assumption lied essentially in the fact that transport did not induce glycerol intracellular accumulation against its chemical gradient and was not affected by the action of an ionophore [10]. According to the same authors, mutants defective on *FPS1* gene did not display this uptake, the reason why it was attributed to Fps1p activity.

The *FPS1* gene codes for a channel-type protein of the MIP family [11,12], despite its unusual topology with two long terminal hydrophilic extensions [13–15]. The main physiological role of Fps1p, according to Tamás et al. [15], rather than taking up glycerol, is to regulate glycerol export under sudden osmotic down-shock, or in reaction to changes in cell turgor [16]. Yet, the channel closes upon osmotic up-

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shock, and can thus contribute to immediate glycerol retention [11,15,16]. Another ORF, with a high degree of homology to the *FPS1* gene [13,17], *YFL054c*, has been identified, but no related phenotype has been described so far [10,15], although both genes have been shown to be genetically close to aquaglyceroporins [13].

The physiological characterisation of *S. cerevisiae* glycerol H^+/symport [8] was performed using a diploid wild-type strain from the YPCC (IGC3507), cultivated in MM and collected in mid-exponential growth phase. Under these strict growth conditions, the low affinity glycerol activity attributed to Fps1p was not detected [8,10]. On the contrary, *fps1* mutants from W303-1A genetic background, grown under derepression conditions, using either ethanol or glycerol as carbon and energy sources, presented active glycerol H^+/symport, without a further detectable second-order kinetic uptake component [10]. This way, it was possible to establish that one and the other types of transport were unrelated, differently regulated and most likely used by the cell for different purposes [10].

This work presents evidence that the Fps1p channel does not mediate transport of a saturable nature, being the facilitated diffusion previously described [10], dependent on the presence and activity of glycerol kinase, encoded by *GUT1* gene. The interference of this enzyme on glycerol uptake measurements has been first described in *Escherichia coli* [19]. More recently, Holst et al. [18] also described this transport artefact in *S. cerevisiae* when the *GUP1* and *GUP2* genes were identified as putative glycerol transporters.

Further experimental data aims to clarify the relationship between Fps1p-mediated glycerol entry and passive diffusion changes, according to cell physical reaction to stress. A weak phenotype for *YFL054c*, suggesting that it can also mediate glycerol entry, is shown for the first time. Concomitantly, the long accepted concept of glycerol's *liposoluble nature* is re-visited and re-analysed.

2. Materials and methods

2.1. Yeast strains and growth conditions

S. cerevisiae strains, listed in Table 1, were maintained at 4 °C in YNB without amino acids (Difco) supplemented with 2% (w/v) glucose and amino acids, according to the strains demands, or YEP (peptone, 2% w/v and yeast extract, 1% w/v) supplemented with 2% (w/v) glucose (YEPD) and at −70 °C in glycerol 30% (w/v). Batch cultures were performed using YNB (Difco) (MM) or complete medium (YEP) supplemented with 2% (w/v) glucose or 2% (v/v) ethanol as carbon sources. Incubation conditions were standardised at 30 °C and 180 rpm orbital shaking, in 500 ml Erlenmeyer flasks containing 200 ml of growth medium. Growth was followed spectrophotometrically at 600 nm.

Table 1
S. cerevisiae strains used in this work

Strain designation	Genotype	Origin/description
IGC3507	wild-type (diploid)	PYCC[a]
W303-1A	*MATa leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100*	Thomas and Rothstein [59][b]
YSH 6.36.-3B	*Matα leu 2-3/112 trp1-92 ura 3-52 GAL SUC mal0*	Hohmann et al. [60][b]
–	W303-1A *fps1*::LEU2	van Aelst et al. [12][b]
–	W303-1A *fps1*::LEU2 *YLF054c*::TRP1	van Aelst et al. [12][b]
YSH 294	YSH 6.36.-3B yEplac195 FPS1::URA3	Luyten et al. [11][b]
–	W303-1A yEplac195 FPS1::URA3	Luyten et al. [11][b]
Cly1	W303-1A *gut1*Δ	Holst et al. [18][c]
BHY40	W303-1A *gut2*Δ	Holst et al. [18][c]

[a] Portuguese Yeast Culture Collection (New University of Lisbon, Portugal).
[b] S. Hohmann (Göteborg University, Sweden) and J. Thevelein (Katholieke Universiteit te Leuven, Belgium).
[c] Carlsberg Laboratory, Yeast Physiology Department, Copenhagen, Denmark.

2.2. Measurements of glycerol uptake

Glucose- or glycerol-grown cells were harvested by centrifugation, washed twice and resuspended to a final concentration of ±30 mg dry weight ml^{-1} in ice-cold distilled water. Initial uptake rates of glycerol were determined as described before [8]. [^{14}C]-glycerol ethanol-free solutions ranged from 0.2 to 40 mM, with variable specific activity (s.a.), respectively, from 2800 to 250 dpm $nmol^{-1}$. Uptake was linear up to at least 20 s for all glycerol concentrations used. Assays were composed of three replicas and one *blank* for each solute concentration. Computer regression analysis program GraphPad PRISM® (1994–1997 Copyright GraphPad Software, Inc.) was used to determine transport kinetic parameters and evaluate their statistical validity.

2.3. Measurement of intracellular volume

The intracellular volume was measured as previously described [20,21]. Measurements were performed in the absence and in the presence of sodium chloride mixed with the reaction buffer using different incubation periods.

2.4. Measurement of [^{14}C]-glycerol accumulation

To measure [^{14}C]-glycerol accumulation, the same method described before was used [22]. The experiments were started by the addition of 10 mM [^{14}C]-glycerol (s.a. ±700 dpm $nmol^{-1}$). Parallel experiments were performed, adding to the reaction buffer from the start of the experiment, 50 μM of the ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). The capacity to induce efflux

of intracellular accumulated radiolabelled glycerol was assayed with 50 mM *cold* (non-radioactive) glycerol.

2.5. Measurements of intracellular and extracellular compounds

Compound identification and quantification was performed by chromatography (HPLC), using the same methodology applied before [8].

2.6. Enzymatic assays

Cell-free extracts were prepared according to Neves et al. [23]. Glycerol kinase activity and total protein concentration determinations were done according to the methodology previously described [18,24].

2.7. Mutant strains integrity control

Considering that the results we are presenting contradict previous results and concepts, special care was taken with the control of the mutant interruptions of both *gut1* and *gut2*, and most important, of *fps1*. This was done checking the correspondent auxotrophic marks, as well as testing PCR amplification of the deleted DNA strains with specific primers for each gene (not shown). Furthermore, the *fps1* mutant strains were assayed as to arsenite and antimonite survival ability in comparison to parental strain W303-1A, according to Wysocki et al. [25] (see Acknowledgement).

2.8. Northern analysis of FPS1 expression

For RNA isolation, cells were harvested by centrifugation in mid-exponential growth phase (A_{600} 0.4–0.5) and during diauxic-shift (A_{600} 1.1–1.2), and washed twice with ice-cold distilled water. RNA was isolated with hot acidic phenol according to standard procedures [26–28]. Probes for *FPS1* and internal standard, rRNA 18S, were obtained by PCR, using genomic DNA as template for *FPS1* and cDNA for internal standard. Primers designed for *FPS1*, were: forward, 5′CCTACAGTCTTGCCCTCCAC3′, and reverse, 5′AACATTCCCGCAACACTTTC3′. Primers designed for internal standard were: forward, 5′AGGAATTGACGGAAGGGCAC3′, and reverse 5′GGACATCTAAGGGCATCACA3′. Probes were labelled with alkaline phosphatase with CDP-Star™ (Amersham Pharmacia Biotech) for subsequent chemiluminescent detection with CDP-Star™ detection reagent. Membranes were revealed using Hyperfilm™ ECL. Images were digitalised and densitometric analysis was performed using NIH Image 1.60 software.

2.9. Ethanol effect over glycerol entry

Cells were cultured as mentioned above in YEPD up to mid-exponential growth phase, ≈ 0.5 A_{600}. Uptake was measured incubating the cells for 5 min at 30 °C in Tris–citrate buffer adjusted to pH 5.0 and containing 12% (v/v) ethanol (reagent grade). Uptake was performed using ethanol-free [^{14}C]-glycerol solutions, ranging from 10 to 60 mM, with variable s.a., ranging, respectively from ± 3900 to ± 300 dpm nmol^{-1}. Diffusion constants (K_d) were calculated using computer regression analysis software GraphPad PRISM® as mentioned above.

2.10. Reproducibility of results

All the transport and enzyme assays presented are results of at least three independent assays. and the data reported are either average or representative values. The number of experiments was mentioned whenever considered relevant.

3. Results

3.1. Glycerol transport studies in MM glucose-grown cells

The wt strain IGC3507 was cultivated in MM with glucose and collected along the growth curve until stationary phase. During all growth phases, including diauxic-shift, in which glucose had been reduced to less than 0.02% (w/v) and considerable amounts of ethanol accumulated in the medium, glycerol uptake followed first-order kinetics, according to what is generally classified as passive diffusion [8]. In spite of the partial derepression that happens during glucose exhaustion, glycerol saturation kinetics uptake was not detected (not shown). These results were identical to the ones found previously in W303-1A, as well as in the correspondent *fps1* mutant [10]. *S. cerevisiae* cells cultivated in MM with glucose do not display glycerol-mediated uptake.

3.2. Glycerol transport studies in YEPD-grown cells

According to the results presented by Sutherland et al. [10], glycerol uptake in W303-1A YEPD-grown cells presented a low affinity Michaelis–Menten kinetic component, together with a first-order kinetic branch. Experimental results should thus fit the following equation:

$$V_{app} = [V_{max}[S]/(K_m + [S])] + K_d[S] \quad (1)$$

The mediated uptake was attributed to Fps1p and classified as facilitated diffusion [10]. We repeated these experiments, using YEPD-grown IGC3507 and results were identical, except for the fact that the glycerol uptake low affinity Michaelis–Menten component was not detected during exponential growth, but rather in cells collected during diauxic-shift (Fig. 1). Experimental raw data was: K_m 24.6 ± 3.6 (3) mM and V_{max} 288.4 ± 77.3 μmol h^{-1} g^{-1} dry weight. Iteration by computer regression analysis, transformed these into: K_m 7.7 ± 2.2 mM and V_{max} 91.1 ±

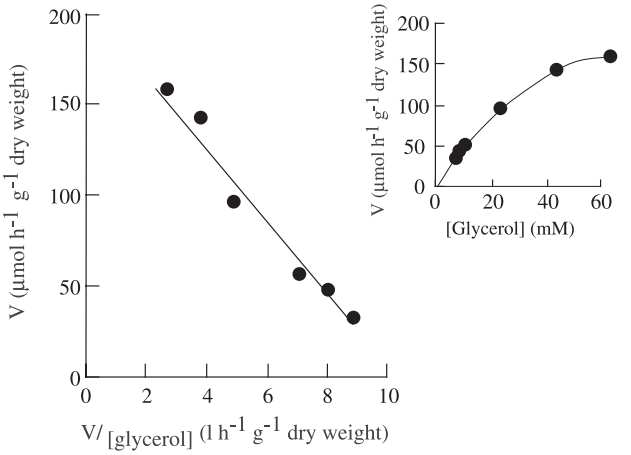

Fig. 1. Eadie–Hofstee plot of [^{14}C]-glycerol initial uptake rates of IGC3507 YEPD-grown cells collected in diauxic-shift and assayed at 30 °C, in Tris–citrate buffer, at pH 5.0. Insert: Michaelis–Menten presentation of the same experimental results.

30.9 µmol h^{-1} g^{-1} dry weight and suggested the existence of a non-saturable branch with a K_d of 0.003 ± 0.001 l h^{-1} g^{-1} dry weight, which was nevertheless not detected experimentally. The K_m value thus estimated was almost 10 times higher than the one determined before for glycerol H^+/symport [8] in the same strain, and V_{max} was considerably lower. Moreover, K_d lied around 0.003 l h^{-1} g^{-1} dry weight, approximately four times lower the value published for W303-1A [10]. These results are thus apparently consistent with the existence of a facilitated diffusion for glycerol, together with an underlying passive diffusion as previously published [10], but they suggest that this might be exclusively present in cultures collected during fermentation/respiration transition phase.

3.3. Glycerol passive diffusion and cell volume changes in salt-shocked MM glucose-grown cells

Transferring MM-glucose-grown cells to MM-glucose with increasing NaCl concentrations, resulted, as expected, in a proportional increase in intracellular accumulated glycerol (Fig. 2). This accumulation, must be, at first hand, the consequence of an increase in glycerol production, due to *GPD1* and *GPP2* transcription stimulation by salt stress [1,2,4] through specific signal transduction pathways [3,29]. On the other hand, glycerol accumulation must also be the consequence of an increase in glycerol retention ability. This is mostly unlikely connected to active transport, since, besides other results [8] cells transferred to medium containing 1 M NaCl, after a 3-h incubation period did not present proton symport activity. Glycerol retention could more likely be the consequence of both (i) the closing of Fps1p channel [15,16] and a reduction in passive diffusion, since it reduces to ± 1/3 when cells are shocked with 1 M NaCl [8].

Nevertheless, since cell volume decrease is one of the most direct and visible changes occurring as a consequence of osmotic shock [8,9,30–32], glycerol retention could also be an indirect consequence of cell shrinking, The percentage of decrease of *S. cerevisiae* intracellular volume in relation to the degree of salt stress present during growth has been reported before for IGC3507 strain [8,9,31] and is directly proportional to the amount of salt used. We repeated these determinations using W303-1A, and similar results were obtained (not shown). Thus considering, and taking also into consideration that Fps1p channel, in W303-1A, has been shown to close upon hyperosmotic salt shock, reducing glycerol uptake to ± 20–30% [15], we decided to measure W303-1A volume reduction after salt shock. Cells were shocked for 2 min with 500 mM and 1 M NaCl. Volume was reduced to 70% and 40%, respectively.

We compared these results with glycerol passive diffusion K_d changes under the same experimental conditions. K_d measured in 1 M salt-shocked cells reduced from 0.005 ± 0.001 (n = 5) to 0.002 ± 0.0004 l h^{-1} g^{-1} dry weight (n = 3), i.e. ± 40%. Therefore, the percentage of reduction of *S. cerevisiae* cell volume was identical to the percentage of reduction of passive diffusion K_d under the same experimental conditions, and very similar to the percentage of glycerol uptake left after Fps1p closing [15].

3.4. Glycerol transport studies in salt-shocked YEPD-grown cells

Being aware that MM is a very stringent medium and that the previously published results concerning Fps1p kinetic properties were obtained cultivating the cells in

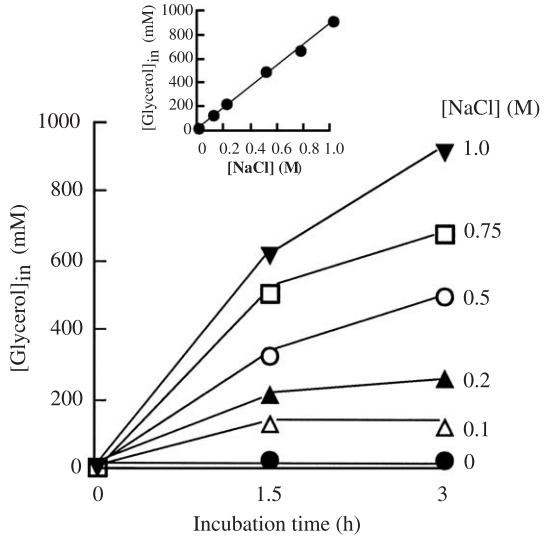

Fig. 2. Variation in intracellular glycerol as a function of extracellular salt concentration in the growth medium. *S. cerevisiae* IGC3507 was cultivated on MM with 2% glucose without salt and transferred medium with salt and incubated at 30 °C. Initial medium pH was adjusted to 4.5. Insert: Variation of glycerol concentration achieved after 3 h incubation as a function of external salt concentration.

YEPD [10], glycerol uptake study was repeated in cells cultured in YEPD and shocked in salt as above.

IGC3507 YEPD-grown cells were submitted to 2 min salt-shock with 500 mM and 1 M NaCl. Glycerol uptake was assayed using only three labelled glycerol concentrations 6, 14 and 50 mM. These concentrations were chosen for representing, according to Eq. (1), increasingly higher contribution of passive diffusion to the experimental uptake velocity measured. Results showed a reduction of uptake proportional to salt concentration, only perceptible for the higher substrate concentration. The degree of uptake reduction using 50 mM glycerol in the presence of 1 M NaCl was 40%, while the uptake of 6 and 14 mM were not affected by salt. This shows that only very high glycerol concentrations, beyond doubt in the range of glycerol passive diffusion, are affected by salt. Again, the degree of reduction in uptake in 1 M salt-shocked cells was identical to the degree of reduction presented above.

Furthermore, using the corresponding experimentally measured velocities for each substrate concentration, it was possible to estimate different $K_{m\ app}$ constants for glycerol uptake, 21.6, 16.6 and 13.7 mM at 0, 500 mM and 1 M NaCl, respectively. These follow a linear reduction with salt (corr. − 0.998). This means that artefacts in K_m determination can easily be created by using substrate concentrations which considerably increase the importance of the first-order kinetics component in Eq. (1)'s global result.

Results suggest that glycerol retention under salt shock could, besides being the result of Fps1p closure, since this should happen at a sub-minute time scale under such circumstances [11,15], also happens through a decrease in passive diffusion, as a consequence of cell shrinkage. This way, the K_d measured after salt-shock would be the remaining glycerol entry which is not Fps1p-mediated.

At this stage, we can thus state that *S. cerevisiae* cells repressed by growth on glucose, both in MM and YEP, display first-order kinetics glycerol uptake, characteristic of passive diffusion, not affected by growth medium composition, but affected by the presence of salt. Furthermore, cells collected during diauxic-shift, and thus partially derepressed, present a low affinity saturable kinetic branch for glycerol entry, which, unlike passive diffusion, is not affected by salt stress, but depends on growth media stringency, since it could be detected in YEP but not in MM.

3.5. Glycerol kinase putative interference in uptake measurements

Glycerol kinase is induced during diauxic-shift [24,33], and it has been proven to be able to interfere with glycerol uptake measurements in *S. cerevisiae* [18]. We measured glycerol kinase levels of activity in IGC3507 cells collected along growth in MM-glucose and YEPD (Fig. 3). Additionally, we also measured glucose consumption and extracellular accumulation of glycerol and ethanol (Fig. 3). Regardless to growth phase, *S. cerevisiae* cultivated in MM-glucose presented more glycerol than cells cultivated in YEPD. Consistently, glycerol kinase levels were much higher in cells cultivated in YEPD than in MM (Fig. 3A,B). In both media, ethanol levels achieved in late exponential phase were rather similar and glucose disappeared from the medium at a similar rate. These results are consistent with a recent publication [24], which shows that glycerol kinase activity is not as strictly regulated by glucose as formerly thought, and that growth in less strictly repressed conditions (YEP), though in the presence of glucose (YEPD), could induce significant levels of this enzyme (Fig. 3). Furthermore, these results evidence a coincidence between the high glycerol kinase levels of activity achieved during diauxic-shift and the measurements of the supposed Fps1p-mediated facilitated diffusion showed above.

3.6. Glycerol kinase measurements in fps1 mutants

The levels of glycerol kinase activity were determined in YEPD-growing cells of *fps1* mutant comparing with the parental strain and with *gut1* mutant (W303-1A genetic background). For control, the enzyme activity was also measured in W303-1A ethanol and glycerol exponentially growing cells: 61–68 mU mg protein^{-1}. Regardless to the growth phase, enzyme activity levels were higher in *fps1* mutant than in the parental strain (Fig. 4). During diauxic-shift, these were approximately half the activity measured in ethanol- and glycerol-grown cells. The cell samples collected to measure kinase activity were also used to measure *FPS1* expression by Northern analysis (Fig. 4, insert). As expected [34], *FPS1* expression was constant along growth on YEPD. Therefore, the saturable kinetics characteristically detected during diauxic-shift growth phase, must apparently not be connected with *FPS1* expression. It might instead be the result of, as previously suggested [18], an artefact created by a glycerol kinase-driven uptake, whatever the means by which glycerol enters the cell.

These results evidence a coincidence between (i) growth on complex media (YEPD), (ii) growth phase (diauxic-shift), (iii) high glycerol kinase levels and (iv) the measurements of the so-called facilitated diffusion attributed to the functioning of Fps1p.

3.7. Uptake measurements in gut1 and gut2 mutants

We measured glycerol uptake in W303-1A cells grown on YEPD up to diauxic-shift as well as in mutants defective for glycerol kinase (Gut1p), or mitochondrial glycerol 3-phosphate dehydrogenase (Gut2p) (from W303-1A genetic background) (Table 2). Wild-type cells present identical transport results as *gut2* mutant but not as *gut1* mutant as predicted. We further compared these two mutants collected during diauxic-shift on glucose (Fig. 5A) with ethanol-grown cells (Fig. 5B). Also, here the results clearly distinguish the two mutant strains. The low affinity saturable kinetic component

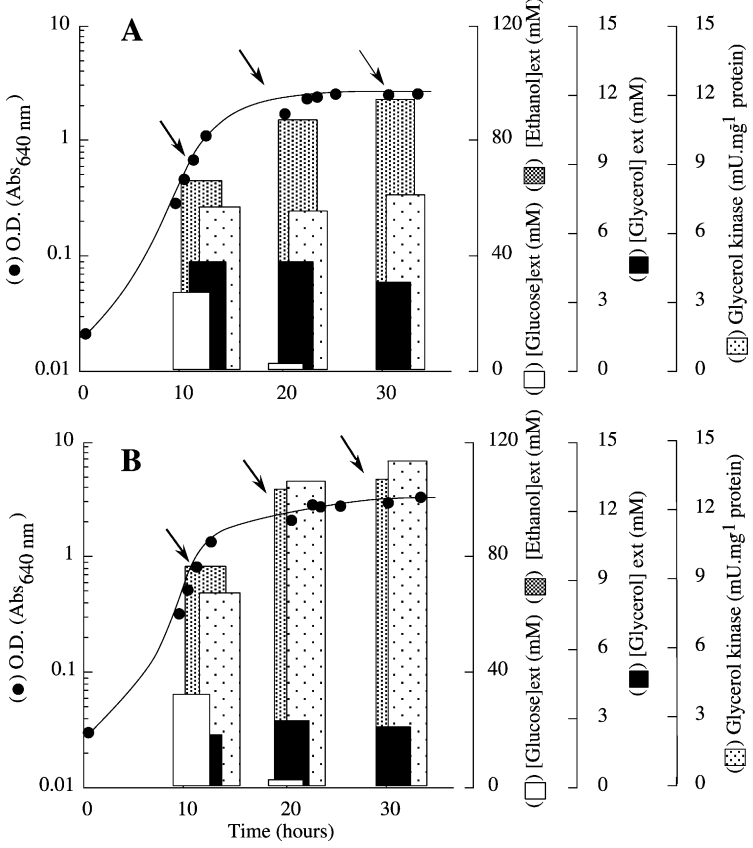

Fig. 3. Extracellular concentrations of glucose, ethanol and glycerol as well as glycerol kinase activity, determined in IGC3507 cells cultivated in MM with 2% (w/v) glucose (A) and in YEPD (B), and collected in three different growth phases (arrows).

of glycerol uptake is not present in the *gut1* strain. Instead, this mutant shows a first-order kinetics glycerol uptake. Furthermore, in the same cells, glycerol accumulation capacity did not exceed equilibrium and the uncoupler CCCP had no effect on in/out ratio (Fig. 5A, insert). Instead, *gut2* diauxic-shift cells presented the saturable low affinity

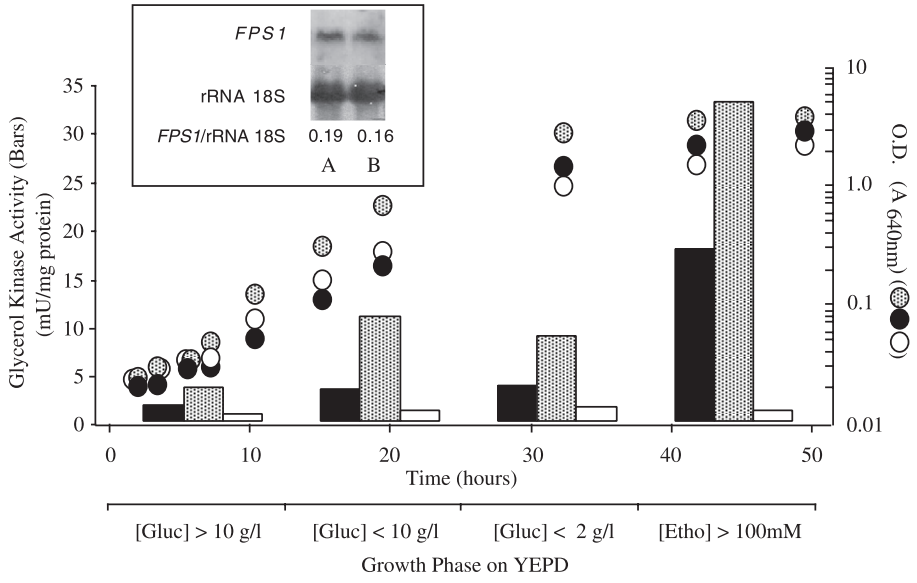

Fig. 4. Growth curves on YEPD at 30 °C of *S. cerevisiae* W303-1A (●), *fps1* (◍) and *gut1* (W303-1A background) (○). Glycerol kinase activity in the same cells is shown (black, grey and white bars, respectively). Growth phases are defined according to glucose and ethanol extracellular concentrations. Insert: Northern results of *FPS1* expression obtained in cells collected in (A) [gluc]>10 g l^{-1} and (B) [etho]>100 mM. Relative fluorescence intensity of both samples is shown.

Table 2
Kinetic parameters of initial uptake rates of [^{14}C] glycerol at 30 °C and pH 5.0 in cells grown in glucose-rich medium and harvested in exponential and post-diauxic growth phases

Growth medium	YEPD					
Growth phase	Exponential			Diauxic-shift		
Strain	K_m (mM)	V_{max} (μmol h^{-1} g dry weight^{-1})	K_d (l h^{-1} g dry weight^{-1})	K_m (mM)	V_{max} (μmol h^{-1} g dry weight^{-1})	K_d (l h^{-1} g dry weight^{-1})
IGC3507[a]	–	–	0.006 (1)	7.7 ± 2.2 (3)	91.1 ± 30.9 (3)	0.003 ± 0.001 (3)
gut1	–	–	0.010 (2)	–	–	0.009 (2)
gut2	n.d.	n.d.	n.d.	7.0 ± 7.5 (3)	94 ± 29 (3)	0.005 ± 0.001 (3)
fps1	–	–	0.003 (2)	6.2 (2)	104.5 (2)	0.005 (2)

(–) No glycerol uptake saturation kinetics detected ($n \geq 3$).
(n.d.) Not determined.
[a] Sutherland et al. [10]: W303-1A YEPD grown cells K_m 5.0 ± 0.3 mM; V_{max} 100 ± 5 μmol h^{-1} g dry weight^{-1}; K_d 0.013 ± 0.0004 l h^{-1} g dry weight^{-1}.

component and a slightly higher accumulation ratio than *gut1* mutants, not significantly different from equilibrium but slightly affected by the uncoupler CCCP.

Furthermore, ethanol-grown cells of both mutants presented the biphasic kinetics characteristic of Eq. (1)—an active transport plus a non-mediated component (Fig. 5B).

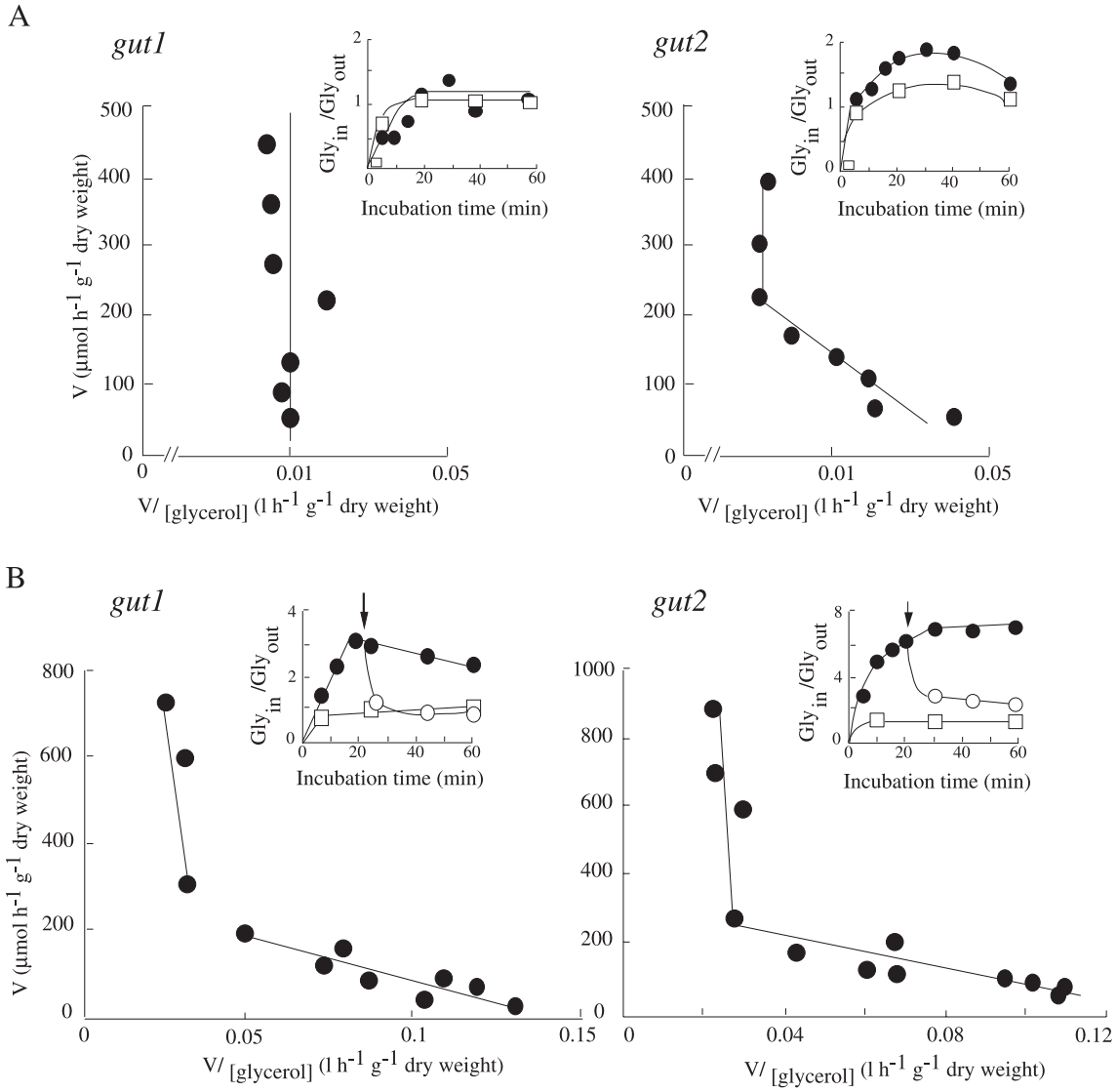

Fig. 5. Eadie–Hofstee plots of [^{14}C]-glycerol initial uptake rates in *gut1* and *gut2* mutants (W303-1A background), grown on YEPD and collected during diauxic-shift (A) and grown on YEPE and collected during exponential growth (B). Inserts: Accumulation curves of the same cells in the absence (●) and in the presence (□) of the ionophore CCCP. Efflux obtained using cold glycerol (○).

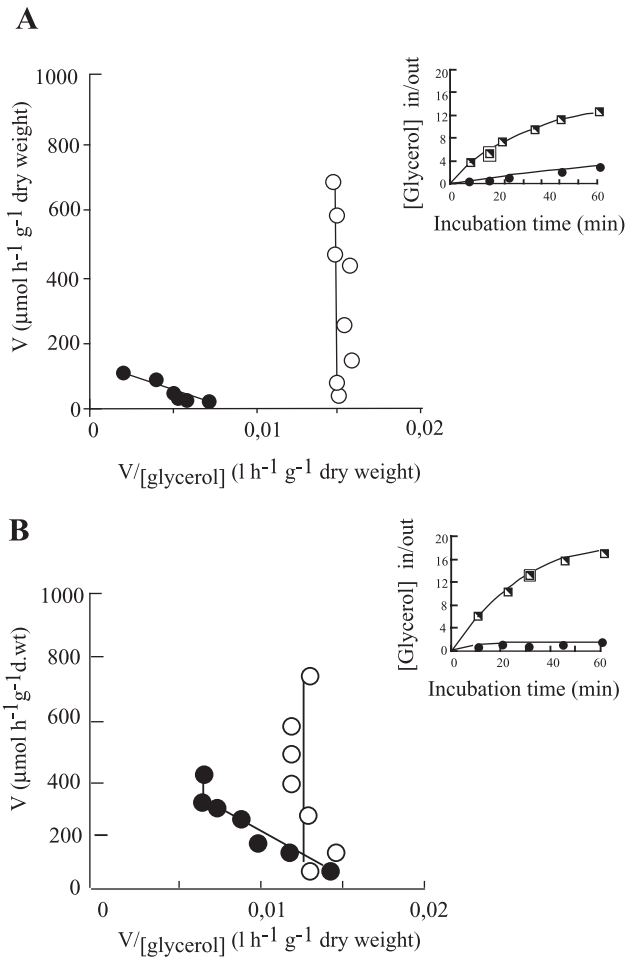

Fig. 6. Eadie–Hofstee plots of [^{14}C]-glycerol initial uptake rates of YEPD-grown cells of W303-1A (A) and *fps1* mutant (W303-1A background) (B), collected in exponential growth phase (○) and during diauxic-shift (●). Cells were assayed at 30 °C, in Tris–citrate buffer, at pH 5.0. Raw experimental data kinetic constants for diauxic-shift cells were K_m 27 and 12 mM, V_{max} 179 and 102 µmol h^{-1} g^{-1} dry weight for, respectively, W303-1A and *fps1* mutant. Inserts: Glycerol accumulation ratios of the same YEPD–diauxic-shift collected cells (●), comparing with cells cultivated in ethanol and collected during exponential growth (◥).

Accumulation ratios varied according with the correspondent deletions: *gut1* mutant accumulated less than *gut2* mutant, but glycerol was 100% free inside the cell and label efflux was total. In *gut2* mutant, efflux was partial, most probably due to glycerol phosphorylation and deviation to other pathways. In both cases, CCCP prevented accumulation (Fig. 5B, insert). The results show that glycerol proton symport is present in both *gut1* and *gut2* mutants growing on ethanol as expected [18]. More importantly, results also sustain the hypothesis of the so-called *facilitated diffusion* being an experimental artefact created by glycerol kinase substrate-driven uptake.

3.8. Re-evaluation of glycerol uptake in fps1 mutants

In spite of previous reports that mutants defective on *FPS1* do not present facilitated diffusion [10,11], taking into account the results presented above, the involvement of the *FPS1* gene on glycerol uptake was re-analysed. The experimental approach aimed to confirm the absence of facilitated diffusion activity on *fps1* mutants previously published [10].

When W303-1A and the correspondent *fps1* strain were cultivated in YEPD and collected during diauxic-shift, both presented, as IGC3507 in Table 2, the low affinity transport mentioned above (Fig. 6). Inserts in this figure compare the levels of glycerol accumulation (in/out ratios) obtained on glucose diauxic-shift cells with ethanol-grown cells, i.e. cells taking up glycerol actively. As it can be seen, diauxic-shift cells do not overcome in/out equilibrium, while ethanol-grown cells accumulate high amounts of glycerol against its chemical gradient, according to the presence of the active transporter [8,10].

Surprisingly, ethanol-grown *fps1* mutant cells present a ± 35% higher accumulation ratio than the parental strain, though neither strain reached the values obtained previously using IGC3507 [8]. No result so far indicated that the interruption of *FPS1* gene could interfere with H^+/glycerol symport kinetics [10]. Nevertheless, in view of the increase in glycerol accumulation ratio obtained on the *fps1* mutant compared to wt, *fps1* mutant grown on YEPE was further studied.

This was done, as before for W303-1A [10], testing the uncoupling effect of CCCP over a rather broad substrate concentration range, which covers active uptake range and passive diffusion substrate range (Fig. 7). As before [10], the higher the contribution of passive diffusion to global glycerol uptake, the lower the uncoupling effect of CCCP observed. Accordingly, the higher glycerol concentration used to measure uptake, the higher the contribution of passive diffusion in experimental velocity (Eq. (1)), and the smaller the effect of CCCP. The effect observed on *fps1* mutant was identical to the effect observed in wild-type, in the substrate range of active transport, but stabilised around

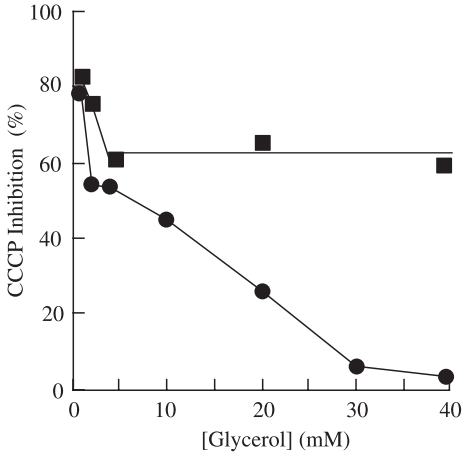

Fig. 7. Percentage of the ionophore CCCP inhibitory effect over [^{14}C]-glycerol initial uptake rates in YEPE-grown cells of W303-1A (●) and *fps1* mutant (W303-1A background) (■). (100% glycerol uptake rate determined without CCCP).

60% inhibition for the higher substrate concentrations. This suggests that *fps1* mutation most probably has no effect over active uptake. But this also shows that in *fps1* mutant, glycerol uptake, under CCCP action, remained unchanged in the range of substrate concentrations which are used to measure passive diffusion. This result suggests that in *fps1* mutant, glycerol uptake does not follow Eq. (1) as wt, i.e. the so-called *passive diffusion* apparently does not exist.

According to Sutherland et al. [10], *fps1* strain was grown overnight and subsequently collected for assay. Although wild-type and mutant strains lag phase duration on YEPD are rather similar, specific growth rates (μ_g) are very different (Fig. 8). *fps1* has a higher specific growth rate than wild-type, being able to reach stationary phase while the parental strain is still in late exponential phase or around diauxic-shift. In the presence of salt, although the growth rates decrease and the lag phase duration increases as expected, the differences between the two strains are considerable. The mutant lag phase is half the one of the parental strain (Fig. 8). After a 24-h growth, wild-type and *fps1* present, respectively, OD_{600} of 1.4 and 3.0. Besides other possible factors, this might justify why we now present such different results for glycerol uptake in *fps1* mutant than the ones published before [10].

3.9. The effect of the presence of multiple copies of FPS1

Both YSH 6.36-3B and W303-1A parental strains (Table 1) were transformed with the multicopy plasmid YEplac195 containing the *FPS1* gene, and used to measure glycerol active transport. These strains were grown on ethanol or glycerol and collected in mid-exponential growth phase, presenting, as expected, glycerol H^+/symport activity. Kinetic constants from radiolabelled glycerol uptake as well as glycerol-driven proton uptake, and the correspondent stoicheiometry (Table 3) were similar to before [8] but V_{max} was ± three to four times higher than in parental strains or even in the previously used IGC3507 (Table 3, Fig. 9A). On the other hand, passive diffusion K_d approximately doubled: 0.027 ± 0.006 l h^{-1} g^{-1} dry weight (n>3).

Strains overexpressing *FPS1*, growing on MM with glucose and harvested in mid-exponential growth phase, unlike wild-type cells, showed a transient alkalinization upon glycerol addition, although very small and thus not quantifiable. They also accumulated radiolabelled glycerol against the gradient as exemplified in Fig. 9B. According to the previously described stimulation of proton symport activity by salt stress [8,9], accumulation ratios were higher when the cells were incubated in 1 M NaCl.

Although Fps1p-mediated glycerol transport and glycerol H^+/symport are distinct issues, this result suggests that the presence of extra copies of *FPS1* influence either active transport activity or the correspondent gene expression/regulation. Furthermore, extra copies of *FPS1* did increase passive diffusion K_d.

3.10. Studies on passive diffusion

Fps1p, as a channel, in the absence of stress, should be opened. Once opened, it is not awkward to postulate that glycerol-free entrance, measured as a first-order kinetic component, i.e. passive diffusion, could be done through the channel. Nevertheless, whatever the strain or the growth or experimental conditions, passive diffusion K_d values always indicate a rather slow entry, which is not too consistent with a channel-mediated substrate entry. Nevertheless, the main function attributed to Fps1p has been shown to regulate glycerol retention in relation to turgor changes [16], and to close/open upon sudden osmotic up- or down-shock [15]. The channel is thus specialised in retaining glycerol rather that allowing it in. This way, the results above give consistency to a hypothesis postulating that the first-order kinetic component of Eq. (1) might correspond to Fps1p-mediated diffusion. This idea, however, is in conflict with the widely spread concept of glycerol being a liposoluble component permeating freely through yeast plasma membrane [6,7].

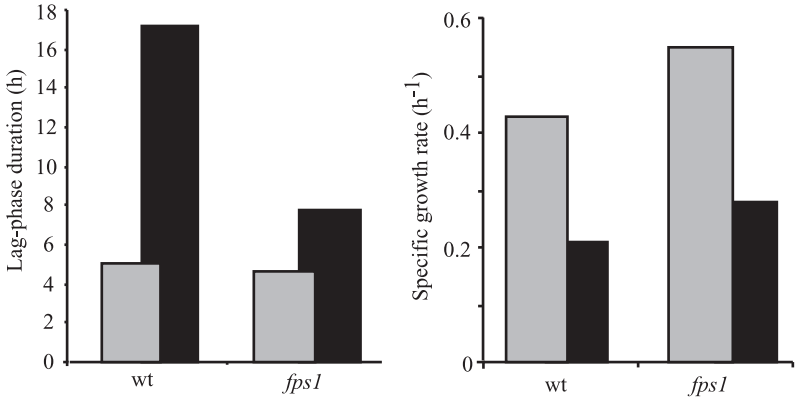

Fig. 8. Comparison of lag phase duration and specific growth rate (μ_g) and in YEPD-grown cells of W303-1A and the correspondent *fps1* mutant, at 30 °C, in the absence (grey bars) and in the presence of 1 M NaCl (black bars).

Table 3
Glycerol uptake kinetic constants determined in *FPS1* overexpressed strains in two different genetic backgrounds grown on YEPE and collected in mid-exponential growth phase

Kinetic constants	[^{14}C]-Glycerol uptake		Glycerol-driven proton uptake		Passive diffusion
Strains	K_m^a	V_{max}^b	K_m^a	V_{max}^b	K_d^c
YSH 6.36.-3B yEp lac195 FPS1::URA3	1.02 ± 0.09 (3)	651 ± 42 (3)	1.64 ± 1.06 (4)	996 ± 241 (4)	0.027 ± 0.006 (3)
W303-1A yEp lac195 FPS1::URA3	1.47 ± 0.52 (3)	519 ± 77 (3)	+	+	0.020 ± 0.002 (3)

Results obtained by computer regression analysis.
(+) Presence of glycerol-driven proton uptake (kinetic constants not determined).
[a] (mM).
[b] (μmol h^{-1} g^{-1} dry weight).
[c] (l h^{-1} g^{-1} dry weight).

To test this hypothesis, we measured glycerol passive diffusion in cells incubated in ethanol. Ethanol has often been used as a membrane solubiliser [35]. Its primary target is plasma membrane, and passive diffusion has been shown to be exponentially enhanced by ethanol [36–38]. On the other hand, ethanol is generally an inhibitor of protein-mediated transport [38], helping to destabilise protein functional insertion in the membrane and/or acting as an uncoupler [38,39]. Therefore, glycerol passive diffusion was measured again, using *fps1*, and the double mutant *fps1/yfl054c*, as well as W303-1A for control, cultivated in YEPD and incubated in buffer with ethanol (Fig. 10). Ethanol concentration used was rather high: 12% (v/v). It was chosen after preliminary experiments which showed that lower ethanol concentrations did not induce significant and reproducible changes in glycerol entry.

Surprisingly, the results clearly show an increase in glycerol uptake in the parental strain, and in the *fps1* mutant (Fig. 10). K_d values measured on wt cells were ± 0.009 and ± 0.024 l h^{-1} g^{-1} dry weight (≈ 3-fold), respectively, in the absence and in the presence of ethanol. On *fps1*, K_d changed from ± 0.0024 to ± 0.005 l h^{-1} g^{-1} dry weight (≈ 2-fold). In the double mutant *fps1/YFL054c*, instead, no ethanol-induced changes were observed. Furthermore, K_d was identical to the one measured in *fps1* mutant: 0.0025 ± 0.0002 l h^{-1} g^{-1} dry weight ($n=4$) in the absence of ethanol. These results, in spite of their several possible interpretations, confirm that glycerol first-order kinetics is

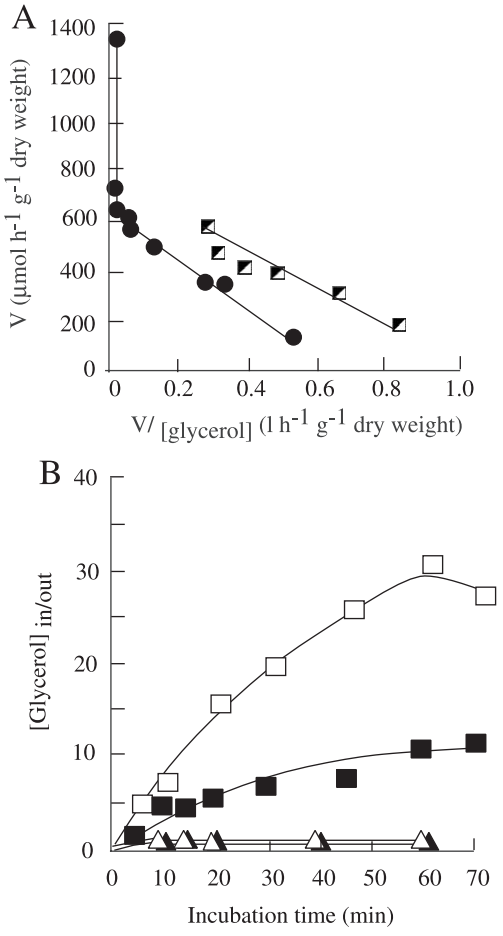

Fig. 9. (A) Eadie–Hofstee plot of experimental data of [^{14}C]-glycerol (●) initial uptake rates and glycerol-driven initial external alkalinization rates (◩) of YSH294 cells grown on MM with 0.2% glucose and 2% glycerol, collected in exponential growth phase and assayed at 30 °C, in Tris–citrate buffer, at pH 5.0. (B) Accumulation ratios of cells cultivated in MM with 2% glucose and collected in mid-exponential phase: YSH294 (■,□) and IGC3507 (▲,△). Cells were assayed in the absence (■,▲) and in the presence of 1 M NaCl (□,△).

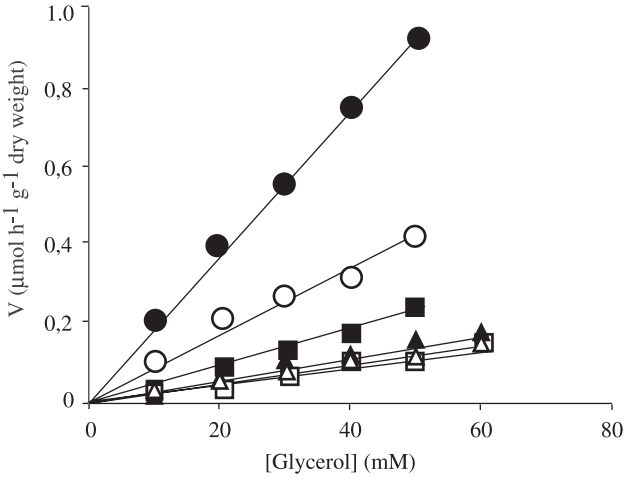

Fig. 10. Effect of 12% (v/v) ethanol 5 min shock on passive diffusion K_d on W303-1A (○,●) *fps1* (□,■) and *fps1/yfl054c* (△,▲). Absence (○,□,△) and presence (●,■,▲) of ethanol.

dependent on the channel presence and evidence that YFL054cp also mediates glycerol entry and, apparently slightly more prone to ethanol stimulation than Fps1p.

4. Discussion

4.1. Kinetic characteristics of Fps1p-mediated glycerol uptake

The results presented in this article aim to clarify the physiological role of the Fps1p channel in what regards its ability to allow glycerol to permeate into the cell in the absence of salt/osmotic stress or turgor changes. This entry has been classified as a *facilitated diffusion*, a transport obeying Michaelis–Menten kinetics, though not concentrative. Results have been presented before, suggesting that *fps1* mutant strain did not present this saturable glycerol uptake [10,11]. In spite of being an unusual member of the MIP gene family [13–15], Fps1p was proved to act as a glycerol channel [11], and has since been known as the *yeast glycerol facilitator* [10]. This classification has been used to mean that the substrate can cross the membrane through this intrinsic protein in a fast and controlled manner, though this has not been totally clarified.

Our results contradict published ones [10,11]. We show that both parental strain and *fps1* mutant present the same type of saturable kinetics. This can be measured in glucose-growing cells provided they are collected during diauxic-shift. This way, glycerol permeabilisation into the cell through Fps1p and the so-called *facilitated diffusion* become different issues. As are also different issues, the channel activity and the active transport activity in derepressed cells, as had been suggested before [8,10,18]. Fps1p's role keeps being the one of a channel, according to Tamás et al. [15,16], devoted to the main function of controlling glycerol efflux when cells suffer a sudden osmotic shock [15] or in response to turgor changes [16]. The problem arises when trying to explain how the channel works at large time scales. Meaning we do not know for how long it keeps closed or opened when the cells are growing in the presence or the absence of stress, respectively.

The low affinity uptake kinetics (facilitated diffusion) could either be a glycerol kinase-mediated artefact as demonstrated before [18] or it could be caused by Fps1p closing and opening activity, which in the long-term, could be a more dynamic process than predicted till now. The most straightforward interpretation of these results would be that the so-called Fps1p-mediated facilitated diffusion might be under glucose regulation, explaining why it could only be detected in the end of exponential growth phase on YEPD. Yet, *FPS1* is constitutively expressed [10–12]. Furthermore, if Fps1p is supposed to be closed under salt-stress, the correspondent facilitated diffusion should be measured only in the absence of salt, which is not the case, although K_d reduces substantially [8].

Following this reasoning, glycerol entry in ethanol-grown cells, i.e. under derepression conditions, should fit a complex, three component, equation, including H^+/symport (1), facilitated diffusion (2) plus a first-order component, passive diffusion:

$$V_{app} = [V_{max1}[S]/(K_{m1} + [S])] + [V_{max2}[S]/(K_{m2} + [S])] + [K_d[S]] \quad (2)$$

This equation does not fit the results obtained before [8,10], neither experimentally, nor by computer regression analysis. On the contrary, results obtained on ethanol-grown cells fit a two-component equation like Eq. (1). Taking altogether the results and rationales above, we can suggest that the so-called Fps1p-mediated facilitated diffusion might be an experimental artefact of uptake measurements.

4.2. The interference of Gut1p in glycerol uptake measurements

Glycerol kinase activity has been suggested to play an important role in the determination of labelled glycerol initial uptake rates, being able to create uptake artefacts which suggest low affinity mediated transport. This is the case of *E. coli* glycerol transport system through the GlpF channel [19]. The illusive existence of a facilitated diffusion was the consequence of a fast glycerol *catch* by the enzyme and subsequent saturation [40–42]. As a glycerol channel, it mediates the substrate entry into the cell at a flow rate of $\pm 2 \times 10^6$ molecules s^{-1} per cell ($\approx$ 1 μmol min^{-1} mg cell protein) [19]. It was more recently described as a non-saturable and very unspecific pore-like channel [41,42]. Fps1p from *S. cerevisiae* like GlpFp is a monomer. Thorough and complex molecular manipulations were done to establish an interchange of specific amino acid signatures between the two channels [14]. Despite the similar broad substrate acceptance, which includes other polyols as well as very unrelated drugs [25], several working specificities of either channel were not interchanged [14] and no clue, so far, exists on how Fps1p is gated and how it senses and reacts to osmotic or turgor changes [14]. Furthermore, Fps1p cannot oligomerise and thus form tetramer-like pores similar to aquaporins [43]. A channel/pore-like protein should allow transport of $\pm 10^7$ molecules s^{-1}, while permeases allowing equilibrating facilitated diffusion characterised so far, allow transport with $V_{max} \leq 10^5$ molecules s^{-1} [44] and active transporters generally present lower V_{max}, $10–10^3$ molecules s^{-1} [44]. Fps1p glycerol uptake rates [10,15] are far too low to be compatible with either, which is an argument more in favour of the channel being specialised in glycerol efflux rather than influx [15,16]. This is consistent with Fps1p being less efficient, or else, less specialised than GlpF for mediating glycerol entry.

The involvement of the first reaction of glycerol metabolism in glycerol transport measurements, was suggested for

the first time in yeasts to justify residual uptake of low V_{max} which could be measured in mutants defective in *GUP1* and *GUP2* genes, both putative glycerol active transporters [18]. We observed that both wild-type and *fps1* cells, grown on YEPD and collected during diauxic-shift, presented considerable levels of glycerol kinase activity. This is in accordance with more recent results on *GUT1* regulation [24]. Glycerol kinase activity levels were higher in *fps1* mutants than in wild-type cells. Northern analysis of *FPS1* gene in wild-type cells revealed, as predicted from the microarray data [34], an approximately constant level along growth curve in YEPD with very slight changes during diauxic-shift. For this reason, one could doubt that *facilitated diffusion* detection could be caused by Fps1p activity. Thus considering, we hypothesised again that kinase activity might be responsible for the uptake saturation kinetics. This was confirmed by the results obtained using *gut1* and *gut2* mutants, grown on YEPD and collected in diauxic-shift. Clearly, *GUT1* interruption led to the disappearance of the saturation kinetics, while it was still present in *gut2* mutants. In cells grown in YEPE, the active transport and the passive diffusion maintained identical values as in wild-type cells. As expected from previous results [18], in *gut1* strain, active transport V_{max} is somewhat smaller than in *gut2*. According to the absence of the first step on glycerol metabolism, in the *gut1* strain, maximum accumulation ratios were shown to correspond to the true strength of the transporter to create a chemical gradient, and all the radiolabel was expelled after *cold* glycerol addition.

Therefore, our results strongly suggest that glycerol facilitated diffusion is a Gut1p-dependent experimental artefact, and glucose growing cells only present passive diffusion. Thus, Fps1p should not be considered a glycerol facilitator, in the sense that it does not mediate glycerol low affinity, non-concentrative, facilitated diffusion type of transport.

4.3. Fps1p mediates the major part of glycerol passive diffusion

Glycerol enters glucose-repressed cells by first-order kinetics, which at first hand, should indicate passive diffusion through the lipid bilayer. Nevertheless, we propose that this might be a misconception, since the chemical properties of glycerol are not compatible with free passage through plasma membrane.

Glycerol oil/octanol partition coefficient (logP), according to Leo et al. [45] is -4.15. Comparing this value with the one of an obviously liposoluble component like ethanol, which logP is -1.33, we can see that glycerol should not easily penetrate a lipid environment. Actually, sugars, which supposedly are not able to cross yeast plasma membrane through the lipid bilayer, have logP values higher than glycerol, like, for example, -1.96 for glucose and -1.77 for fructose [45] (just as a curiosity, Ref. [46] simply states that glycerol is insoluble in oils). According to Gennis [44], glycerol permeability coefficient in egg phosphatidylcholine planar membranes is $\pm 10^{-6}$ cm s^{-1}, and $\pm 10^{-7}$cm s^{-1} in red blood cells. This is 10^3–10^4 times lower than the permeability coefficient for water in the same membranes [44]. Thus considering, we could suppose that yeast plasma membrane should be, if not completely, almost impermeable to glycerol. Nevertheless, a plasma membrane is obviously a very complex and dynamic system and its physico-chemical properties cannot be resumed by partition or permeability coefficients.

The similarity of K_d values when determined in glucose-, ethanol- or glycerol-grown cells [8–10,18], which should have considerable different lipid composition, supports the idea and suggests that Fps1p, once opened, should be responsible for the permeation of at least a part of glycerol *passive diffusion*. Moreover, K_d reduction observed under osmotic stress is consistent with the reduction observed in cell volume, due to the transient cell shrinkage upon osmotic shock. This comparison favours *passive diffusion* being done through the lipids rather than through a channel. However, glycerol uptake, measured by Tamás et al. [15] in cells presenting Fps1p closed after being submitted to an osmotic shock, showed identical percentages of remaining first-order kinetics glycerol uptake as did *fps1* mutants. This way, we can suggest that Fps1p is mediating the major part of the first-order kinetics glycerol entry according to changes in cell volume, which, in spite that Fps1p is not a mechanosensitive channel [13], is compatible with its ability to react to cell turgor changes [16].

Glycerol entry in *fps1* mutants, though very slow, could still be measured: ≈ 0.1 mmol h^{-1} g^{-1} dry weight, around 10–20% uptake rates in wild-type cells [15]. The correspondent passive diffusion K_d: 0.004 ± 0.0002 l h^{-1} g^{-1} dry weight, is approximately 60% the value measured in wild-type cells [10]. Furthermore, when these cells were subjected to 1 M NaCl shock, the channel should be closed [15] and thus, postulating that the non-saturable branch we have measured ubiquitously [8,9,18] representing passive diffusion is channel mediated, it should not be measurable in the presence of salt. The remaining glycerol entry, although very slow and maybe almost not significant, can eventually be done across another channel, like, e.g. a less specific porin. This has been reported before for other biological systems [13,47,48]. We cannot completely disregard the hypothesis that at least a small part of glycerol's passive diffusion might occur through the lipids, eventually taking advantage of changes caused in the physico-chemical environmental conditioning. This corresponds to thermodynamic changes in lipid fluidity [38,49,50], or else, in membrane lipid organisation, which influences intrinsic proteins' biochemical behaviour and molecular structure in a way that is yet poorly understood. Apparently, proteins undergo drastic structural and functional changes under different environmental rigor states [51]. The specific functional consequences are unclear. It is also not clear what role the lipid environment surrounding the protein might have on its activity regulation [44]. Furthermore, the lateral heterogeneity of the bilayer in terms of lipid composition and the

localised interchanges it suffers by flip-flop movements, can create microcompartments in the membrane with specific vectorial properties. These can affect the orientation of membrane components and thus protein insertion/activity as well as localised changes in lipid solubility properties for non-charged compounds [44]. Glycerol is one such case, and we have to admit that very little is known about its interaction with lipids.

On *S. cerevisiae*, former studies [52] have compared glycerol permeability using much different methodologies from ours, like gel permeation chromatography using [^{14}C]ethylene glycol as internal standard. These suggested an inverse correlation between the molecular radius and permeation ability, e.g. glycerol being a small molecule with twice as much molecular radius as a water molecule, it should enter the cell at approximately half the speed. This reinforces the idea that glycerol-free diffusion is most unlikely. Several attempts have been made to determine glycerol permeability coefficients in other biological systems. In the algae *Dunalliela salina*, in the cell envelope of pig erythrocytes and in egg phosphatidylcholine vesicles [53]; in the yeast *Debaryomyces hansenii* [54] as well as in *S. cerevisiae* as mentioned above [10,52]. Nevertheless, in all these cases, with the methodologies used, if glycerol was entering through any type of channel/porin, it would not have been possible to discriminate from lipid through passive diffusion. Also, some attempts have been made to determine glycerol permeability changes according to salt stress, and relate these with changes in lipid composition. Apparently, only minor changes in the overall phospholipid fatty acid composition were found [10,52] and these could not be related with glycerol permeability. The only exception in the literature, to our knowledge, is the case of the ergosterol-less nystatin-resistant mutants of the yeast *Candida albicans*, whose glycerol permeability was considerably reduced in relation to wild-type [55].

In our opinion, the concept of free entrance and leakage of glycerol in yeasts [6,7] has to be re-considered. Intracellular glycerol concentrations regulation might be tightly connected to the regulation of the entire glycerol pathway activity, important for several vital global regulation processes from cell metabolism. *FPS1* gene was first described as a suppressor of *fdp1* [12], allelic to *GGS1*/*TPS1* [11], a mutant unable to grow on fermentable carbon sources, presenting high glycerol intracellular concentrations [11]. The same suppression was obtained by overexpressing *GPD1* gene, provided *FPS1* gene was present [11]. Either case present an excess in glycerol accumulation which, most probably, creates turgor problems to the cell, which can be overcome by Fps1p export activity [16]. The authors suggested that *FPS1* might be important in the control of cytosolic glycerol concentrations through controlling the expression of *GPD1* an/or *GPD2* genes. But control could, in a much simpler way, be explained just by the channel function without having to postulate molecular interactions. On the other hand, glycerol production has been suggested to replace trehalose production for P_i supply in *ggs1*/*tps1* mutant, thus contributing for glycolysis to function [11,56]. This model fits the suppression phenotypes mentioned above. Blomberg [5] suggested that glycerol pathway could function as a futile cycle under stress. This way, glycerol cycle would (1) contribute to a P_i intracellular pool compatible with optimal glycolytic flux [56] and provide some protection against oxidative stress through Gpp1p and Gpp2p activity [4], (2) provide cytosolic redox balance during glucose consumption mainly through Gpd1p activity [1,2] and coupling cytoplasmic to mitochondrial redox balance through the glycerol-3-phosphate shuttle [57], (3) interfere with lipid production through the regulation of glycerol-3-phosphate levels, (4) allow survival upon osmotic shock and growth at low a_w, increasing intracellular glycerol production through Gpd1p and Gpp2p activity [1–4], and reducing the speed of the upper part of glycolytic flux under stress [5] by deviating glucose for glycerol production. All this complexity centred in glycerol production/consumption demands a tightly controlled transport across the plasma membrane.

Our results suggest that glycerol, according to its chemical properties, cannot freely permeate the yeast lipid bilayer. They also demonstrate that passive diffusion is a mediated entry, essentially done through Fps1p, eventually together with other less specific channel/porins, not necessarily responsive to stress.

4.4. Overexpression of FPS1 influence on H^+/glycerol symport

An increase in accumulation ratio was found in ethanol-grown *FPS1* mutant. Also, an increase to the double was observed on K_d when extra copies of *FPS1* were expressing in a recipient cell. This is consistent with most of the diffusion being Fps1p-mediated. On the other hand, higher intracellular glycerol concentration in *FPS1* overexpressing strains has been reported before [11]. This is consistent with the higher H^+/symport V_{max} we measured. Furthermore, glucose repression over glycerol proton symport was probably alleviated, since some residual transport activity was detected in glucose-growing cells. This could be due to a number of reasons, involving expression regulation, either at the molecular or biochemical levels. Eventually, the most straightforward cause would be an unbalance in intracellular glycerol levels, due to the increased leakage as a direct consequence of the outnumbered opened Fps1p (since these experiments were made in the absence of stress) with subsequent consequences in the carrier protein amount. Active transport would thus compensate for increased leakage through more channel proteins.

4.5. A phenotype for FPS1 homologous gene, YFL054C

Finally, we should comment on the effect of ethanol on *FPS1* and *YFL054c* phenotypes. This ORF encodes a

putative channel-like protein [12], very similar to Fps1p. Apparently, it does not mediate glycerol uptake under standard conditions, since glycerol uptake in *fps1* and *fps1/yfl054c* is identical [10,13,15,17].

According to the literature [38], substrate passage through the lipid bilayer is stimulated by ethanol, while protein-mediated transports are inhibited. Nevertheless, to our knowledge, it is not known what effect ethanol might have on transport mediated by channels. Nevertheless, the fact that we had to raise ethanol concentrations up to 12% to be able to see some effect on glycerol uptake is consistent with glycerol passage not being done through the lipids, which would have been affected by much smaller amounts of ethanol [35,37,38]. This indirectly indicates that ethanol is rather acting at the level of an extremely ethanol-resistant membrane component rather than the lipid fraction.

Passive diffusion through Fps1p was confirmed, since the interruption of this gene reduced K_d from wt to $\pm$ 30%. Moreover, further interruption of *YFL054c* did not change K_d value, showing that apparently only Fps1p is mediating glycerol entry. Nevertheless, in the presence of ethanol, results were very different. *YFL054c* gene product like Fps1p were shown to mediate glycerol entry, being stimulated by ethanol. This, considerably reduces a_w, creating a strong osmotic-like type of stress [58]. But, essentially, its effects as membrane fluidizer [38] and as an uncoupler [39] are considered more important. Certain channels are gated by protons, like, for example the aquaglycerol porin (AQ3) from red blood cells [47]. One such behaviour could explain why Yfl054cp-mediated glycerol passive diffusion was, so far, exclusively detected in the presence of ethanol. This is the first time that a phenotype could be attributed to the absence of the *YFL054c* ORF.

Fps1p has been extensively studied in what relates cell response to osmotic shock and turgor changes, but not in what relates to ethanol stress. Our results emphasise that, in spite that *YFL054c* phenotype was a first-order kinetics glycerol uptake identical to Fps1p-mediated passive diffusion, these two genes must be used for different purposes, since YFL054cp permeates less glycerol and Fps1p appears to be less sensitive to ethanol. Taking into consideration the multiple and central roles glycerol plays in the cell metabolism, this is not an improbable suggestion.

Acknowledgements

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