Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species

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A comparison of 42 yeast species with respect to growth in the presence of high NaCl concentration and characteristics of glycerol uptake is presented. The yeast species were classified into four classes on the basis of their ability to grow in the presence of 1, 2, 3 or 4 M NaCl. Considering that two different types of active-transport systems for glycerol uptake have been described, Na⁺/glycerol and H⁺/glycerol symports, glycerol transport was investigated by testing for proton uptake upon glycerol addition in cells incubated in the absence and in the presence of NaCl. Only strains belonging to the two higher classes of salt tolerance showed constitutive active glycerol uptake, and could accumulate glycerol internally against a concentration gradient. Five of these strains exhibited a H⁺/glycerol symport. All the other strains showed evidence of the activity of a salt-dependent glycerol uptake similar to that described in the literature for *Debaryomyces hansenii*. The strains within the two lower classes of salt tolerance showed, to varying degrees, glycerol active uptake only when glycerol was used as the carbon and energy source, suggesting that this uptake system is involved in glycerol catabolism. The results within this work suggest that active glycerol uptake provides a basis for high halotolerance, helping to maintain a favourable intracellular concentration of glycerol. The relation between the constitutive expression of such carriers and a higher level of salt-stress resistance suggests that this may be an evolutionary advantage for growth under such conditions.

Keywords: Halotolerance, glycerol active transport, intracellular volume

INTRODUCTION

The so-called osmotolerant yeasts and, among them, the halotolerant ones, constitute a heterogeneous group of yeasts belonging to very different genera and sharing the common capacity to resist low water-activity environments (Tokuoka & Ishitani, 1991; Tokuoka, 1993; van Eck *et al.*, 1993). Nevertheless, the difference that may exist between the adaptation to osmotic stress produced by high quantities of sugars, which generated the classification of osmotolerant yeast, and the one produced by high quantities of salt, especially sodium chloride, has been rather poorly studied. Tolerance to either stress condition, in some yeast strains, is quite specific and sometimes mutually exclusive (Tokuoka &

Ishitani, 1991; Tokuoka, 1993; van Eck *et al.*, 1993; M. Silva-Graça & C. Lucas, unpublished). At the moment, the most generalized mechanism identified in microorganisms in response to osmotic stress is the production and intracellular accumulation of low-molecular-mass molecules that function as compatible solutes (Brown, 1977; Brown *et al.*, 1986; Reed *et al.*, 1987). In yeasts, this role is played almost exclusively by glycerol, followed by arabinol and other polyhydroxy alcohols to a lesser extent (Gustafsson & Norkrans, 1976; Jennings & Burke, 1990; van Eck *et al.*, 1993).

Glycerol production has been reported to be proportional to the osmotic stress the cell is subjected to during growth (Nobre & da Costa, 1985; van Eck *et al.*, 1989; van Zyl & Prior, 1990; Ölz *et al.*, 1993). Besides the enhancement in production observed under stress, and taking into account that glycerol is a liposoluble molecule that leaks through the yeast plasma membrane, its retention should thus require an active response from

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; MM, mineral medium; YEP, complete medium; YEPD, yeast extract/peptone/glucose medium.

the cell. In two different osmotolerant yeast species, *Debaryomyces hansenii* and *Pichia sorbitophila*, two different types of constitutive active-transport systems for glycerol have been described and characterized: a Na⁺/glycerol co-transport (Lucas *et al.*, 1990) and a H⁺/glycerol symport (Lages & Lucas, 1995). Also in *Zygosaccharomyces rouxii*, an active-transport system for glycerol has been described (van Zyl *et al.*, 1990). On the other hand, the model yeast *Saccharomyces cerevisiae*, also considered an osmotolerant species, shows an inducible active uptake (Lages & Lucas, 1997), apparently not associated with the stress response. In turn, glycerol retention is secured by Fps1, a constitutively expressed protein belonging to the MIP channel family that is able to control glycerol leakage under conditions of osmotic stress (Sutherland *et al.*, 1997; Tamas *et al.*, 1999).

The purpose of this work was to investigate the presence of active-transport systems for glycerol in different yeast species in an attempt to identify major common mechanisms that might be involved in halotolerance. A series of yeast strains from 42 different species was selected as representative of different responses to salt stress, including spoilage yeasts frequently found in high-salt or high-sugar food products and yeasts used for the production of different processed foods, brines and beverages, and thus with biotechnological importance.

METHODS

Yeast strains and growth conditions. Yeast strains (Table 1) were obtained from internationally credited yeast culture

collections. Strains were maintained at 4 °C in solid medium containing glucose (2%, w/v), peptone (2% w/v) and yeast extract (1% w/v) (YEPD) and at –70 °C in glycerol (30%, w/v). Growth was assayed in batch cultures, using mineral medium with vitamins (van Uden, 1967) (MM) or complete medium, 2%, w/v, peptone and 1%, w/v, yeast extract (YEP), supplemented with 2%, w/v, glucose or glycerol, at 25 °C and 160 r.p.m. orbital shaking. Specific growth rates were calculated by monitoring the optical density at 640 nm.

Determination of maximum NaCl tolerance. Growth in the presence of different NaCl concentrations was tested in MM and YEP in 1 M increments. For this purpose, test tubes with 10 ml growth medium were incubated at 25 °C and 200 r.p.m. orbital shaking. Each tolerance experiment was repeated at least three times (three replicates each) and growth was monitored according to van der Walt & Yarrow (1984) every 2 d, up to a maximum of 40 d.

Measurement of initial uptake rates. Glucose- or glycerol-grown cells were harvested in the mid-exponential growth phase (variable optical density according to the strain) by centrifugation, washed twice and resuspended to a final concentration of ~30 mg ml⁻¹ (dry wt) in ice-cold distilled water. The methods used to determine (i) initial rates of proton uptake upon glycerol addition, (ii) initial uptake rates of [¹⁴C]glycerol (Amersham) and (iii) the inhibitory effects of the protonophore CCCP (carbonyl cyanide *m*-chlorophenyl-hydrazine) and ethanol on uptake, have been described before (Lucas *et al.*, 1990; Lages & Lucas, 1995, 1997). Computer regression analysis program GraphPad PRISM (GraphPad Software) was used to determine Michaelis–Menten kinetic parameters (maximum velocity, V_{max} , and Michaelis constant, K_m).

Measurement of radiolabelled glycerol accumulation. Cell suspensions were obtained as described above. [³H]Glycerol

Table 1. Maximum tolerance to NaCl obtained in batch cultures (MM with 2% glucose) in 1M salt increments.

Sacch. cerevisiae, *Deb. hansenii* and *P. sorbitophila* were included as controls. CBS (Centraalbureau voor Schimmelcultures, Oosterstraat 1, Baarn, The Netherlands) and PYCC [Portuguese Yeast Culture Collection, New University of Lisbon, Portugal (referenced as IGC and ISA)].

1 M (5.8%, w/v)	2 M (11.7%, w/v)	3 M (17.5%, w/v)	4 M (23.4%, w/v)
<i>Dekkera anomala</i> IGC 5153	<i>Candida tropicalis</i> IGC 3097*	<i>Candida famata</i> IGC 3056	<i>Candida cacaioi</i> § IGC 3422*
<i>Dekkera bruxellensis</i> IGC 4179	<i>Debaryomyces castellii</i> † IGC 2839*	<i>Candida magnoliae</i> § IGC 2903*	<i>Candida halonitratophila</i> § CBS 5240*
<i>Lipomyces kononenkoae</i> IGC 4051*	<i>Fellomyces penicillatus</i> CBS 5492*	<i>Candida silvicultrix</i> § CBS 6269*	<i>Candida halophila</i> § CBS 4019*
<i>Pichia jadinii</i> †‡ IGC 2541*	<i>Hanseniaspora osmophila</i> † CBS 313*	<i>Citeromyces matritensis</i> § CBS 4116*	<i>Candida nodaensis</i> § IGC 3198*
<i>Pichia stipitis</i> † IGC 4374	<i>Issatchenkia orientalis</i> † IGC 3806	<i>Pichia etchellsii</i> § IGC 3811*	<i>Candida versatilis</i> § CBS 1752*
<i>Rhodotorula minuta</i> † IGC 4790*	<i>Kluyveromyces marxianus</i> † IGC 3886	<i>Pichia farinosa</i> § IGC 2459*	<i>Sterigmatomyces halophilus</i> § IGC 4178*
<i>Schizosaccharomyces pombe</i> § IGC 2769	<i>Octosporomyces octosporus</i> § IGC 4180	<i>Pichia membranaefaciens</i> † IGC 3796*	
<i>Zygosaccharomyces bailii</i> IGC 4806	<i>Pichia angusta</i> †‡ IGC 4129	<i>Stephanoascus ciferrii</i> § IGC 4164*	<i>Pichia sorbitophila</i> CBS 7064*
<i>Zygosaccharomyces florentinus</i> IGC 4169*	<i>Pichia anomala</i> †§ IGC 4121	<i>Torulasporea delbrueckii</i> † ISA 1229	
	<i>Pichia haplophila</i> IGC 2818*	<i>Wingea robertsiae</i> § IGC 3804*	
	<i>Rhodotorula mucilaginosa</i> IGC 4791	<i>Debaryomyces hansenii</i> § IGC 2968*	
	<i>Saccharomyces exiguus</i> IGC 2543*		
	<i>Schwanniomyces occidentalis</i> IGC 2829*		
	<i>Zygosaccharomyces rouxii</i> § IGC 4194		
	<i>Saccharomyces cerevisiae</i> † IGC 3507		

* Type strain.

† Different strains show variable tolerance to 50% (w/v) glucose stress (Barnett *et al.*, 1990).

‡ *Pichia angusta* is *Hansenula polymorpha*; *Pichia anomala* is *Hansenula anomala*; *Pichia jadinii* is *Candida utilis*.

§ Different strains show variable tolerance to 60% (w/v) glucose stress (Barnett *et al.*, 1990).

|| Tolerant to 50% glucose (Barnett *et al.*, 1990).

§ Tolerant to 60% glucose (Barnett *et al.*, 1990).

(Amersham) accumulation ratios were determined according to Lages & Lucas (1995).

Measurement of intracellular volumes. The intracellular volume was measured as previously described by de la Peña *et al.* (1982) and Rottenberg (1979), utilizing $^3\text{H}_2\text{O}$ as total marker and ^{14}C methoxyinulin (Amersham) as external marker. Measurements were made in the absence and in the presence of sodium chloride in the assay buffer (30 min incubation in 1 M NaCl). The intracellular volumes were determined in five strains, each one belonging to a different class of salt-stress tolerance. For all the other strains, the generally accepted value for intracellular volumes in yeasts [$2\ \mu\text{l (mg dry wt)}^{-1}$] was assumed.

Reproducibility of the results. Assays were repeated at least three times, and the data reported are mean values. The exact number of independent experiments, as well as replicates, are mentioned whenever relevant.

RESULTS

Determination of the level of tolerance to NaCl

The maximum tolerance to NaCl in MM allowed us to establish four phenotypic classes of salt-stress tolerance. The species in each class are shown in Table 1, which also shows the species able to resist high sugar concentrations according to Barnett *et al.* (1990). The survey was repeated in YEP: the results (not shown) did not differ significantly from those in Table 1 for most of the strains. However, *Z. rouxii*, *Schiz. pombe* and *P. membranaefaciens* showed a marked decrease in the level of tolerance: from 2 to 0.5 M, from 1 to <0.5 M and from 3 to 1 M, respectively. *Z. rouxii* and *Schiz. pombe* exhibit different salt tolerance in relation to the medium pH (Watanabe & Tamai, 1992; Jia *et al.*, 1992), which is different in MM and YEP (pH 4.3 and 6.5, respectively). Considering this, the salt tolerance of *P. membranaefaciens* was investigated in MM at initial pH 4.0, 5.0, 6.0 and 6.5. No growth beyond 1 M NaCl was observed at $\geq$ pH 6.0, while the resistance level in Table 1 was maintained at lower pH.

The survey was repeated using cells pre-adapted to salt stress as inocula. Most of the strains maintained their maximum level of resistance (not shown), but *Can. halophila*, *Can. halonitratophila* and *Sterig. halophilus* showed growth in the presence of 5 M NaCl.

Survey for constitutive glycerol active uptake

The first phase of the survey was the search for proton uptake upon the addition of glycerol in glucose-grown cells of all the strains in Table 1. The assays were done in the absence and in the presence of 1 M NaCl. Results are shown in Table 2, where we have included *Deb. hansenii*, *P. sorbitophila* and *Sacch. cerevisiae* as strains with well-characterized active-transport systems. In the strains from the two higher salt-tolerance classes, the initial rates of proton disappearance from the medium followed saturation kinetics and accumulation of labelled glycerol against the gradient was observed. Five strains showed proton uptake in both the absence and presence of NaCl, as exemplified by *W. robertsiae* (Fig.

1), while in the others, proton uptake was exclusively detected in cells incubated in 1 M NaCl, as exemplified in *Can. magnoliae* (Fig. 1). All the strains belonging to the two lower salt-tolerance classes, with *Deb. castellii* as the single exception, did not exhibit proton uptake. One of those strains, *Schiz. pombe*, was chosen to assay labelled glycerol uptake (from 6 to 50 mM) and simple diffusion was measured, in cells incubated either without or with salt. The diffusion constant (K_d) was affected by salt, as evidenced in Fig. 1, being K_d 0.011 and 0.003 $\text{l h}^{-1} (\text{g dry wt})^{-1}$, in the absence and in the presence of salt, respectively.

The in/out ratio from the accumulation of labelled glycerol against the gradient varied from strain to strain (not shown) and was prevented by the action of the protonophore CCCP as illustrated in Fig. 1. In the case of the five strains that showed salt-independent proton uptake, the in/out ratio obtained in the absence of salt was very similar to that obtained in its presence (Table 2). In the case of the strains that showed salt-dependent proton uptake, the in/out ratio obtained in the presence of 1 M NaCl was much higher than in its absence (Table 2). In all the other strains, showing neither salt-independent nor salt-dependent proton uptake, in/out ratios either did not exceed equilibrium, like *Schiz. pombe*, or instead followed a linear increase with incubation time like *P. jadinii* (not shown).

Prior to performance of accumulation assays, and to enable the calculation of the intracellular concentration of glycerol, intracellular volume was assessed for one strain of each tolerance class: *Schiz. pombe*, *W. robertsiae*, *Sacch. cerevisiae*, *Can. halophila* and *P. sorbitophila* (Table 3). When the cells were incubated in 1 M NaCl, a reduction in cell volume was observed (Table 3), the percentage of which was higher for the less-resistant strains. The percentage volume reduction observed in each strain was generalized to all the others within same tolerance class, and applied to a standard value of $2\ \mu\text{l (mg dry wt)}^{-1}$, assumed for the intracellular volume in the absence of stress.

The activity of a constitutive salt-independent active glycerol transport, as has been reported for the H^+ /glycerol symport of *P. sorbitophila* (Lages & Lucas, 1995), should be consistent with the ability to grow on glycerol as the single carbon and energy source. Specific growth rates on glycerol of the five strains presenting evidence of activity of such a type of carrier, *Can. halophila*, *Can. nodaensis*, *Steph. ciferrui* and *W. robertsiae*, varied from 100 to 50% of the rates measured on glucose growth medium (Table 4).

Survey for inductive glycerol active uptake

The strains from the two classes of lower salt tolerance were grown on glycerol to assay glycerol transport (Table 5). In MM supplemented with glycerol, growth was generally slow and was almost undetectable for some strains. Except for *Sch. occidentalis*, all the strains that showed growth also accumulated labelled glycerol against the gradient. The in/out ratio, as above, varied

Table 2. Kinetic parameters of H⁺ uptake upon glycerol addition and accumulation of [³H]glycerol against the gradient in glucose-grown cells

Deb. hansenii, *P. sorbitophila* and *Sacch. cerevisiae* were included as controls. –, No proton uptake detected: glycerol accumulation not exceeding equilibrium; +, accumulation ratio in/out > 1; ++, accumulation ratio in/out at least two times higher than the ratio observed in the absence of salt; ND, not done.

Species	H ⁺ uptake				[³ H]Glycerol accumulation	
	K_m (no NaCl)*	V_{max} (no NaCl)†	K_m (with 1 M NaCl)*	V_{max} (with 1 M NaCl)†	Without NaCl	With 1 M NaCl
Tolerant to 4 M NaCl						
<i>Can. cacaioi</i>	–	–	0.9	683	+	++
<i>Can. halonitratophila</i>	–	–	1.2	707	+	++
<i>Can. halophila</i>	1.7	319	2.4	827	+	+
<i>Can. nodaensis</i>	ND	242	0.5	1230	+	+
<i>Can. versatilis</i>	–	–	1.0	1367	+	++
<i>P. sorbitophila</i> ‡	1.3	559	1.1	1050	+	+
<i>Sterig. halophilus</i>	–	–	ND	7360	+	++
Tolerant to 3 M NaCl						
<i>Can. famata</i>	–	–	0.3	356	+	++
<i>Can. magnoliae</i>	–	–	1.7	1528	+	++
<i>Can. silvicultrix</i>	–	–	0.7	113	+	++
<i>Can. matritensis</i>	–	–	ND	463	+	++
<i>Deb. hansenii</i> ‡	–	–	0.5	135	+	++
<i>P. etchellsii</i>	–	–	ND	666	+	++
<i>P. farinosa</i>	–	–	0.8	558	+	++
<i>P. membranaefaciens</i>	–	–	ND	564	+	++
<i>Steph. ciferrii</i>	ND	712	ND	690	+	+
<i>T. delbrueckii</i>	–	–	2.0	692	+	++
<i>W. robertsiae</i>	2.1	285	1.9	692	+	+
Tolerant to 2 M NaCl						
<i>Can. tropicalis</i>	–	–	–	–	–	–
<i>Deb. castellii</i>	–	–	ND	285	+	++
<i>F. penicillatus</i>	–	–	–	–	–	–
<i>H. boscophila</i>	–	–	–	–	–	–
<i>I. orientalis</i>	–	–	–	–	–	–
<i>K. marxianus</i>	–	–	–	–	–	–
<i>O. octosporus</i>	–	–	–	–	–	–
<i>P. angusta</i>	–	–	–	–	–	–
<i>P. anomala</i>	–	–	–	–	–	–
<i>P. haplophila</i>	–	–	–	–	–	–
<i>R. mucilaginosus</i>	–	–	–	–	–	–
<i>Sacch. cerevisiae</i> ‡	–	–	–	–	–	–
<i>Sacch. exiguus</i>	–	–	–	–	–	–
<i>Sch. occidentalis</i>	–	–	–	–	–	–
<i>Z. rouxii</i>	–	–	–	–	–	–
Tolerant to 1 M NaCl						
<i>Dek. anomala</i>	–	–	–	–	–	–
<i>Dek. bruxellensis</i>	–	–	–	–	–	–
<i>L. kononenkoae</i>	–	–	–	–	–	–
<i>P. jadinii</i>	–	–	–	–	–§	–
<i>P. stipitis</i>	–	–	–	–	–	–
<i>R. minuta</i>	–	–	–	–	–	–
<i>Schiz. pombe</i>	–	–	–	–	–	–
<i>Z. bailii</i>	–	–	–	–	–	–
<i>Z. florentinus</i>	–	–	–	–	–	–

* mM.

† $\mu\text{mol h}^{-1} (\text{g dry wt})^{-1}$.‡ Published results (Lucas *et al.*, 1990; Lages & Lucas, 1995, 1997).

§ Steep linear increase with incubation time.

Fig. 1. (a–c) $[^3H]$ Glycerol-accumulation ratios and (d–f) Eadie–Hofstee plots of initial proton-uptake rates elicited by glycerol (*W. robertsiae*, *Can. magnoliae*) and initial uptake rates of $[^{14}C]$ glycerol (*Schiz. pombe*). Assays were performed on glucose-grown cells incubated in the absence (white symbols) and in the presence (black symbols) of 1 M NaCl. Accumulation ratios obtained in the absence (●, ○) and in the presence (▲, △) of 50 μM CCCP are also shown.

Table 3. Intracellular volume of strains representative of each class of tolerance to NaCl upon osmotic shock by incubation in 1 M NaCl at pH 5.0

Cells were cultivated in MM and glucose without salt stress. Numbers in parentheses are the number of replicates/number of independent batches of cells.

Species	Tolerance to NaCl (M)	Intracellular volume [μl (mg dry wt) $^{-1}$]		
		Without NaCl	With 1 M NaCl	Reduction (%)
<i>Can. halophila</i>	5	1.26 ± 0.18 (12/6)	0.82 ± 0.13 (10/1)	35
<i>P. sorbitophila</i>	4	3.17 ± 0.33 (31/18)	2.48 ± 0.29 (20/11)	22
<i>W. robertsiae</i>	3	1.38 ± 0.18 (14/7)	0.76 ± 0.11 (10/6)	45
<i>Sacch. cerevisiae</i>	2	1.95 ± 0.15 (18/9)	0.78 ± 0.47 (16/9)	60
<i>Schiz. pombe</i>	1	1.47 ± 0.12 (6/3)	0.63 ± 0.07 (6/3)	57

from strain to strain and was prevented by the action of the protonophore CCCP (not shown). In *P. anomala*, *Dek. anomala* and *P. jadinii*, as reported for *Sacch. cerevisiae* (Lages & Lucas, 1997), the accumulation ratio was higher in the absence than in the presence of salt.

Proton uptake upon glycerol addition was observed in the strains that accumulated glycerol, except in *P. jadinii* (Fig. 2). However, in this strain, in/out ratios, both in the presence and in the absence of salt, exceeded equilibrium and could be prevented by the action of CCCP (Fig. 2). Subsequently, the detailed study of *P.*

Table 4. Specific growth rates of the yeast strains showing salt-independent proton uptake and glycerol accumulation, grown on MM with 2 % (w/v) glucose or glycerol

Species	Specific growth rate [μ_g (h^{-1})] on:	
	Glucose	Glycerol
<i>Can. halophila</i>	0.12 ± 0.01	0.09 ± 0.003
<i>Can. nodaensis</i>	0.22 ± 0.02	0.13 ± 0.004
<i>P. sorbitophila</i>	0.25 ± 0.02	0.24 ± 0.02
<i>Steph. ciferrii</i>	0.25 ± 0.007	0.13 ± 0.009
<i>W. robertsiae</i>	0.22 ± 0.004	0.16 ± 0.01

Table 5. Specific growth rates (μ_g) on glucose and glycerol of the strains from the lower classes of resistance to NaCl, and accumulation of [3H]glycerol against the gradient in glycerol-grown cells incubated in the absence and in the presence of 1 M NaCl

The strains which could not grow on glycerol (–) were not assayed (NA). +, Accumulation ratio in/out > 1; ++, accumulation ratio in/out at least two times higher than the ratio observed in the other incubation condition.

Species	μ_g (h^{-1})		[3H]Glycerol accumulation	
	Glucose	Glycerol		
			Without NaCl	With 1 M NaCl
Tolerant to 2 M NaCl				
<i>Can. tropicalis</i>	0.54	0.11	+	++
<i>F. penicillatus</i>	0.13	–	NA	NA
<i>H. osmophila</i>	0.28	–	NA	NA
<i>I. orientalis</i>	0.34	–	NA	NA
<i>K. marxianus</i>	0.39	0.15	+	++
<i>O. octosporus</i>	0.10	–	NA	NA
<i>P. angusta</i>	0.27	0.19	+	++
<i>P. anomala</i>	0.36	0.29	++	+
<i>P. haplophila</i>	0.29	0.08	+	++
<i>R. mucilaginisosa</i>	0.36	0.19	+	++
<i>Sacch. cerevisiae</i>	0.37	0.11	++	+
<i>Sacch. exiguus</i>	0.33	–	NA	NA
<i>Sch. occidentalis</i>	0.36	0.05	–	–
<i>Z. rouxii</i>	0.18	0.09	+	++
Tolerant to 1 M NaCl				
<i>Dek. anomala</i>	0.19	0.10	++	+
<i>Dek. bruxelensis</i>	0.09	–	NA	NA
<i>L. kononenkoae</i>	0.21	–	NA	NA
<i>P. jadinii</i>	0.35	0.32	++	+
<i>P. stipitis</i>	0.23	0.07	+	++
<i>R. minuta</i>	0.37	–	NA	NA
<i>Schiz. pombe</i>	0.14	0.10*	–	–
<i>Z. bailii</i>	0.33	0.13	+	++
<i>Z. florentinus</i>	0.19	–	NA	NA

* Cells cultivated in YEP.

Fig. 2. Eadie-Hofstee plot of initial uptake rates of [^{14}C]glycerol in glycerol-grown cells of *P. jadinii*, assayed in the absence (white symbols) and in the presence (black symbols) of 1 M NaCl. Inset: accumulation ratio of labelled glycerol in the absence (●, ○) and in the presence (▲, △) of 50 μM CCCP.**Table 6.** Comparison between labelled-glycerol uptake kinetic parameters on glucose- and glycerol-grown cells of *P. jadinii* incubated in the absence and in the presence of salt

	Carbon source	
	Glucose	Glycerol
Without NaCl		
K_m (mM)	0.6 ± 0.2	0.7 ± 0.1
V_{max} [$\mu mol h^{-1} (g dry wt)^{-1}$]	2271 ± 252	3964 ± 180
K_d [$l h^{-1} (g dry wt)^{-1}$]	0.11 ± 0.03	0.15 ± 0.01
With 1 M NaCl		
K_m (mM)	2.3 ± 0.9	1.1
V_{max} [$\mu mol h^{-1} (g dry wt)^{-1}$]	719 ± 186	1845
K_d [$l h^{-1} (g dry wt)^{-1}$]	0.04 ± 0.01	0.05

jadinii cells grown on glucose or glycerol revealed that, even without any detectable proton uptake, this substrate was taken up following biphasic kinetics (Fig. 2). The transport was affected by salt as shown in Fig. 2. The experimental data, analysed by computer regression analysis, was best fitted by one Michaelis-Menten term plus one first-order kinetics term. The kinetic parameters (V_{max} , K_m and K_d) obtained in the absence and in the presence of salt are shown in Table 6. Furthermore, CCCP (20 μM) and ethanol (3 %, v/v) both inhibited V_{max} by ~ 70 %.

As a control for the strains that could not grow on MM with glycerol, *Schiz. pombe* was assayed after growth on YEP supplemented with glycerol. It still did not show either proton uptake or labelled-glycerol accumulation against the gradient, revealing instead simple diffusion with a K_d of $0.013 l h^{-1} (g dry wt)^{-1}$, identical to the K_d measured in glucose-grown cells (above).

DISCUSSION

The set of 42 strains from different yeast species chosen to survey tolerance to NaCl included 34 of the so-called osmotolerant species, 20 from 91 described as able to grow on 60% glucose and 14 from 202 described as able to grow on 50% glucose (Barnett *et al.*, 1990). Another 8 strains from non-osmotolerant species were included as controls. A few species are known for their strain heterogeneity; however, it did not correspond to the objectives of this survey to explore this variability. *Z. rouxii* is undoubtedly one such case (Tokuoka & Ishitani, 1991). This is a species in which some strains would be expected to survive a higher salt stress than the 2 M NaCl we observed. In an attempt to decrease as much as possible the variation of salt-tolerance phenotypic expression due to the ecological origin of different strains from the same species, we tried to use type strains whenever possible.

Results from salt tolerance revealed that the yeast strains which were able to resist more than 2 M NaCl are the ones described as being able grow on at least 50% glucose, most of them on 60% glucose (Barnett *et al.*, 1990). The opposite was not evident. Some strains from the species reported as tolerant to high glucose did not tolerate more than 1 or 2 M NaCl, suggesting that the physiological response to sugar stress may not be as effective for salt stress, as has been reported previously (Tokuoka & Ishitani, 1991; van Eck *et al.*, 1993; Tokuoka, 1993; M. Silva-Graça & C. Lucas, unpublished).

On the other hand, and in spite of what has been reported before by Tokuoka (1993), pre-adaptation of inocula to salt-stress did not introduce a detectable difference in the capacity to grow at higher molarities for the majority of the strains, as published for *Deb. hansenii* (Neves *et al.*, 1997). However, the level of resistance of any strain may also be temperature dependent (Trollmo *et al.*, 1988; Prista & Madeira-Lopes, 1995; Tokuoka, 1993) or pH dependent (Tokuoka, 1993). So, we cannot disregard the possibility that other growth conditions might provide different results, since some of the strains might not grow at a certain salt concentration, but may be kept in latency, as reported for *Sacch. cerevisiae* (Morris *et al.*, 1986) and *Deb. hansenii* (L. Neves, personal communication). In particular, the requirement for low pH values to grow in the presence of salt observed in *P. membranaefaciens* could be, as described in *Z. rouxii* and *Schiz. pombe* (Jia *et al.*, 1992; Rodríguez-Navarro *et al.*, 1994; Nishi & Yagi, 1995), the consequence of sodium extrusion being performed by a Na⁺/H⁺ antiporter, thus dependent on ΔpH and plasma membrane H⁺/ATPase activity (Watanabe & Tamai, 1992; Nishi & Yagi, 1995).

Observing extracellular alkalinization of a cell suspension elicited by the addition of any substrate can be a strong indication of the activity of an active-transport system of the proton-symport type (Loureiro-Dias, 1988). Accumulation of labelled substrate against the gradient may, in turn, be the consequence of either an

active transport or passive diffusion through plasma membrane followed by catabolism (Gancedo *et al.*, 1968; Lages & Lucas, 1997). The action of a protonophore, eliminating ΔpH and lowering intracellular pH (Serrano, 1991), can affect active uptake and prevent the consequent accumulation, but we cannot disregard the possibility that it also affects enzymes from the first steps of catabolism, creating artefacts. Diagnosis of transport cannot be achieved exclusively by this type of assay, which is the reason why such a survey had to be supported by pre-existing knowledge of the model yeasts *Deb. hansenii* (Lucas *et al.*, 1990), *P. sorbitophila* (Lages & Lucas, 1995) and *Sacch. cerevisiae* (Lages & Lucas, 1997). Each represented one level of tolerance to salt and a different type of active transport for glycerol.

The results present consistent evidence of constitutive active uptake in all the strains belonging to the 3 and 4 M salt-stress tolerance classes. This, in our opinion, revealed a general physiological mechanism underlying salt-stress resistance, enabling glycerol intracellular levels to be maintained. *Can. halophila*, *Can. nodaensis*, *Steph. ciferri* and *W. robertsiae* showed, like the H⁺/glycerol symport from *P. sorbitophila*, proton uptake as well as intracellular accumulation of glycerol independent of salt. Also, as seen in *P. sorbitophila*, this type of glycerol transport is constitutive. Otherwise, most strains behaved very similarly to *Deb. hansenii*, showing salt-dependent proton uptake and glycerol accumulation, which have been attributed to the functioning of a Na⁺/glycerol symport. Nevertheless, this transport system is still a matter of discussion, since in *Deb. hansenii* the proton-motive force may eventually be found to be regulated differently from *P. sorbitophila* (Lages & Lucas, 1995), especially in the presence of high-salt stress, as suggested by the results of Prista *et al.* (1997), misleading transport characterization.

As has been published for *Sacch. cerevisiae* (Lages & Lucas, 1997), most of the strains which were able to grow on glycerol as the single carbon and energy source showed evidence of inductive active transport. The existence of a H⁺/glycerol symport induced by growth on glycerol is consistent with a role of such a carrier in glycerol catabolism. In contrast, a constitutive transporter can more easily be associated with both the salt-stress response and glycerol assimilation.

Glycerol kinase activity has been described in *Sacch. cerevisiae* as being reduced by approximately 50% when cells were incubated in 1 M salt (Albertyn *et al.*, 1994). Although two different enzymic pathways have been described for glycerol consumption in yeasts (Gancedo *et al.*, 1986), and considering that many of these strains can show constitutive metabolism for glycerol, the probability of avoiding its interference in transport assays could be increased incubating the cells in 1 M NaCl. This was obviously even more important in the case in glycerol-grown cells. An actual interference of metabolism in uptake measurements was probably the case of glycerol-grown cells of *P. anomala*, *Dek. anomala* and *P. jadinii*. By analogy with *Sacch. cerevisiae*

(Lages & Lucas, 1997), this possibility is supported by the observation that, in these strains, maximum accumulation ratios were higher in the absence than in the presence of salt.

The results obtained with *P. jadinii*, unlike what has been published in the literature (Gancedo *et al.*, 1968) and in spite of not observing proton uptake, suggest the activity of a proton-motive-force-dependent carrier for glycerol. The calculated K_d was very high, approximately 20 times higher than the one of *Sacch. cerevisiae* glucose-grown cells (Lages & Lucas, 1997). A K_d of this order of magnitude could indeed support growth, as suggested by Gancedo *et al.* (1968). Nevertheless, computer-assisted kinetic analysis clearly revealed a saturation curve with high V_{max} and a K_m similar to the ones found for other glycerol active transports. Ethanol should stimulate uptake if it were exclusively due to passive diffusion (van Uden, 1985). In *P. jadinii* instead, a strong inhibition was observed, thus supporting the idea that uptake is performed through a transporter (van Uden, 1985). Consistently, the accumulation ratio exceeded equilibrium. Gancedo *et al.* (1968) also reported saturation in glucose-grown cells of *P. jadinii*, attributing this to the activity of constitutively expressed glycerol kinase. Their conclusions are based on long-term transport experiments, which is the reason why the kinetic parameters are difficult to compare.

As final remark, we emphasize that in yeasts with different degrees of halotolerance, glycerol active transport appears to be differently affected by the presence of salt. As an example, in *P. jadinii* (1 M salt-tolerance class) the transport maximum velocity decreases more than 50% when cells are incubated in salt. Under the same circumstances, *Sacch. cerevisiae* (2 M salt-tolerance class) glycerol uptake maximum velocity remains approximately constant (Lages & Lucas, 1997), while in *P. sorbitophila* (4 M salt-tolerance class) it is stimulated by salt by about 50% (Lages & Lucas, 1995). Together with an efficient glycerol production, glycerol active transport could justify the intrinsic capacity of a strain to resist salt stress, besides of other requirements like, for instance, the capacity to extrude efficiently sodium ions. The results obtained in this survey indicate that the activity of an active glycerol transport system may be an evolutionary advantage for growth under high-salt stress, since it is constitutively expressed in all the strains that can grow on glucose at more than 2 M NaCl.

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