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Differential uptake of fumarate by *Candida utilis* and *Schizosaccharomyces pombe*

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Abstract The dicarboxylic acid fumarate is an important intermediate in cellular processes and also serves as a precursor for the commercial production of fine chemicals such as L-malate. Yeast species differ remarkably in their ability to degrade extracellular dicarboxylic acids and to utilise them as their only source of carbon. In this study we have shown that the yeast *Candida utilis* effectively degraded extracellular fumarate and L-malate, but glucose or other assimilable carbon sources repressed the transport and degradation of these dicarboxylic acids. The transport of both dicarboxylic acids was shown to be strongly inducible by either fumarate or L-malate while kinetic studies suggest that the two dicarboxylic acids are transported by the same transporter protein. In contrast, *Schizosaccharomyces pombe* effectively degraded extracellular L-malate, but not fumarate, in the presence of glucose or other assimilable carbon sources. The *Sch. pombe* malate transporter was unable to transport fumarate, although fumarate inhibited the uptake of L-malate.

Introduction

The C_4-dicarboxylic acid fumarate serves as an intermediate of the tricarboxylic acid (TCA) cycle that allows for the metabolic flow of carbon between various metabolic pathways. Yeast species differ remarkably in their ability to transport and utilise one or more intermediates of the TCA cycle (Barnett and Kornberg 1960). Previous studies have shown that L-malate can be utilised by *Candida utilis* (Cássio and Leão 1993), *Candida sphaerica* (Côrte-Real et al. 1989), *Hansenula anomala* (Côrte-Real and Leão 1990) and *Kluyveromyces marxianus* (Queiros et al. 1998) as their only source of carbon and energy. In these species, the dissociated form of L-malate is transported across the plasma membrane by a H^+-symport system that is substrate-inducible and subject to glucose repression. In contrast, *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii* can degrade L-malate only in the presence of an assimilable carbon source (Rodriguez and Thornton 1990; Osothsilp and Subden 1986a). Other yeasts such as *Saccharomyces cerevisiae* can import L-malate and other dicarboxylic acids only via simple diffusion (Salmon 1987) and is therefore unable to effectively degrade or utilise extracellular L-malate.

In *Sch. pombe*, the dissociated form of L-malate is actively transported via a H^+-symport system that operates constitutively, whereas the undissociated acid enters the cell via simple diffusion (Baranowski and Radler 1984; Osothsilp and Subden 1986b; Sousa et al. 1992). The dicarboxylic acids fumarate, D-malate, succinate, oxaloacetate, maleate, malonate and α-ketoglutarate acted as competitive inhibitors for the uptake of L-malate (Sousa et al. 1992), suggesting a common transporter for the uptake of dicarboxylic acids in fission yeast. However, Grobler et al. (1995) showed that L-malate, succinate and malonate, but not α-ketoglutarate, were actively transported by *Sch. pombe* cells.

In addition to its role in metabolic processes, fumarate is also an important precursor for the commercial production of fine chemicals such as L-malate. The D,L-malate racemic mixture is routinely used in a variety of foods and beverages whereas the L-isomer is used for the treatment of conditions such as hyperammonaemia (Rosenberg et al. 1999). The racemic mixture is commercially produced via chemical hydratation of maleate or fumarate, and the L-isomer through the enzymatic conversion of fumarate using fumarase-containing

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microbial cells. The bioconversion of fumarate to L-malate has been obtained by strains of *Brevibacterium* (Takata et al. 1980), *Candida rugosa* (Yang et al. 1992), *Pichia* (Keruchen'ko et al. 1995) and *Dipodascus* (Rosenberg et al. 1999) that exhibit high fumarase activities. Over-expression of the *Sac. cerevisiae* fumarase gene, *FUM1*, also resulted in an increased conversion rate of fumarate to L-malate (Peleg et al. 1990). Since *Sac. cerevisiae* can only import fumarate through diffusion, the introduction of a fumarate transporter gene into *Sac. cerevisiae* could enable this yeast to actively transport fumarate and consequently improve the bioconversion of fumarate.

Heterologous expression of the *Sch. pombe* malate transporter gene, *mae1*, in a strain of *Sac. cerevisiae* resulted in the active transport and efficient degradation of L-malate (Volschenk et al. 1997a, b). Our first approach was therefore to determine whether expression of the *mae1* gene in *Sac. cerevisiae* would also enable the recombinant strain to transport fumarate. We found that neither the recombinant *Sac. cerevisiae* strain nor the wild type *Sch. pombe* strain could transport fumarate. In search of an alternative fumarate transporter, several yeast species were evaluated for their ability to degrade extracellular fumarate. Since *C. utilis* proved to be able to degrade both fumarate and L-malate, the transport of these dicarboxylic acids was further investigated in this yeast.

Materials and methods

Microorganisms and culture media

The yeast strains used in the transport studies included *C. utilis* ATCC 9950 T, *Sch. pombe* 972 h^- (Osothsilp 1987), *Sch. pombe* 972 h^- *leu1*-32 TR^- *mae1*$^-$ (Osothsilp and Subden 1986b) and *Sac. cerevisiae* YPH259 (MATα *ura*3-52, *lys*2-801$_a$, *ade*2-101$_o$, *his*3Δ200, *leu*2-Δ1) (Sikorski and Hieter 1989). The strains used for the screen on fumarate/malate indicator plates are listed in Table 1. Unless otherwise stated, the growth media contained 0.17% YNB (yeast nitrogen base without amino acids and ammonium sulphate [Difco Laboratories, Detroit, Mich.]), 0.5% $(NH_4)_2SO_4$, supplemented with amino acids and buffered at pH 3.5. Different concentrations of fumarate, L-malate and/or a carbon source were added as indicated for the different experiments.

Degradation of extracellular fumarate and L-malate

Indicator agar plates were used to screen different yeast species for the degradation of fumarate and L-malate in the presence of different carbon sources. The yeast strains were streaked onto YNB agar plates containing 0.05% bromocresol-green, 0.3% fumarate or L-malate, together with 2% glucose, fructose, galactose, glycerol, maltose, raffinose or sucrose as carbon source. The plates were incubated at 30 °C for 2 days and evaluated for a colour change from yellow at pH 3.3 to blue at pH 5.2.

The utilisation of extracellular fumarate and L-malate by *C. utilis* and *Sch. pombe* was determined after growth in liquid YNB media containing 2% glucose, raffinose or glycerol/ethanol as carbon source, supplemented with either 0.5% fumarate or L-malate. Cells were harvested at different time intervals and high performance liquid chromatography (HPLC) was used to determine the residual levels of fumarate and L-malate. Glucose concentrations were measured with the glucose oxidase method

Table 1 Utilisation of L-malate and fumarate in various yeast species grown on indicator agar with different carbon sources

	Malate							Fumarate						
	Fructose	Galactose	Glucose	Glycerol	Maltose	Raffinose	Sucrose	Fructose	Galactose	Glucose	Glycerol	Maltose	Raffinose	Sucrose
Saccharomyces cerevisiae 228	−	−	−	ng[a]	−	−	−	−	−	−	ng	−	−	−
Schizosaccharomyces pombe 972 h^-	+	ng	+	+	+	+	+	−	ng	−	−	−	−	−
Candida utilis ATCC 9950 T	+	+	−	+	+	+	+	+	+	+	+	+	+	+
Hansenula anomala UOFS YW207 HT	+	−	+	−	−	−	−	+	−	+	−	+	+	−
Rhodosporidium toruloides CBS 0014	−	−	−	−	+	+	−	−	+	−	+	+	+	−
Rhodotorula graminis CBS 2826T	−	−	−	−	+	+	−	−	−	−	+	+	+	−
Tremella fuciformis CBS 6970T	−	−	−	+	+	+	−	−	+	+	+	+	+	−
Yarrowia lypolitica CBS 2073	−	+	−	−	−	+	+	−	+	+	−	+	+	+

[a] No growth

(Glucose [Trinder], Sigma, St Louis, Mo.) and cell growth was determined spectrophotometrically at OD_{600}. All assays were done in triplicate.

Transport assays

Cells of *Sac. cerevisiae* YPH259 transformed with plasmid pHV3 containing the *Sch. pombe mae1* gene (Volschenk et al. 1997b), and wild type *Sch. pombe* 972 cells were grown in YNB media containing 2% glucose. For *C. utilis*, cells were cultured in 0.5% fumarate, 0.5% L-malate, 2% glucose, 2% raffinose or 2% glycerol/ethanol as the only source of carbon. To further investigate the effect of different carbon sources on the transport of fumarate and L-malate, *C. utilis* cells were cultured to OD_{600} of 0.6 in media containing 0.5% fumarate, 0.5% L-malate, 2% glucose or 2% glycerol/ethanol and divided into two batches. One batch of cultures was assayed immediately while the other was transferred to fresh medium containing either 0.5% L-malate or 2% glucose as carbon source and incubated for another 6 h.

Cells were harvested in the exponential growth phase (OD_{600} of 0.6), washed twice with ice-cold distilled water and resuspended in 0.1 M KH_2PO_4 (pH 3.5) to a final concentration of approximately 20 mg dry weight ml^{-1} (adapted from Grobler et al. 1995). Cell suspensions were pre-incubated for 5 min at 30 °C in a shaker waterbath at 100 rpm. Assays were initiated by adding 10 µl of an aqueous solution of [1-^{14}C]-fumarate (6.62 µCi/µmol; ICN Pharmaceuticals, CA) or L-[1,4(2,3)-^{14}C]-malate (55 µCi/µmol; Amersham, Bucks, UK). Non-specific binding of ^{14}C-fumarate or ^{14}C-malate to the yeast cells was determined by pre-boiling the cells for 5 min at 100 °C. Samples of 0.5 ml were withdrawn at different time intervals and the reactions were stopped by dilution with 5 ml ice-cold distilled water. The cells were rapidly filtered through 0.45 µm membranes (Millipore Corporation, Bedford, Mass.) and immediately washed with 5 ml ice-cold distilled water. The filters were air dried for 10 min and placed in scintillation vials with 5 ml scintillation reaction mixture (EcoLite, ICN Pharmaceuticals, Calif.). Levels of radioactivity were measured with a Beckman LS 3801 scintillation counter (Beckman Instruments, Calif.).

Cellular fractionation for localisation of dicarboxylic acids

Cultures of *Sch. pombe* in 10 ml YNB medium containing 2% glucose were harvested at OD_{600} of 0.6 and resuspended in 1 ml of 0.1 M KH_2PO_4 (pH 3.5). Cultures were incubated for another hour with 1 µl of either ^{14}C-fumarate (6.62 µCi/µmol) or ^{14}C-malate (55 µCi/µmol). A final concentration of 0.5% non-labelled fumarate or L-malate was added to the ^{14}C-malate or ^{14}C-fumarate cultures, respectively. Cells were harvested and the supernatant was transferred to scintillation vials containing 5 ml scintillation reaction mixture. The cells were resuspended in 300 µl of 0.1 M KH_2PO_4 (pH 3.5) together with 0.3 g glass beads (106 µm diameter). Cells were broken with 10 pulses of 15 s with 1 min on ice between pulses. The supernatant and cell debris were separated through centrifugation and transferred to scintillation vials containing 5 ml scintillation reaction mixture. The levels of radioactivity were determined as described above.

Kinetic parameters for protein-mediated transport

Cells of *C. utilis* were cultured in YNB medium containing 0.5% fumarate as the only carbon source. Cells were harvested in the exponential growth phase (OD_{600} of 0.6), washed twice with ice-cold distilled water and resuspended in 0.1 M KH_2PO_4 (pH 3.5) to a final concentration of 7 mg dry weight ml^{-1}. Transport assays were initiated by adding increasing concentrations of ^{14}C-fumarate (0.015–2 mM) in the presence or absence of 2 mM non-labelled L-malate. Estimates of kinetic parameters were obtained from Lineweaver-Burk plots of the initial uptake rates of ^{14}C-fumarate. The K_m for total dicarboxylic acids was based on the concentrations of both anionic and undissociated dicarboxylic acids.

Results

Lack of fumarate transport by recombinant *Sac. cerevisiae* and wild type *Sch. pombe*

Strains of *Sac. cerevisiae* cannot transport extracellular dicarboxylic acids such as L-malate or fumarate (Salmon 1987). However, transport studies with a recombinant *Sac. cerevisiae* strain expressing the *mae1* gene of *Sch. pombe*, showed that active transport of ^{14}C-malate was obtained, whereas fumarate was not transported (Fig. 1a). The active transport of ^{14}C-malate by the wild type *Sch. pombe* 972 h^- strain was confirmed (Fig. 1b), but not in the *Sch. pombe mae1*$^-$ mutant strain that has a defective malate transporter. No transport of ^{14}C-fumarate was observed in either strain (Fig. 1b). However, increasing concentrations of fumarate progressively inhibited L-malate uptake by the recombinant *Sch. pombe* malate transporter (Fig. 1c). The HPLC analyses confirmed that *Sch. pombe* cells removed a significant portion (approximately 65%) of the L-malate from the glucose-containing growth media within 28 h (Fig. 2a), whereas less than 15% of the fumarate was removed (Fig. 2b). Similar results were obtained for cells grown in media containing raffinose or glycerol/ethanol as carbon source (data not shown).

Since fumarate inhibited the transport of L-malate in the recombinant *Sac. cerevisiae* strain without being transported itself, the uptake and subsequent cellular localisation of ^{14}C-malate and fumarate was further investigated in wild type *Sch. pombe* cells (Table 2). An hour after the addition of ^{14}C-malate to glucose-grown cells, approximately 30% of the ^{14}C-malate was removed from the extracellular fraction. The majority of this was already further metabolised to pyruvate and CO_2 with only 0.36% and 0.32% retained in the cell debris and intracellular fractions, respectively. The addition of unlabelled fumarate decreased the uptake of ^{14}C-malate by 20% and reduced the localisation of ^{14}C-malate in the cell debris and intracellular fractions by 50% and 28%, respectively.

When ^{14}C-fumarate was added to the *Sch. pombe* cells, only 1.9% was removed from the extracellular fraction after 1 h, but almost 10% of this was retained in the cell debris (Table 2). Although the addition of unlabelled L-malate did not significantly influence the uptake of ^{14}C-fumarate, it decreased its localisation in the cell debris by more than 50%. These results suggested that fumarate competes for the uptake of L-malate by inhibiting its binding to the malate transporter, although only L-malate is actively transported by the protein.

Screening of yeast species for degradation of extracellular fumarate and L-malate

In a screen for yeasts capable of transporting fumarate and L-malate, several yeast species were screened for

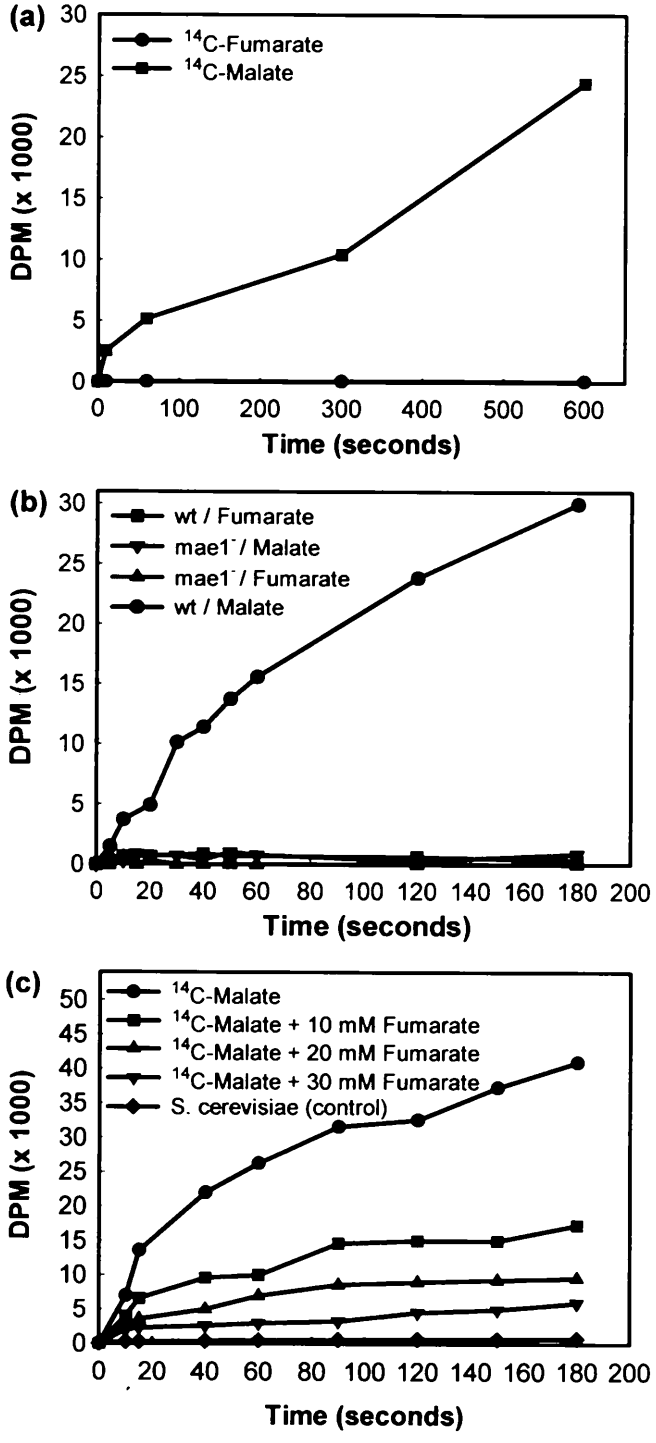

Fig. 1 Transport studies to determine the uptake of L-malate and fumarate by strains of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. **a** Uptake of ^{14}C-malate and ^{14}C-fumarate in *Sac. cerevisiae* cells transformed with the *Sch. pombe mae1* gene. **b** Uptake of ^{14}C-malate and ^{14}C-fumarate by *Sch. pombe* 972 h$^-$ (wt) and *Sch. pombe mae1*$^-$ (*mae1*$^-$) grown in 2% glucose. **c** Competition by fumarate for the transport of 4 mM ^{14}C-malate at pH 3.5 by the *Sac. cerevisiae* YPH259 host strain (control) or transformed with the *Sch. pombe mae1* gene. The yeast strains were grown in 2% glucose without fumarate, or with 10 mM, 20 mM or 30 mM non-labelled fumarate added simultaneously with the ^{14}C-malate

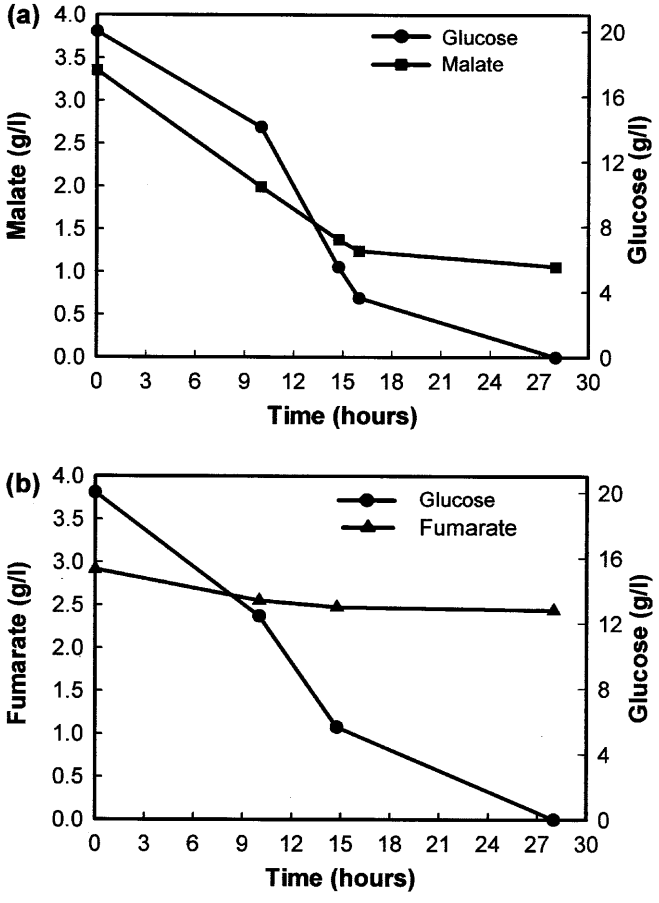

Fig. 2 HPLC analyses of extracellular concentrations of **a** L-malate and **b** fumarate during growth of *Sch. pombe* 972 h$^-$ on medium containing 2% glucose

their ability to degrade extracellular fumarate or L-malate incorporated into fumarate/malate indicator agar plates (Table 1). The yeasts *Sch. pombe* and *Sac. cerevisiae* are not able to utilise intermediates of the TCA cycle as their only source of carbon (Barnett and Kornberg 1960), whereas the other species that were investigated are known for their ability to utilise TCA cycle intermediates. No degradation of either fumarate or L-malate was found for *Sac. cerevisiae*, since the yeast is unable to transport either of the dicarboxylic acids. In *Sch. pombe*, L-malate was effectively degraded in the presence of all the carbon sources that sustained growth, but no degradation of fumarate was observed. For *C. utilis*, degradation of both fumarate and L-malate were found in all the carbon sources investigated, except for glucose (Table 1). The other yeast species showed varying abilities to utilise fumarate or L-malate that seemed to be dependent on the available carbon source. Since the indicator plates only provided limited information, further investigation was required to better understand the regulatory mechanisms involved in the degradation and transport of fumarate and L-malate by *C. utilis*.

Table 2 Distribution of ^{14}C-labelled fumarate and L-malate in different cellular fractions in *Sch. pombe* cells after incubation for 60 min with or without unlabelled L-malate or fumarate

Culture conditions	Cellular distribution (% of total ^{14}C added)		
	Cell debris	Intracellular	Extracellular
Grown in 2% glucose, add ^{14}C-malate	0.36	0.32	69.36
Grown in 2% glucose, add fumarate and ^{14}C-malate	0.18	0.09	83.19
Grown in 2% glucose, add ^{14}C-fumarate	0.18	0.14	98.10
Grown in 2% glucose, add L-malate and ^{14}C-fumarate	0.06	0.07	98.41

Degradation and transport of fumarate and L-malate by *C. utilis*

Cells of *C. utilis* effectively degraded extracellular fumarate when grown in YNB medium containing fumarate as the only carbon source (Fig. 3a). However, the degradation of fumarate was less efficient when grown in the presence of either raffinose or glycerol/ethanol as carbon source, suggesting that other assimilable carbon sources may result in catabolite repression of fumarate transport. In support of this, the degradation of fumarate by cells grown in glucose/fumarate media only commenced once the glucose had been depleted (Fig. 3b). Similar results were obtained for L-malate (data not shown), indicating that the degradation of both fumarate and L-malate is subject to catabolite repression.

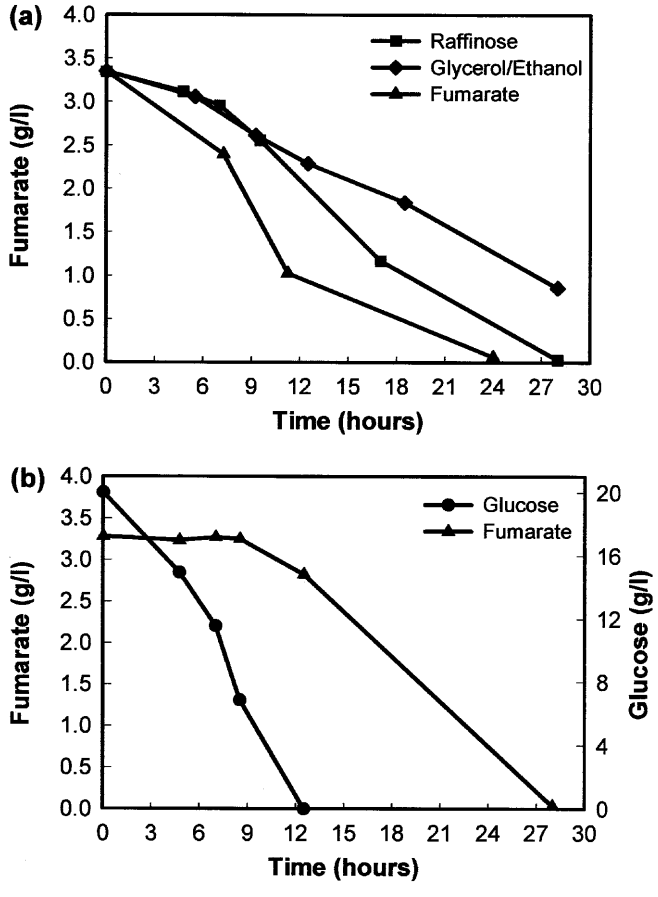

Fig. 3 HPLC analyses showing the residual levels of fumarate after growth of *C. utilis* on **a** 0.5% fumarate, 2% raffinose or 2% glycerol/ethanol or **b** 2% glucose as carbon source (residual concentration of glucose is also indicated)

When *C. utilis* cells were pre-cultured in either fumarate or L-malate as the only carbon source, most of the ^{14}C-fumarate was taken up within 10 s of addition (Fig. 4a). However, the uptake of ^{14}C-fumarate by cells grown on either glucose or raffinose as the only carbon source was almost non-detectable, with only a small amount transported by cells grown on glycerol/ethanol. Similar results were obtained for the transport of L-malate in *C. utilis* (data not shown), indicating that active transport of both fumarate and L-malate was subject to substrate induction by either dicarboxylic acid.

The transport of ^{14}C-malate by *C. utilis* was further investigated by shifting cultures grown on different carbon sources to fresh medium containing either 0.5% L-malate or 2% glucose (Fig. 4b, c). ^{14}C-malate was quickly transported by cells grown on either fumarate or L-malate, but transport ceased when cells were transferred to glucose-containing medium (Fig. 4b). Cells were unable to transport ^{14}C-malate when grown on glucose or glycerol/ethanol medium, not even when 0.5% L-malate was included in the glucose medium (Fig. 4c). However, cells grown on glucose medium regained their ability to transport ^{14}C-malate when transferred to medium containing L-malate as the only carbon source (Fig. 4c).

Preliminary kinetic studies were done to determine whether *C. utilis* uses the same transporter protein for the uptake of fumarate and L-malate. Lineweaver-Burk plots of the initial rates of uptake of ^{14}C-labelled fumarate at pH 3.5 were linear over the concentration range of 0.08–2 mM (Fig. 5). The following kinetic parameters were calculated: $V_{max(fumarate)}$(pH 3.5) = 1.058 nmol s^{-1} mg (dry weight) $cells^{-1}$; K_m(pH 3.5) = 0.11 mM. These results indicated that fumarate and L-malate were mutually competitive inhibitors, suggesting that they might share the same carrier protein in *C. utilis*.

Discussion

The dicarboxylic acid L-malate is widely employed in both the pharmaceutical and food industries. Due to its industrial importance, several groups have investigated the bioconversion of fumarate to L-malate using microbial cells (Takata et al. 1980; Yang et al. 1992; Keruchen'ko et al. 1995; Rosenberg et al. 1999). Increased bioconversion of fumarate to L-malate (80.4 mmol fumaric acid/h per g of cell wet weight) was

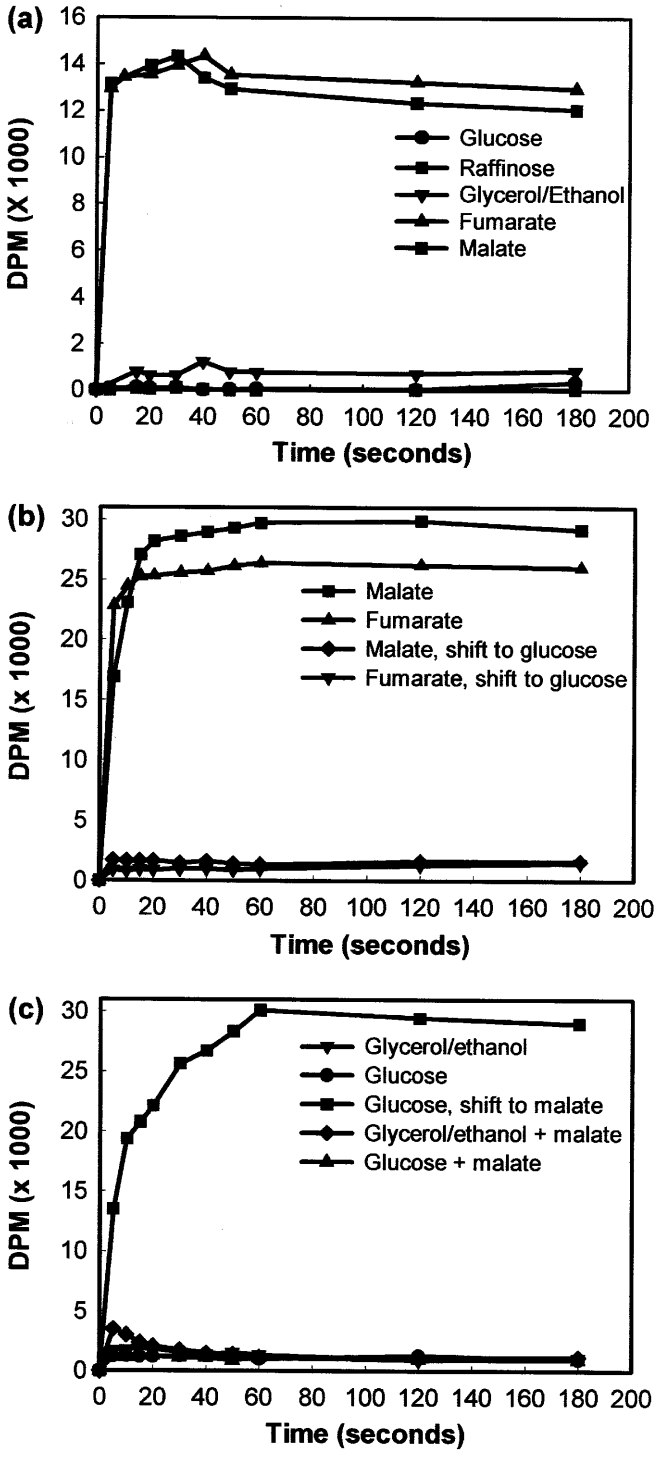

Fig. 4 Transport studies to determine the uptake of ^{14}C-labelled L-malate and fumarate by *C. utilis*. **a** Uptake of ^{14}C-fumarate after growth on 2% glucose, 2% raffinose, 2% glycerol/ethanol, 0.5% fumarate or 0.5% L-malate as only carbon source. **b** Uptake of ^{14}C-malate after growth on 0.5% fumarate or 0.5% L-malate as only carbon source, and shifted to fresh medium containing 2% glucose. **c** Uptake of ^{14}C-malate after growth on 2% glucose or 2% glycerol/ethanol with or without 0.5% L-malate. Glucose-grown cells were also shifted to fresh medium containing 0.5% L-malate

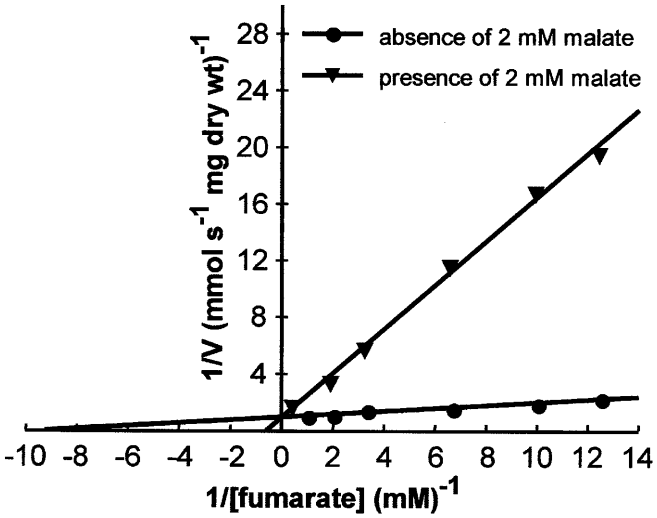

Fig. 5 Lineweaver-Burk plots of the initial uptake rates of 4 mM ^{14}C-fumarate by fumarate-grown cells as a function of the fumarate concentration in the media. Assays were done in the presence or absence of 2 mM L-malate

obtained by over-expression of the *Sac. cerevisiae* fumarase gene, *FUM1* (Peleg et al. 1990). This efficiency may be further improved if the *Sac. cerevisiae* cells were able to actively transport fumarate and not have to rely only on diffusion of the substrate. This could be realised through heterologous expression of a suitable fumarate transporter from another yeast in *Sac. cerevisiae*.

A screen for yeast strains that could degrade extracellular fumarate showed significant differences in the regulation and specificity for the uptake of fumarate and L-malate between yeast species. A common dicarboxylic acid transporter was suggested for *Sch. pombe* strain ICV'M (Sousa et al. 1992), but results presented here showed that neither the wild type *Sch. pombe* 972 h$^-$ strain nor a recombinant strain of *Sac. cerevisiae* containing the *Sch. pombe* malate transporter gene was able to transport fumarate (Fig. 1). However, increasing concentrations of fumarate were able to progressively inhibit the uptake of L-malate by the recombinant strain. Cellular fractionation of glucose-grown cells (Table 2) showed that the addition of unlabelled fumarate decreased both the uptake and membrane localisation of ^{14}C-malate. The data suggested that fumarate can also bind to the malate transporter and therefore inhibit the uptake of L-malate. The binding of both fumarate and L-malate to the *Sch. pombe* malate transporter can be ascribed to the structural relatedness of the two dicarboxylic acids. Similarly, Grobler et al. (1995) reported that α-ketoglutarate was not transported by *Sch. pombe*, although it competed for the uptake of L-malate (Sousa et al. 1992).

The results presented here indicate a significant difference in the transport of fumarate and L-malate by *C. utilis* and *Sch. pombe*. Cells of *Sch. pombe* 972 effectively transported L-malate, but not fumarate, and no evidence for substrate induction or glucose repression for the uptake of L-malate was found. In contrast, the

C. utilis ATCC 9950 T strain effectively transported both fumarate and L-malate and the uptake of both dicarboxylic acids was induced by either of the substrates. The kinetic data suggest that fumarate and L-malate are transported by the same carrier protein in *C. utilis*, which explains the similar regulatory mechanisms observed for the transport of fumarate and L-malate.

The degradation of either fumarate or L-malate by *C. utilis* was sensitive to the presence of glucose (Fig. 3). This supports previous reports that the utilisation of L-malate in *C. utilis* strain IGC 3092 was subject to glucose repression (Cássio and Leão 1993). In addition, we observed that the transport of either fumarate or L-malate was also insignificant in the presence of other carbon sources such as raffinose and glycerol/ethanol (Fig. 4). This confirmed that *C. utilis* employs a double regulatory mechanism for the transport of L-malate and fumarate with the dicarboxylic acids only being transported in the presence of either of the inducers and when no alternative carbon source is available.

The carbon sensitivity and substrate induction observed for the uptake of fumarate and L-malate by *C. utilis* could be interpreted in the context of its ability to utilise intermediates of the TCA cycle as the only source of carbon and energy. The yeast *C. utilis* is Crabtree-negative and can therefore ferment sugars only under oxygen-limited conditions (Van Dijken et al. 1993). Under aerobic growth conditions, the yeast tended to channel most of its pyruvate into the TCA cycle, resulting in an adequate supply of intracellular TCA cycle intermediates such as fumarate and L-malate. Since the degradation of glucose, raffinose or glycerol/ethanol can provide pyruvate for the TCA cycle, the dicarboxylic acids will most likely only be utilised if a more efficient carbon source is not available. The results presented here support the notion that *C. utilis* cells allow the transport of fumarate and L-malate only in the presence of the inducers and when an alternative carbon source is not available. Furthermore, the results presented in Fig. 3 indicate that the catabolite repression is stronger when cells are grown on glucose than on the less favourable carbon source glycerol/ethanol.

Although the transport of dicarboxylic acids has been described for a number of yeast species, the *Sch. pombe mae1* gene is the only malate transporter gene cloned and sequenced thus far (Grobler et al. 1995). In this study, we demonstrated significant differences between *Sch. pombe* and *C. utilis* concerning the uptake of fumarate and L-malate and the regulation thereof. However, a proper investigation into the molecular basis for the transport of fumarate and L-malate by *C. utilis* can only be done once the fumarate/malate transporter gene from *C. utilis* is cloned.

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References

Baranowski K, Radler F (1984) The glucose-dependent transport of L-malate in *Zygosaccharomyces bailii*. Antonie Van Leeuwenhoek 50: 329–340

Barnett JA, Kornberg HL (1960) The utilisation by yeast of acids of the tricarboxylic acid cycle. J Gen Microbiol 23: 65–82

Cássio F, Leão C (1993) A comparative study on the transport of l(-)malic acid and other short-chain carboxylic acids in the yeast *Candida utilis*: evidence for a general organic acid permease. Yeast 9: 743–752

Côrte-Real M, Leão C (1990) Transport of malic acid and other dicarboxylic acids in the yeast *Hansenula anomala*. Appl Environ Microbiol 56: 1109–1113

Côrte-Real M, Leão C, Van Uden N (1989) Transport of L-malic acid and other dicarboxylic acids in the yeast *Candida sphaerica*. Appl Microbiol Biotechnol 31: 551–555

Grobler J, Bauer F, Subden RE, Van Vuuren HJJ (1995) The *mae1* gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C_4 dicarboxylic acids. Yeast 11: 1485–1491

Keruchen'ko YS, Kerucheno'ko ID, Gladilin KL (1995) Formation of malate by yeast of the genus *Pichia*. Prikl Biochim Mikrobiol 31: 143–144

Osothsilp C (1987) Genetic and biochemical studies of malic acid metabolism in *Schizosaccharomyces pombe*. PhD dissertation, University of Guelph, Ontario, Canada

Osothsilp C, Subden RE (1986a) Isolation and characterisation of *Schizosaccharomyces pombe* mutants with defective NAD-dependent malic enzyme. Can J Microbiol 32: 481–486

Osothsilp C, Subden RE (1986b) Malate transport in *Schizosaccharomyces pombe*. J Bacteriol 168: 1439–1443

Peleg Y, Rokem JS, Goldberg I, Pines O (1990) Inducible overexpression of the *FUM1* gene in *Saccharomyces cerevisiae*: Localization of fumarase and efficient fumaric acid bioconversion to L-malic acid. Appl Environ Microbiol 56: 2777–2783

Queiros O, Casal M, Althoff S, Moradas-Ferreira P, Leão C (1998) Isolation and characterization of *Kluyveromyces marxianus* mutants deficient in malate transport. Yeast 14: 401–407

Rodriguez SB, Thornton RJ (1990) Factors influencing the utilisation of L-malate by yeasts. FEMS Microbiol Lett 72: 17–22

Rosenberg M, Miková H, Krištofíková L (1999) Formation of L-malic acid by yeasts of the genus *Dipodascus*. Lett Appl Microbiol 29: 221–223

Salmon JM (1987) L-malic acid permeation in resting cells of anaerobically grown *Saccharomyces cerevisiae*. Biochim Biophys Acta 901: 30–34

Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19–27

Sousa MJ, Mota M, Leão C (1992) Transport of malic acid in the yeast *Schizosaccharomyces pombe*: evidence for a proton–dicarboxylate symport. Yeast 8: 1025–1031

Takata I, Yamamoto K, Tosa T, Chibata I (1980) Immobilization of *Brevibacterium flavum* with carrageenan and its application for continuous production of L-malic acid. Microb Technol 2: 30–36

Van Dijken JP, Weusthuis RA, Pronk JT (1993) Kinetics of growth and sugar consumption in yeasts. Antonie Van Leeuwenhoek 63: 343–352

Volschenk H, Viljoen M, Grobler J, Bauer F, Lonvaud-Funel A, Denayrolles M, Subden RE, Van Vuuren HJJ (1997a) Malolactic fermentation in grape musts by a genetically engineered strain of *Saccharomyces cerevisiae*. Am J Enol Vitic 48: 193–197

Volschenk H, Viljoen M, Grobler J, Petzold B, Bauer F, Subden RE, Young RA, Lonvaud-Funel A, Denayrolles M, Van Vuuren HJJ (1997b) Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. Nat Biotechnol 15: 253–257

Yang LW, Wang XY, Wei S (1992) Immobilization of *Candida rugosa* having high fumarase activity with polyvinyl alcohol. Ann NY Acad Sci 672: 563