

ACCELERATED PUBLICATION

Identification of the mitochondrial pyruvate carrier in *Saccharomyces cerevisiae*

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Mitochondrial pyruvate transport is fundamental for metabolism and mediated by a specific inhibitable carrier. We have identified the yeast mitochondrial pyruvate carrier by measuring inhibitor-sensitive pyruvate uptake into mitochondria from 18 different *Saccharomyces cerevisiae* mutants, each lacking an unattributed member of the mitochondrial carrier family (MCF). Only mitochondria from the YIL006w deletion mutant exhibited no

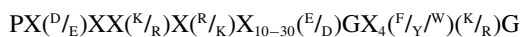
inhibitor-sensitive pyruvate transport, but otherwise behaved normally. YIL006w encodes a 41.9 kDa MCF member with homologous proteins present in both the human and mouse genomes.

Key words: mitochondrial carrier family, mitochondrial pyruvate transport, pyruvate metabolism, *Saccharomyces*.

INTRODUCTION

The transport of pyruvate into mitochondria is essential for glucose oxidation, lipogenesis and gluconeogenesis, and is also required for the metabolism of some amino acids [1,2]. The existence of a specific mitochondrial carrier for pyruvate was unequivocally demonstrated in this laboratory by the discovery of the specific and potent inhibitor of transport α -cyano-4-hydroxycinnamate (CHC) [3]. This reagent, and the more potent analogue α -cyano- β -(1-phenylindol-3-yl)acrylate (UK5099), specifically inhibit mitochondrial pyruvate transport by modifying a thiol group on the carrier [4]. Subsequent studies in this laboratory and that of Professor Giuseppe Paradies at the University of Bari, Bari, Italy, led to a detailed description of the kinetic properties and substrate and inhibitor specificity of the carrier, and revealed that it also plays an important role in the transport of ketone bodies, such as acetoacetate and β -hydroxybutyrate, across the mitochondrial inner membrane (MIM) [5–9]. The amount of pyruvate carrier present in heart and liver mitochondria was estimated from inhibitor titrations and radioactive binding studies to be in the range of 50–100 pmol/mg of protein [10,11].

The mitochondrial carrier family (MCF) is characterized by the possession of a tripartite structure: three tandem repeated homologous sequences of approx. 100 amino acids each, carrying two transmembrane helices connected by an extensive (matrix-facing) loop, and also by possession of between one and three copies of the consensus motif:



(one-letter amino acid code; X = any amino acid) [12]. However, attempts to identify and clone the mammalian mitochondrial pyruvate carrier have so far failed. Work in this laboratory demonstrated that α -cyanocinnamate protects a 15 kDa MIM protein from labelling by the non-specific thiol reagent *N*-phenylmaleimide, and we originally proposed that this might be the carrier protein or a proteolytic degradation product of it [13]. However, subsequently, using biotinylated maleimide derivatives,

we have isolated this protein on immobilized avidin and sequenced it using MS (J. Hildyard and A. P. Halestrap, unpublished work). Our results revealed the protein to be the 15 kDa subunit of cytochrome oxidase (COXIV) and therefore very unlikely to be the mitochondrial pyruvate carrier. These data also provide an explanation of why the amount of *N*-phenylmaleimide-labelled protein in heart mitochondria was found to be fivefold greater than the amount of carrier protein estimated from inhibitor titrations of transport [10]. Nalecz et al. [14] pursued an alternative strategy to identify the protein, which involved purification and reconstitution of active carrier from solubilized MIMs. This tentatively identified a protein of the expected molecular mass (about 30 kDa), but no sequence was ever published.

In view of the lack of success with identifying the mitochondrial pyruvate carrier in mammalian mitochondria, we turned our attention to bakers' yeast (*Saccharomyces cerevisiae*), where sequencing of the genome has identified 35 members of the mitochondrial carrier family. Knockout mutants of all these are freely available from the EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis) (Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany) index (<http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html>) and, at the time that experimental work commenced, 16 members had already been functionally characterized (Table 1; in this Table, recently characterized functions, and functions suggested by homology, are listed in *italics*). Of the remaining 19, one (YBR192w, RIM2) is essential for viability under respiratory growth conditions and is thus not accessible to our approach. We therefore decided to isolate mitochondria from all 18 of the remaining mutants to establish which demonstrate a total lack of CHC-sensitive mitochondrial pyruvate transport. With this approach, we have identified YIL006w (41.9 kDa) as the mitochondrial pyruvate carrier in yeast. A BLAST search of the human and mouse genome revealed that the closest human member of the mitochondrial carrier family was MGC4399 (accession no. NP_115691). This is widely expressed, but no function has yet been attributed to it.

Abbreviations used: abf2, ARS-binding factor 2; CHC, α -cyano-4-hydroxycinnamate; DTT, dithiothreitol; MCF, mitochondrial carrier family; MIM, mitochondrial inner membrane; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, UK5099: α -cyano- β -(1-phenylindol-3-yl)acrylate.

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Table 1 The 35 *S. cerevisiae* MCF genes

Initially unknown or unconfirmed functions are listed in *italics*. Growth rates in pyruvate medium (see under 'Methods' in the Experimental section) are given relative to wild-type and categorized according to the time taken to reach an attenuation (*D*) of 2.5: normal, < 4 days; slow, 4–6 days; very slow, > 6 days. (ND, not determined). The 18 deletion mutants used in the present study are collected together at the bottom and indicated by *. Other abbreviations: SGD ID, *Saccharomyces* Genome Database Identifier; mtRNA, mitochondrial RNA.

Gene name	SGD ID	Amino acid residue	Function	Pyruvate growth rate
AAC2/PET9	YBL030c	318	ADP/ATP	ND
AAC3	YBR085w	308	ADP/ATP	ND
CTP1	YBR291c	299	Citrate	ND
UGO1	YDR470c	502	Mitochondrial fusion	ND
	YER053c	300	Vacuolar phosphate	ND
FLX1	YIL134w	311	Flavin	ND
MIR1	YJR077c	311	Phosphate	ND
SFC1	YJR095w	322	Succinate/fumarate	ND
OAC1	YKL120w	324	Oxaloacetate	ND
DIC1	YLR348c	298	Dicarboxylate	ND
AAC1	YMR056c	309	ADP/ATP	ND
ORT1	YOR130c	292	Ornithine	ND
ODC2	YOR222w	307	Oxodicarboxylate	ND
CRC1	YOR100c	327	Carnitine/acylcarnitine	ND
ANT1	YPR128c	328	Peroxisomal ATP/AMP	ND
ODC1	YPL134c	310	Oxodicarboxylate	ND
RIM2	YBR192w	377	Unknown	ND
TPC1	YGR096w*	314	<i>Thiamine pyrophosphate</i>	Very slow
LEU5	YHR002w*	357	<i>CoA</i>	Slow
	YNL083w*	545	<i>Aspartate/glutamate</i>	Normal
	YPR021c*	902	<i>Aspartate/glutamate</i>	Slow
MRS3	YJL133w*	296	<i>mtRNA splicing/iron transport</i>	Normal
MRS4	YKR052c*	296	<i>mtRNA splicing/iron transport</i>	Normal
YMC2	YBR104w*	329	Unknown	Normal
YHM1	YDL198c*	300	Unknown: <i>mtDNA maintenance?</i>	Very slow
	YDL119c*	307	Unknown	Normal
	YEL006w*	335	Unknown	Normal
	YFR045w*	309	Unknown	Normal
	YGR257c*	366	Unknown	Very slow
	YIL006w*	373	Unknown	Normal
YHM2	YMR241w*	314	Unknown	Very slow
	YMR166c*	368	Unknown	Slow
PET8	YNL003c*	284	Unknown	Slow
YMC1	YPR058w*	307	Unknown	Normal
	YPR011c*	326	Unknown	Normal

EXPERIMENTAL

Materials

Lyticase (recombinant; from *Arthrobacter luteus*) was obtained from Sigma (Gillingham, Dorset, U.K.). Protease-inhibitor tablets (Complete™) were obtained from Roche Diagnostics Ltd. (Lewes, East Sussex, U.K.). [2-¹⁴C]Pyruvate and [³H]acetate were obtained from PerkinElmer Life Sciences (Beaconsfield, Bucks., U.K.), and ³H₂O and [U-¹⁴C]sucrose were from Amersham Biosciences (Chalfont St. Giles, Bucks., U.K.). Dithiothreitol (DTT) was purchased from Apollo Scientific (Stockport, Cheshire, U.K.). All other chemicals and biochemicals were obtained from Sigma or Merck via VWR International Ltd (Poole, Dorset, U.K.).

Methods

Yeast strains and growth conditions

All yeast MCF knockouts were obtained from EUROSCARF except ΔYHR002w, which was prepared as described [15] and kindly donated by Professor Dr Johannes Hegemann (Institut für Mikrobiologie, Heinrich-Heine-Universität, 40225 Duesseldorf, Germany). All EUROSCARF strains were BY4741 (MATa, his3Δ1, leu2Δ0, ura3Δ0) with the relevant MCF gene disrupted and replaced with a kanamycin-resistance cassette (KanMX4) where indicated. ΔYHR002w was strain YM4585 (MATa,

CAN^S, his3Δ200, lys2-801, leu2-3112, trp1-903, tyr1-501) with YHR002w disrupted and replaced with a *GFP HIS3* cassette (*GFP* is green fluorescent protein; the *HIS3* gene controls imidazoleglycerolphosphate dehydratase). Yeast strains, grown overnight at 30 °C on YP medium [1% (w/v) yeast extract/2% (w/v) peptone, supplemented with 2% (w/v) glucose] were diluted 100-fold into YP medium supplemented with 0.5% (w/v) sodium pyruvate, or 0.5% pyruvate plus 0.1% (v/v) ethanol, and were cultured aerobically at 30 °C. Cultures were grown to mid-exponential phase before harvesting for the preparation of mitochondria.

Preparation of mitochondria

Spheroplasts were prepared using lyticase, as follows. Yeast cells were collected by centrifugation (2500 g, 10 min), resuspended in deionized water, followed by two cycles of centrifugation and resuspension in fresh water. Cells were then resuspended in 100 mM Tris/20 mM DTT, pH 9.3, and incubated at 30 °C with gentle shaking for 10 min before washing twice with 100 mM Tris/500 mM KCl, pH 7.0, and final resuspension in lyticase buffer (40 mM trisodium citrate/120 mM Na₂HPO₄/1.35 M sorbitol/1 mM EGTA, pH 5.8, plus lyticase at 3 mg/ml). Cells were then incubated for 30–45 min at 30 °C with gentle shaking, and spheroplasts were harvested by centrifugation (2500 g, 5 min,

4 °C). After washing twice with 10 mM Tris/maleate/0.75 M sorbitol/0.4 M mannitol/2 mM EGTA/0.1 % BSA, pH 6.8, the spheroplasts were resuspended in 40 ml of homogenization buffer (10 mM Tris/maleate/0.6 M mannitol/2 mM EGTA/1 mM EDTA/0.5 mM Na₂HPO₄/2 % BSA, pH 6.8) containing one protease-inhibitor tablet (Roche) added immediately before use. Spheroplasts were lysed using twelve passes of a Dounce–Potter homogenizer with a tight-fitting pestle, and the homogenate centrifuged at 800 g for 10 min (4 °C) to pellet cellular debris and unbroken cells. Pellets were resuspended and centrifuged again at 800 g before pooling the supernatants. A mitochondrial fraction was collected by centrifugation at 11 000 g for 10 min (4 °C) and resuspended in isolation buffer (10 mM Tris/maleate/0.65 M mannitol, pH 6.8). After removal of retained cellular debris by centrifugation at 800 g, the supernatants were centrifuged at 11 000 g and the final mitochondrial pellet was resuspended in a small volume of isolation buffer. The mitochondrial protein concentration was assayed by using the Biuret method.

[2-¹⁴C]Pyruvate transport assays

Pyruvate uptake into mitochondria was monitored using the ‘inhibitor-stop’ technique, with transport being terminated at the required time by addition of excess CHC. Mitochondrial pyruvate transport is proton-linked, and uptake is driven by the pH gradient across the MIM [2]. This pH gradient can be created either by respiratory-chain-driven proton translocation or by equilibrating mitochondria at a high pH before transferring them into a buffer of lower pH. We used the former protocol with ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as respiratory substrate as an initial screen to identify mitochondria lacking a pyruvate transporter, but confirmed positive results using the latter technique. For respiration-driven uptake, mitochondria (about 20 mg of protein/ml of isolation buffer) were pretreated with either 10 μM UK5099 [α-cyano-β-(1-phenylindol-3-yl)acrylate], from a 50 mM stock solution in DMSO, or an equivalent volume of DMSO, for 10 min before centrifugation to remove excess inhibitor. Pelleted mitochondria, were then resuspended in 1.2 ml of uptake buffer [0.65 M mannitol/1 mM EGTA/5 mM Mops/5 mM KH₂PO₄/0.1 % (w/v) BSA/3 μM TMPD/3 mM ascorbate/1 μM antimycin A/1 μM rotenone, pH 7.4] containing ³H₂O (1.0 KBq/ml), and incubated at 4 °C for 3 min to energize the mitochondria. [2-¹⁴C]Pyruvate (0.1 mM; 1.0 KBq/ml) was added to start uptake, and aliquots (200 μl, equivalent to 2–4 mg of protein) were removed at the required time and rapidly transferred into 20 μl of 100 mM CHC solution (final concn. 10 mM) to prevent further uptake. Mitochondria were rapidly sedimented by centrifugation for 1 min in microcentrifuge tubes, the supernatant totally removed and the pellet solubilized in 0.2 ml of 5 % (w/v) SDS. The ¹⁴C and ³H content of both the solubilized mitochondria and a 0.1 ml aliquot of the supernatant were determined by liquid-scintillation counting. Mitochondrial matrix volumes were determined in separate incubations using equivalent amounts of mitochondria and identical buffer, but with [¹⁴C]sucrose replacing the [¹⁴C]pyruvate. These data allowed calculation of the matrix pyruvate corrected for extramitochondrial [¹⁴C]pyruvate present in the pellet as described previously [6,16]. For pyruvate transport driven by an artificially imposed pH gradient, the technique was the same as above, but ascorbate/TMPD was omitted. Transport was initiated by transferring the mitochondria that had been equilibrated for 3 min at 4 °C in pH 7.4 uptake buffer into pH 6.8 uptake buffer containing both ³H₂O and [¹⁴C]pyruvate. Termination of uptake and assay of samples was performed as described above. Acetate uptake driven by the pH gradient

was measured as for pyruvate, but using [³H]acetate (2 kBq/ml, 0.1 mM) in place of pyruvate and in the presence of [¹⁴C]sucrose (1 kBq/ml) to correct for extramitochondrial space. Aliquots (0.2 ml) were collected and uptake terminated by rapid centrifugation, before treatment and counting for radioactivity as described above.

Other techniques

SDS/PAGE was performed by the method of Laemmli [17], and proteins were visualized using Coomassie Blue staining as described previously [13]. Measurement of mitochondrial respiration was performed using a Clark-type oxygen electrode essentially as described previously [6]. Mitochondria were added to oxygen-electrode buffer (0.65 M mannitol/20 mM Tris/maleate/0.5 mM EGTA/2 mM MgCl₂/0.1 % (w/v) BSA/10 mM K₂HPO₄, pH 6.8) in the presence of 0.8 mM ADP and 2 mM potassium L-malate, and rates of oxygen consumption on addition of a variety of substrates were measured.

RESULTS

Growth rates of different mutants

Of the 18 knockout mutants cultured, ten showed growth rates comparable with that of the wild-type yeast when grown on 0.5 % (w/v) pyruvate + 0.1 % (w/v) ethanol, and this combination of carbon sources proved more effective than pyruvate alone (results not shown). We also established that the presence of 50 μM UK5099 sufficient to inhibit mitochondrial pyruvate transport by > 98 %, only inhibited growth rates of the wild-type yeast by about 30 %, indicating that yeast lacking the mitochondrial pyruvate carrier would not show substantial impairment of growth rates. Of the eight remaining mutants, four showed markedly lower growth rates and four showed severe growth defects, with ΔYDL198c the most impaired (Table 1).

Mitochondrial pyruvate uptake studies

The results shown in Figure 1(a) show time courses for the uptake of [¹⁴C]pyruvate by yeast mitochondria energized with ascorbate/TMPD in the presence and absence of the inhibitor UK5099 (10 μM). Results are given for wild-type and two mutants (ΔYIL006w and ΔYFR045w), one of which (ΔYIL006w) showed almost no uptake. The data of Figure 1(b) show the uptake at 2 min in the presence and absence of UK5099 for all the mutants tested. These data demonstrate that, despite considerable variation in total pyruvate uptake, the ascorbate/TMPD-mediated pyruvate transport assays revealed nine MCF deletion mutants with both decreased pyruvate uptake and diminished response to UK5099. These were YDL198c, YDL119c, YIL006w, YMR166c, YKR052c, YJL133w, YNL083w, YPR058w and YPR011c. However, YIL006w stood out by showing the least uptake and no inhibition by UK5099. Perhaps surprisingly, only two of the eight ‘slow growers’ were included in this group, suggesting that the phenotypes observed may be due to more global respiratory defects. Indeed, oxygen-electrode studies showed that ΔYDL198c, the slowest grower included in this group, exhibited no detectable respiration on pyruvate, succinate or glutamate. Studies by Kao et al. [18] demonstrated that the YDL198c protein functions as a multicopy suppressor of an *abf2* (ARS-binding factor 2)-null mutant. *abf2* is a DNA-binding protein involved in mitochondrial DNA maintenance and segregation, and thus these data imply a role for YDL198c in similar processes. As respiring mitochondria require a mitochondrial genome for function, failure to maintain or correctly segregate mitochondrial DNA would

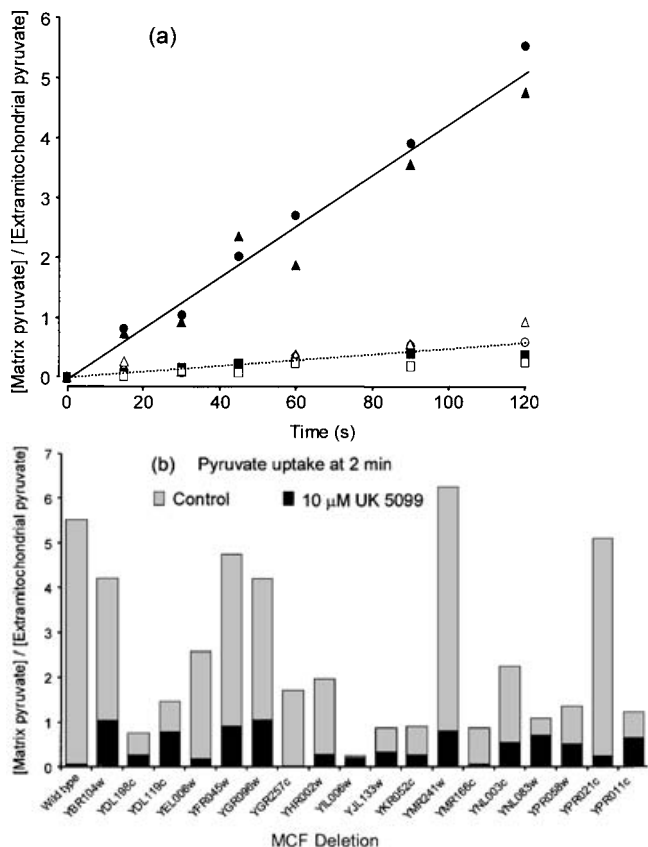


Figure 1 Ascorbate/TMPD-mediated pyruvate uptake by mitochondria from different yeast mutants

Uptake of $[2-^{14}\text{C}]$ pyruvate at the time shown was measured as described in the Experimental section. In (a), time courses are shown for uptake by wild-type (circles), $\Delta\text{YIL006W}$ (squares) and $\Delta\text{YFR045W}$ (triangles) in the absence (closed symbols) and presence (open symbols) of $10\ \mu\text{M}$ UK5099. In (b), data for only the 2 min time point are shown for all mutants, although full-time-course experiments were performed in each case. Data are presented as the ratio of the calculated matrix pyruvate concentration to the extramitochondrial pyruvate concentration ($0.1\ \text{mM}$) in the presence or absence of $10\ \mu\text{M}$ UK5099.

produce the phenotype observed with our $\Delta\text{YDL198c}$ mutant. This gene was therefore discarded from our screen.

In order to eliminate ascorbate/TMPD-related secondary effects, the remaining eight deletion mutants were tested for pyruvate uptake driven by an artificially imposed pH gradient (Figure 2). The lowest uptakes and inhibitor responses were obtained with mitochondria from the YKR052c, YJL133w, YMR166c and YIL006w mutants, but, once again, YIL006w was the only mutant showing no significant inhibition of uptake in the presence of UK5099. It should be noted that the lower pH used in these experiments (6.8) means that a greater proportion of pyruvate transport into the mitochondria occurs via free diffusion than at pH 7.4 [9]. YKR052c and YJL133w encode the MRS4 and MRS3 proteins respectively, and are associated with suppression of mitochondrial splicing defects and/or iron transport [19]. Deletion of these genes would therefore be expected to produce a diminished respiratory capacity, and possible disruption of membrane potential, leading to poor responses in both transport assays. As candidates for pyruvate carriers, these two seem unlikely. $\Delta\text{YMR166c}$, a slow-growing strain, exhibits decreased respiratory activity with a range of substrates when examined by an oxygen electrode (results not shown), suggesting a more severe impairment of mitochondrial function than can be accounted for by the absence of pyruvate transport alone. This was confirmed by

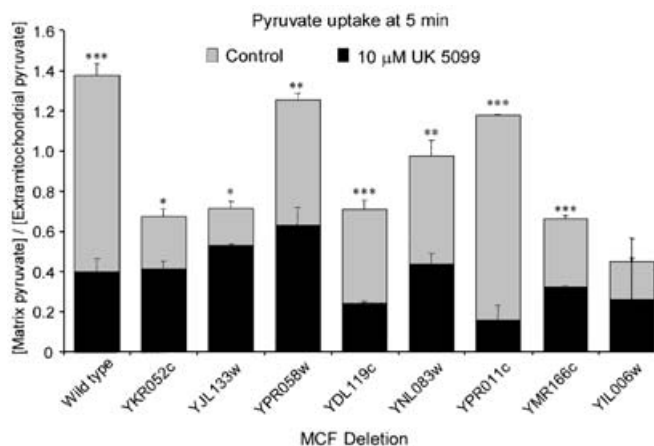


Figure 2 Pyruvate uptake by mitochondria from different yeast mutants driven by an artificially imposed pH gradient

Uptake was measured after 300 s as described in the Experimental section. Data are presented as the ratio of the calculated matrix pyruvate concentration to the extramitochondrial pyruvate concentration ($0.1\ \text{mM}$) in the presence or absence of $10\ \mu\text{M}$ UK5099. Data are given as means \pm S.E.M. (error bars) for two to four separate mitochondrial preparations. For each preparation, three replicate values were obtained. The statistical significance of differences between the control and inhibited uptakes were calculated by the paired Student's *t* test (* $P < 0.02$; ** $P < 0.01$; *** $P < 0.001$).

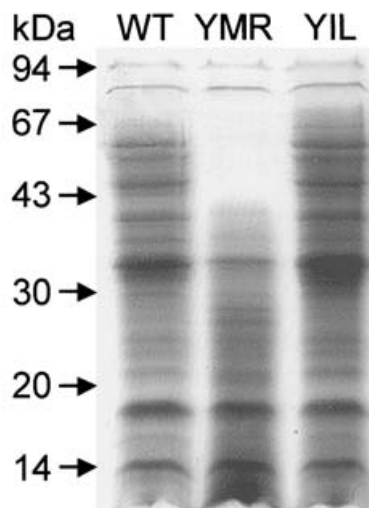


Figure 3 Mitochondrial protein composition of different yeast mutants revealed by SDS/PAGE and Coomassie Blue staining

WT, wild-type; YMR, $\Delta\text{YMR166c}$ mutant; YIL, $\Delta\text{YIL006w}$ mutant.

analysing the protein content of $\Delta\text{YMR166c}$, $\Delta\text{YIL006w}$ and wild-type mitochondria using SDS/PAGE. As shown in Figure 3, in comparison with wild-type and $\Delta\text{YIL006w}$ mitochondria, those from $\Delta\text{YMR166c}$ appear to lack a considerable number of proteins, especially those of higher molecular mass. This suggests that $\Delta\text{YMR166c}$ yeast possesses a defect in either mitochondrial protein import/incorporation or mitochondrial protein stability, with a higher rate of proteolytic breakdown as a consequence. Such a defect would produce the diminished growth rate observed, and might reasonably be expected to decrease levels of mitochondrial carrier proteins successfully incorporated. This would lead to the phenotype observed in the transport assays – namely low, but inhibitable, levels of uptake.

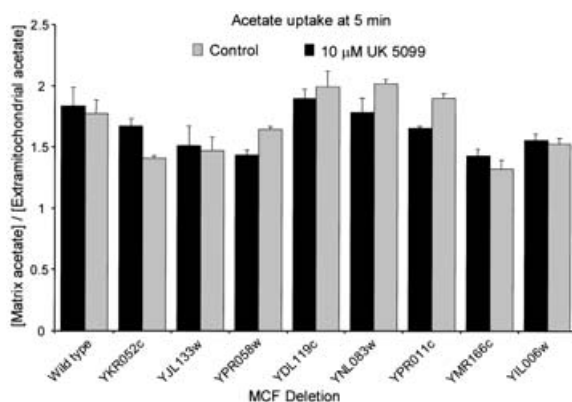


Figure 4 Acetate uptake by mitochondria from different yeast mutants driven by an artificially imposed pH gradient

Uptake was measured after 300 s as described in the Experimental section. Data are presented as the ratio of the calculated matrix acetate concentration to the extramitochondrial acetate concentration (0.1 mM) in the presence or absence of 10 μ M UK5099. Data are given as means \pm S.E.M. (error bars) for two or three separate mitochondrial preparations. For each preparation, three replicate values were obtained.

Mitochondrial acetate uptake studies

Further evidence that the Δ YIL006w mitochondria specifically lack the pyruvate carrier was provided by measurement of acetate uptake. The monocarboxylate acetate can enter mitochondria rapidly by free diffusion, and uptake is not inhibited by CHC or UK5099 [6]. In Figure 4, we show that acetate uptake is similar in all strains tested, and is unaffected by inhibitor, indicating that the pyruvate uptake defect observed in Δ YIL006w is at the level of transport itself.

DISCUSSION

The results presented here demonstrate that mitochondria isolated from the Δ YIL006w strain showed a specific loss of mitochondrial pyruvate transport, but in other ways behaved normally. Mitochondria from none of the other strains lacking any of the other putative mitochondrial carriers exhibited this specific defect. The deleted gene of YIL006w encodes a 41.9 kDa member of the mitochondrial carrier family that we therefore identify as the yeast mitochondrial pyruvate carrier. We have interrogated the human and mouse genomic databases using a BLAST search to identify the nearest mammalian homologue of this protein. These searches reveal that the most closely related proteins in humans are the mitochondrial folate carrier (30% identity) and two putative mitochondrial carriers of unknown function, MGC4399 on chromosome 1 (28% identical) and NP_060625 on chromosome 3 (28% identical with YIL006w and 60% identical with MGC4399). GeneNote expression array data obtained from GeneCards (<http://bioinfo.weizmann.ac.il/cards/index.html>) show both proteins to be ubiquitously expressed at high levels as might be predicted for the mitochondrial pyruvate carrier that is critical for carbohydrate and fat metabolism. Equivalent proteins are expressed in mouse (NP_081736 is 73.5% identical with MGC4399 and NP_620095 is 95% identical with NP_060625), whereas single homologues are present in the fruitfly *Drosophila melanogaster* (CG18317) and the nematode worm *Caenorhabditis elegans* (T09F3.2). The human carrier proteins MGC4399 (35.4 kDa) and NP_060625 (34.1 kDa) are smaller than

that in yeast, with the best alignment giving an about 66-amino-acids-shorter N-terminal extramitochondrial domain. This is not uncommon when comparisons are made between mammalian mitochondrial transporters and their yeast counterparts [20]. Confirmation that MGC4399 and/or NP_620095 are mammalian mitochondrial pyruvate carriers could be achieved if their expression in mitochondria from the Δ YIL006w strain restores CHC-inhibitable pyruvate transport. Work to this end is underway, although expression of mammalian transporters in yeast mitochondria is not always a straightforward task [20].

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