Biochimica et Biophysica Acta 1797 (2010) 81–88

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

# Identification of the mitochondrial carrier that provides *Yarrowia lipolytica* with a fatty acid-induced and nucleotide-sensitive uncoupling protein-like activity

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ARTICLE INFO

*Article history:*
Received 10 July 2009
Received in revised form 2 September 2009
Accepted 8 September 2009
Available online 17 September 2009

*Keywords:*
Uncoupling protein
UCP
Energy dissipation
*Yarrowia lipolytica*
Mitochondria
Heterologous expression
Yeast

ABSTRACT

Uncoupling proteins (UCPs) are mitochondrial carriers distributed throughout the eukaryotic kingdoms. While genes coding for UCPs have been identified in plants and animals, evidences for the presence of UCPs in fungi and protozoa are only functional. Here, it is reported that in the yeast *Yarrowia lipolytica* there is a fatty acid-promoted and GDP-sensitive uncoupling activity indicating the presence of a UCP. The uncoupling activity is higher in the stationary phase than in the mid-log growth phase. The in silico search on the *Y. lipolytica* genome led to the selection of two genes with the highest homology to the UCP family, XM_503525 and XM_500457. By phylogenetic analysis, XP_503525 was predicted to be an oxaloacetate carrier while XP_500457 would be a dicarboxylate carrier. Each of these two genes was cloned and heterologously expressed in *Saccharomyces cerevisiae* and the resulting phenotype was analyzed. The transport activity of the two gene products confirmed the phylogenetic predictions. In addition, only mitochondria isolated from yeasts expressing XP_503525 showed bioenergetic properties characteristic of a UCP: the proton conductance was increased by linoleic acid and inhibited by GDP. It is concluded that the XM_503525 gene from *Y. lipolytica* encodes for an oxaloacetate carrier although, remarkably, it also displays an uncoupling activity stimulated by fatty acids and inhibited by nucleotides.



## 1. Introduction

Uncoupling proteins (UCPs) belong to the mitochondrial carrier superfamily [1]. In mammals, there are five UCP homologs [2]. However, the physiological function is well defined only for UCP1, a protein expressed specifically in brown adipose tissue where it participates in adaptive thermogenesis [3]. Fatty acids act as physiological UCP1 activators through a mechanism still unknown; in turn, cytosolic purine nucleotides inhibit the protein [3]. The physiological role and the molecular mechanism of transport and regulation of the other four homologs are still uncertain [4,5]. Available data suggest that these homologs could be part of the antioxidant defense system of the cells; modulating the mitochondrial transmembrane potential ($\Delta\Psi$), increasing the rate of oxygen consumption and thus decreasing the production of reactive oxygen species (ROS) [4,6,7]. The acute regulation of the mitochondrial activity of UCP2 and UCP3 is not well established and several reports indicate that fatty acids, retinoids, superoxide and lipoperoxides could function as activators [8–12]. The physiological role and regulation of UCP4 and UCP5 (also called BMCP1) is still unclear.

The first UCP gene outside the animal kingdom was identified in *Arabidopsis thaliana* (named PUMP, or Plant Uncoupling Mitochondrial Protein) [13]. Since then UCP homologs have been described in many plants. Most of these UCPs are stress induced and their physiological role seems to be to control the production of ROS. Additionally, some of the UCPs from plants seem to be involved in thermogenic processes related to reproduction and ripening of seasonal fruits [14]. Recently, uncoupling proteins were discovered in unicellular eukaryotes; *Acanthamoeba castellanii* being the first where a UCP activity was reported [15]. Subsequently, there have been reports of UCPs in other fungi such as *Aspergillus fumigatus*, *Candida albicans* and *Candida parapsilosis* [16–18] and in protozoa such as *Plasmodium berghei*, *Plasmodium yoelii yoelii* and *Dictyostelium discoideum* [19–21]. However, none of the genes coding for the UCPs in these unicellular organisms have yet been identified.

The physiological role of UCPs in unicellular organisms is uncertain. Jarmuszkiewicks et al. [22] have reported that *A. castellanii* cells growing at 4 °C exhibited an increase in their UCP levels, resembling what happens in mammalian brown fat during cold adaptation. However, it has been reasoned that in these small unicellular organisms it is not possible to establish a thermal gradient between the cytosol and the external environment, and therefore in

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doi:10.1016/j.bbabio.2009.09.003

lower eukaryotes a thermoregulatory role for these proteins is unlikely [23]. Again, there are evidences that point to a role in the protection against oxidative stress, e.g. it has been shown that in *A. castellanii* the inhibition of the *Ac*UCP increased ROS production while the activation of this protein by fatty acids decreased ROS levels [23,24].

As stated above, and even though a large amount of biochemical information is available, nothing is known about the genes from lower eukaryotes that code for UCPs. Here, we demonstrate the presence of a UCP-like activity in *Yarrowia lipolytica* and report its regulation by fatty acids and nucleotides. The analysis of the *Y. lipolytica* genomic database led to the identification of two mitochondrial carrier sequences that may code for proteins with UCP-like activity. The two candidate genes were cloned, expressed recombinantly in *Saccharomyces cerevisiae* and their bioenergetic properties determined. The characterization indicates that in *Y. lipolytica* XP_500457 encodes for a dicarboxylate carrier, while XP_503525 yields an oxaloacetate carrier which nonetheless exhibits UCP-like activity.

## 2. Materials and methods

### 2.1. Reagents and materials

Mannitol, sorbitol, maleic acid, glucose, galactose, NADH, succinic acid, L-tryptophan, ammonium sulfate, adenine, oligomycin, ammonium hydroxide, nigericin, octyl gallate, linoleic acid, oleic acid, stearic acid, palmitic acid, GDP, safranin O, FCCP and valinomycin were from Sigma Chem. Co. (St. Louis, MO, USA). Carboxyatractyloside was from Calbiochem (La Jolla, CA). Zymolyase was from Seikagaku Co. (Tokyo, Japan). Yeast nitrogen base (YNB), yeast extract and casamino acids were from DIFCO labs (Detroit, MI, USA).

### 2.2. Cell strains and growth

The yeast used in this study were *Y. lipolytica* E150 (*MatB*; *his1-1*; *ura3-302*; *leu2-270*; *xpr2-322*) and *S. cerevisiae* W303 diploid (*Mat a/α*; *ade2-1*; *his3-11,15*; *leu2-3,112*; *ura3-1*; *canl-100*; *trp-*). The *Y. lipolytica* strain was a kind gift from Dr Ulrich Brandt, University of Frankfurt, Germany. *Y. lipolytica* was grown in YD media (yeast extract—dextrose) at 30 °C under vigorous aeration until mid-exponential or stationary phase, as specified in each experiment. *S. cerevisiae* was precultured in SP med (0.67% YNB, 0.1% $KHPO_4$, 0.12% $(NH_4)_2SO_4$, 0.1% glucose, 2% lactate, 1.06 g/L casamino acids supplemented with 40 mg/mL adenine and 20 mg/mL L-tryptophan, pH 4.5). Cells were centrifuged and resuspended in SG (induction) medium (2% galactose, 0.67% YNB, 1.06 g/L casamino acids supplemented with 40 mg/mL adenine and 20 mg/mL L-tryptophan) to an optical density of 0.3; when the culture reached an optical density of 6, the cells were harvested and subjected to mitochondrial isolation.

### 2.3. Isolation of mitochondria

*Y. lipolytica* and *S. cerevisiae* mitochondria were isolated according to Guérin et al. [25]. Briefly, cells were harvested, washed two times and resuspended in 0.5 M β-mercaptoethanol, 0.1 M Tris, pH 9.3. The suspension was incubated for 15 min at 30 °C under agitation and washed three times in Tris–KCl buffer, pH 7. Cells were incubated in digestion buffer supplemented with 1 mg/mL zymolyase for 10 min at 30 °C. The resulting spheroplasts were washed with protoplast buffer (0.75 M sorbitol, 0.4 M mannitol, 10 mM Tris-maleate, 0.1% BSA, pH 6.8), centrifuged and disrupted with 15 passes of a tight pestle in a 50 -mL Potter–Evelheim homogenizer. Mitochondria were obtained by differential centrifugation. Protein concentration was measured by the Biuret method using bovine serum albumin (BSA) as standard.

### 2.4. Oxygen uptake measurements

*S. cerevisiae* mitochondria were incubated in a respiration medium containing 0.6 M mannitol, 10 mM Tris–maleate, 10 mM $K_2HPO_4$, 1 mM $MgCl_2$, 0.5 mM EGTA, 1 mg/mL BSA, 1 μM carboxyatractyloside (adenine nucleotide translocator inhibitor) and 10 μg/mL oligomycin (ATP synthase inhibitor). The rate of oxygen uptake was measured using a YSI model 5300 oxygraph with a Clark- type electrode in a 1. 5-mL water-jacketed chamber at 30 °C. Where indicated, 48 μM linoleic acid (molar ratio 3:1 to albumin) and 1 mM GDP were used to evaluate, respectively, the activation or inhibition effects on the rate of respiration. Respiratory substrate was 1 mM NADH. At the end of the experiment, 1 μM FCCP was added and the rate of oxygen consumption in the uncoupled state was determined. *Y. lipolytica* mitochondria were incubated in the same respiration buffer, except that 100 ng/mL nigericin and 1 μM octyl gallate (alternative oxidase inhibitor) were added. Succinate (10 mM; plus 1 μM rotenone) was used as respiratory substrate. Respiration was modulated by adding different concentrations of cyanide (1 –50 μM). Respiration was stimulated with 48 μM linoleic acid and inhibited with 1 mM GDP.

### 2.5. Transmembrane potential ($\Delta\psi$ measurements and proton conductance kinetics)

Experiments were performed in *Y lipolytica* mitochondria under the same conditions as the oxygen consumption measurements, except 10 μM safranin O was added. Fluorescence was measured in an Aminco/Ollis spectrofluorometer with excitation at 495 nm and emission at 586 nm. Sequential additions of different concentrations of cyanide (1–50 μM) were used to get different steady states. One micromolar valinomycin was used to collapse the $\Delta\Psi$ in order to get a baseline. Fluorescence was calibrated using a free $K^+$ medium with valinomycin. $K^+$ pulses were added to calibrate the membrane potential as described by Akerman and Wikström [26]. Proton conductance kinetics was determined by plotting the rate of oxygen consumption in state 4 against the $\Delta\Psi$ in the presence of increasing concentrations of cyanide. The proton current was calculated assuming a theoretical $H^+/O$ stoichiometry of 6. Free fatty acid concentrations were estimated using the albumin binding constants according to Richieri et al. [27].

### 2.6. Mitochondrial transport of succinate and sulfate

Succinate transport was indirectly determined by measuring the rate of swelling in ammonium succinate. Mitochondria were suspended in iso-osmotic media: 0.125 mM ammonium succinate, 10 mM Tris–maleate pH 6.8, 1 μM antimycin. Swelling was induced by the addition of 5 mM $K_2HPO_4$. Oxaloacetate carriers are capable of transporting sulfate [28]. Therefore, the sulfate-uptake activity was evaluated by following swelling in response to the addition of ammonium sulfate. The reaction mixture was the same as for succinate transport except 0.125 M ammonium sulfate instead of ammonium succinate. In both experiments, the rates of swelling were determined by the absorbance change at 540 nM in an Aminco/Olis DW2000 spectrophotometer.

### 2.7. Mitochondrial permeability to protons

Proton permeability was determined from the rate of swelling in a potassium acetate reaction mixture containing 0.2 M potassium acetate, 10 mM Tris–maleate pH 6.8, 5 mM $K_2HPO_4$, 2 mM $MgCl_2$, 1 mg/mL BSA, 1 μM carboxyatractyloside, 10 μg/mL oligomycin and 1 μM antimycin A. Swelling was initiated by the addition of 1 μM valinomycin and the decrease in absorbance was where indicated: 1 mM GDP and 48 μM linoleic acid (FA: BSA = 3).

### 2.8. Heterologous gene cloning in S. cerevisiae

The cDNA from each of the two genes of interest was synthesized by PCR and cloned in the *S. cerevisiae* diploid strain W303 using the *p*YEDP expression vector according to Rial et al. [9]. Cloned inserts were isolated and sequenced to confirm that the proper nucleotide sequence was obtained and cloned into pGemTeasy between restriction sites *Not*I/*Pst*I for XP_500457 and *Not*I/*Nco*I for XP_503525; the vector was cut with these restriction enzymes to release inserts and the DNA was purified. The inserts were cloned in a *p*YEDP vector previously linearized between *Kpn*I/*Sac*II restriction sites. The modified *p*YEDP was introduced into *S. cerevisiae* W303 diploid by electroporation and transformant yeasts were selected for uracil autotrophy.

### 2.9. Phylogenetic sequence analysis

A total of 400 full-length, non-redundant protein sequences corresponding to phosphate carriers (PiC), adenine nucleotide translocators (ANT), dicarboxylate carriers (DiC), oxoglutarate carriers (OGC), oxaloacetate carriers (OAC), animal uncoupling proteins (UCP) and plant uncoupling proteins (pUCP) were retrieved from NCBI and ENSEMBL public databases (see complete list in Appendix A). According to the evolutionary analysis performed by Rial and Zardoya [29], the sequences were aligned using the program at the Mafft server (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) with the L-INS-i strategy [30]. The maximum likelihood tree was inferred using PhyML v. 2.4.4 [31] with JTT as substitution model. The recovered tree shown was statistically within the confidence set ($P>0.05$) as determined with the RaxML v. 7.0.4 [32] and the Consel v. 0.1j [33] programs. Bootstrap analysis was performed using RaxML at the Cipres Portal (http://www.phylo.org/).

### 2.10. Statistics

Data were analyzed with a two-way ANOVA. $P<0.05$ was considered as a statistically significant difference.

## 3. Results and discussion

### 3.1. Metabolic implications/industrial applications of the modulation of the mitochondrial energetic efficiency in metabolite overproducers such as Y. lipolytica

It has been known since the late seventies that the rate of metabolite production by microorganisms is inversely related to their growth efficiency [34]. The reason behind this apparent paradox lies on the fact that the production of organic acids such as citric or glutamic requires low net amounts of ATP and NADH [35], i.e. the growth yield is lowered as a result of the incomplete oxidation of the energy source (overflow metabolism), bypassing potential sites of energy conservation or activating futile cycles that lead to net hydrolysis of ATP. Industrially, chemical uncouplers such as 2,4-dinitrophenol or benzoic acid successfully increase the production of metabolites [36]. The organisms used for the industrial production of metabolites are generally obligate aerobes and therefore the respiratory chain is (a) their major energy conserving system and (b) the principal reducing equivalent disposal mechanism. The uncoupling proteins (UCPs) are mitochondrial carriers that modulate the energetic efficiency by catalyzing the regulated dissipation of the proton gradient. In this regard, it may be suggested that manipulating the expression and activity of UCPs would optimize efficiency in the production of metabolites of industrial interest. The first uncoupling protein (UCP1) was described in the brown adipose tissue of mammals where it has a thermogenic role [1,3]. Since 1997, genes coding for UCP1 homologs have been described not only in all phyla of the animal kingdom but also in plants [13]. In mitochondria from unicellular eukaryotes, the presence of uncoupling proteins has been evidenced mainly through cross-reaction with antibodies raised against plant UCPs and by detection of GDP-sensitive, fatty acid-promoted uncoupling [15–21]. However, the actual proteins responsible for this activity have not been identified.

### 3.2. Detection of a UCP-like activity in isolated mitochondria from Y. lipolytica

The ascomycetous yeast *Y. lipolytica* has raised a strong commercial interest due to its potential use in the large-scale industrial production of organic acids (2-ketoglutaric acid and citric acid); this yeast can use paraffins, hexadecanes, edible oils and other molecules as carbon sources [37]. In the *Y. lipolytica* cytoplasm, there are large lipid stores known as lipid bodies, and therefore the sensitivity of *Y. lipolytica* mitochondria to those fatty acids known to be present in its cytoplasm was tested [38]. All the fatty acids tested increased the rate of oxygen consumption with the following decreasing order of activity: linoleic>oleic>palmitic>stearic, suggesting an uncoupling effect (Fig. 1A).

To further analyze the possibility that *Y. lipolytica* mitochondria possess a UCP-like activity, it is necessary to demonstrate that the fatty acid effect can be inhibited by purine nucleotides such as ATP or GDP. With this aim, proton leak kinetics were analyzed in mitochondria isolated from yeasts harvested at two different growth stages: the mid-logarithmic phase (Fig. 2A) and the stationary phase (Fig. 2B). The changes in proton conductance were determined under three conditions: (a) basal conditions, without additions; (b) in the presence of linoleic acid; and (c) in the presence of linoleic acid plus GDP. The proton conductance of the two mitochondrial preparations was markedly increased in the presence of linoleic acid and this effect was partially reversed by the addition of GDP. In the stationary phase, the effect of GDP was higher.

The data in Fig. 2A and B were analyzed further by determining the proton flux at the highest common membrane potential under the different conditions of study (131 mV, dotted vertical lines in Fig. 2A and B). The effects are summarized in Fig. 2C: linoleic acid uncoupled to a similar extent both mid-log and stationary mitochondria. However, the inhibitory effect of GDP was higher in mitochondria from stationary cells (65% inhibition) than in those isolated from mid-

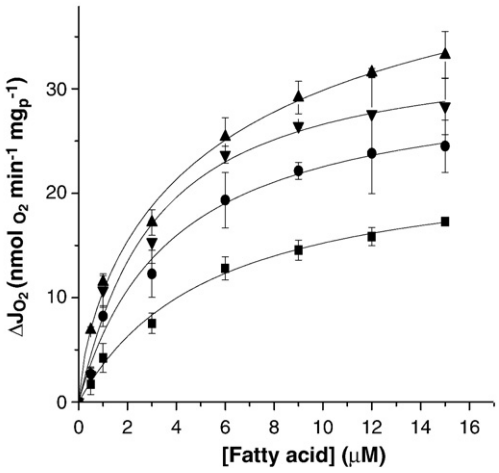


**Fig. 1.** Effect of fatty acids on the rate of respiration of mitochondria isolated from *Yarrowia lipolytica*. Reaction mixture: 0.6 M mannitol, 10 mM Tris–maleate, 10 mM $K_2HPO_4$, 1 mM $MgCl_2$, 0.5 mM EGTA, BSA 1 mg/mL (16 μM), 1 μM carboxyatractyloside, oligomycin 10 μg/mL, 10 mM succinate, 1 μM rotenone. Added fatty acids were as follows: ■, stearic acid; ●, palmitic acid; ▼, oleic acid; ▲ linoleic acid. The basal rate of respiration was 119±2 nmol $O_2$ (min mg protein)$^{-1}$. Data represent the mean±SEM of five to six independent experiments.

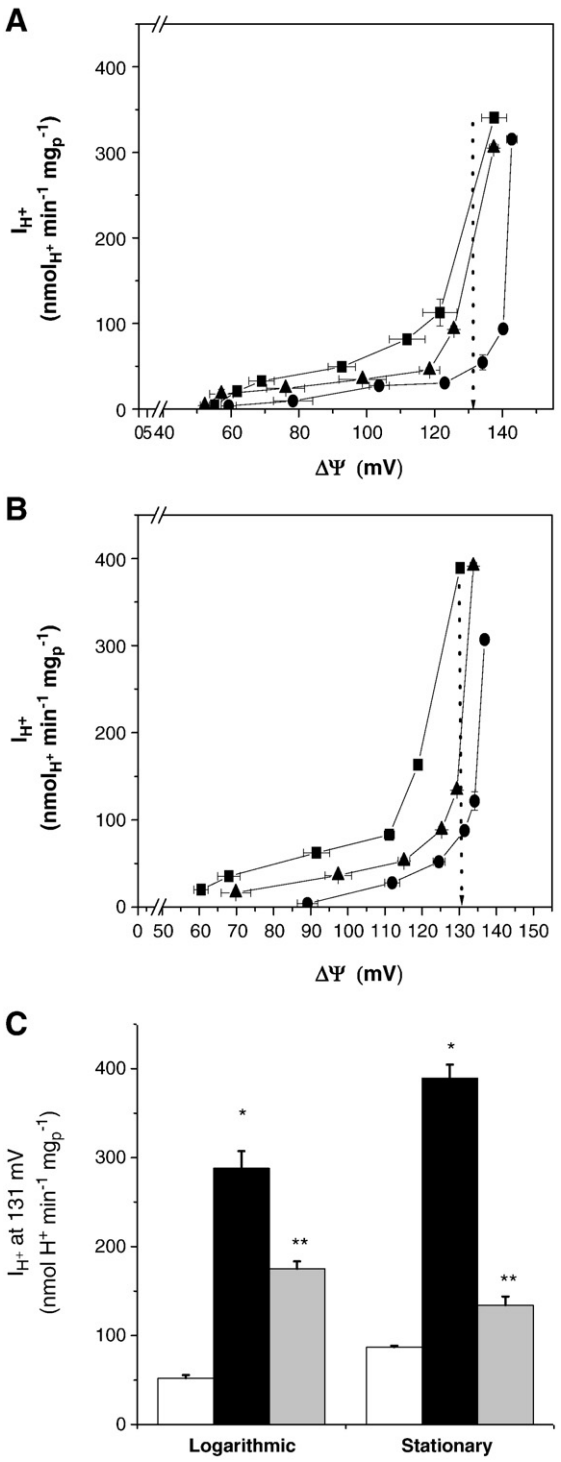


**Fig. 2.** Proton leak kinetics of mitochondria isolated from *Yarrowia lipolytica* in the mid-logarithmic (A) and stationary phase (B). Reaction mixture as in Fig. 1, except KCN was used for titration of the ΔΨ and oxygen flux (see methods). ●, Control; ■, linoleic acid; ▲; linoleic acid + GDP. (C) Estimated proton current ($I_{H+}$) at the highest common Δψ (131 mV, dotted lines in panels A and B). Empty bars, control mitochondria; filled bars, 48 μM linoleic acid; gray bars, 48 μM linoleic plus 1 mM GDP. Data represent the mean ± SEM of five to six independent experiments. *$P$< 0.05 compared to control; **$P$<0.05 compared to uncoupled state without GDP.

log phase cells (39% inhibition). The stimulation of respiration by the uncoupler FCCP was identical in the two preparations (data not shown). The inhibition by GDP of the linoleic-induced increase in proton conductance is indicative of the presence of a UCP in *Y. lipolytica*. Interestingly, when *Y. lipolytica* reaches the stationary phase, the lipid bodies accumulated during the exponential phase are increasingly mobilized. The higher GDP-sensitive activity in mitochondria from cells grown to the stationary phase would imply a higher expression and a resemblance with other eukaryotic cells where UCP2 and/or UCP3 are up-regulated as they shift their catabolism from glucose to fatty acid oxidation [6,39,40]. This UCP-mediated mild uncoupling has been related to the prevention of ROS overproduction [4,6,7,41]. A recent publication has re-emphasized this function clarifying that UCP3 does not play a direct role in fatty acid oxidation or its transport and that UCP3 expression seems to be related to the mitigation of ROS-mediated damage [42].

### 3.3. Search for UCP sequences in Y. lipolytica genome

Once a UCP-like phenotype was detected in *Y. lipolytica* mitochondria, it was decided to analyze its genome in order to identify possible candidates for a gene coding for a UCP-like protein. Since the genome of *Y. lipolytica* as been sequenced [43], a PSI-BLAST search was performed using the sequence profile generated with the available UCP1 sequences. Two candidates were identified: XM_500457 and XM_503525 (Fig. 3). The phylogenetic analysis revealed that XM_500457 appeared as an ortholog of the dicarboxylate carrier (DIC) while XM_503525 is related to the yeast oxaloacetate carriers (OAC) (Fig. 4). Details of the names and sources of the specific proteins in the phylogenetic analysis can be found in the Supplementary material provided (Supplementary Fig. 1 and Supplementary Table 1.

### 3.4. Characterization of the transport properties XP_500457 and XP_503525

The transport activity of the putative carriers was analyzed in mitochondria isolated from *S. cerevisiae* which was chosen as host organism because it lacks UCPs [44,45]. To do this, the candidate genes XM_500457 and XM_503525 were cloned and expressed recombinantly in *S. cerevisiae*. A strain containing the empty plasmid was used as a control.

Mitochondrial swelling in iso-osmotic salts has been widely employed to determine the transport specificity of mitochondrial carriers and particularly those present in yeast [46,28]. Thus, swelling in ammonium succinate (plus phosphate) can be used to establish the presence of a DIC while swelling in ammonium sulfate has been used to identify an oxaloacetate carrier [28]. In ammonium succinate, the swelling rates of mitochondria from the control and the recombinant strains were measured (Fig. 5A): all three strains swell in this medium, indicating the presence of carriers for this dicarboxylic acid. However, mitochondria isolated from the XP_500457-containing *S. cerevisiae* strain exhibited a significant increase ($P$< 0.05) in the uptake of succinate as compared to the control or to XP_503525-containing mitochondria. When the rates of swelling in ammonium sulfate were measured, it was observed that mitochondria containing the XP_503525 protein exhibited higher sulfate transport activity than the control (Fig. 5B). Mitochondria containing the XP_500457 protein exhibited only a marginal increase in activity as compared to the control (Fig. 5B). From the data in Fig. 5, it may be conclusively established that XM_503525 codes for an oxaloacetate carrier while XM_500457 codes for a dicarboxylate carrier. Additionally, it is demonstrated that this recombinant expression system allowed the correct targeting and folding of these *Y. lipolytica* carriers in the mitochondria from *S. cerevisiae*.

### 3.5. The protonophoric activity of the XP_500457 and XP_503525 proteins

Although both the phylogenetic data and the transport experiments demonstrated that XP_500457 is a dicarboxylate carrier while

**A**

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MAVILDKQKK QPPKQISTLG GFVAGAIAAC GAVTVTNPIE LVKTRMQLQG ELAARGEAKK
VYTSPLQALV KIYKSEGIKG LQSGLFSAYV YQIGLNGCRL GLYEPTRKVI ANVCNIDLNK
ENPVGLNVAS GAISGIMGAV AGSPFYLIKT RQQSYSPAFK VGAQTYYKSI GDGFRQIYGA
EGFKGLYRGV DAAILRTGAG SSVQLPIYNW AKELLLKHHI TDPGASTHLV ASAMSGLGVA
VVMNPWDVLM TRMYNQKGNM YKNPFDCLMK TVSIEGPFAL YKGFGAHLLR IAPHTILTLM
FMEQTMKWVK WFEGVPF
```

**B**

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MSSLQKHLPM ESKTKPAEIP LSAVPPSAKI HYPFWYGGFA SVVAGVFTHP LDLAKVRLQT
AKTRGQGLFG TLVNVVKHEG ITGVYSGLSA SMLRLSTYST MRFGMYEYLK ESIAPYYYNP
NKRDQNPPMY VLLPISIIAG ISGGIVGNPA DIINIRMQND QSLPKDQRRN YKHAFDGLIR
MYKEEGVRAM FRGLGPNCTR GVLMTSSQMV SYDSFKALLV NHLGMNPDKK ATHFSASLLA
GLMATTVCSP VDVVKTRIMN AHAHHSKDSA FTIFFNALKQ EGPLFMFRGW LPSFVRLGPQ
TILTYIVLEQ LKFYKIGMRH
```

**Fig. 3.** Protein sequences of the two mitochondrial carriers identified in the *Yarrowia lipolytica* genome as more closely related to the UCP family. (A) XP_503525. (B) XP_500457.

XP_503525 is an oxaloacetate carrier, it was still necessary to determine whether the heterologous expression of one of these proteins conferred a UCP-like phenotype to *S. cerevisiae*. Therefore, we decided to test the uncoupling activity in the strains expressing these proteins. Two controls were performed in order to exclude unspecific effects: (1) a positive control expressing the rat UCP1 and (2) a negative control containing the empty expression vector (*p*YEDP). The rate of oxygen consumption by isolated mitochondria from each *S cerevisiae* strain was measured before and after the addition of linoleic acid or linoleic acid plus GDP (Fig. 6). The rate of oxygen consumption in state 4 was significantly higher in the strain expressing UCP1, reflecting the fatty acid independent basal proton conductance of UCP1 that has been previously reported [47]. In contrast the expression of the *Yarrowia* proteins did not have any

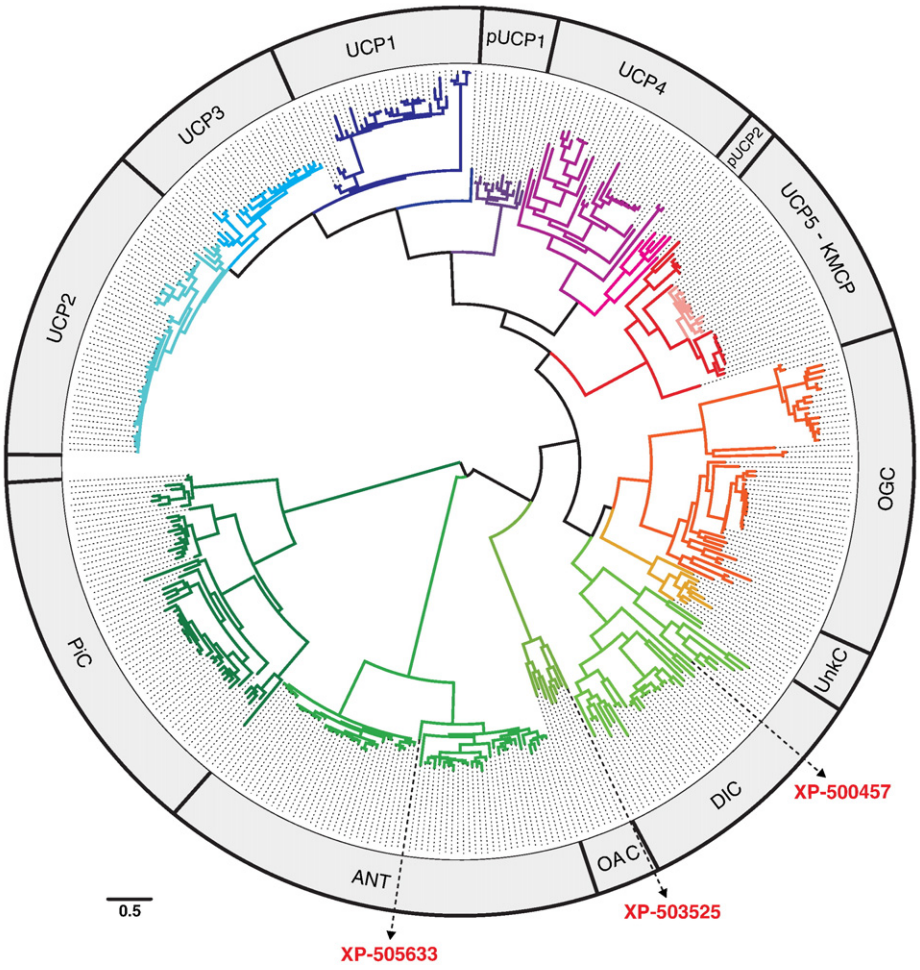


**Fig. 4.** Circular tree representing the phylogenetic relationships of the mitochondrial carrier proteins with highest homology to the uncoupling proteins. The location of the *Yarrowia lipolytica* carriers XP_503525 and XP_500457 are indicated. The ANT from *Yarrowia lipoltyca* (XP_505633) is also indicated. Abbreviations: PiC, phosphate carriers; ANT, adenine nucleotide translocators; OAC, oxaloacetate carriers; DIC, dicarboxylate carriers; UnkC, carriers of yet undefined function; OGC, oxoglutarate carriers; UCP5, uncoupling protein 5 also termed BMCP1 (brain mitochondrial carrier protein 1); KMCP, kidney mitochondrial carrier protein; pUCP2, plant uncoupling protein 2; pUCP1, plant uncoupling protein 1; UCP1-4, uncoupling proteins 1 to 4. A detailed version of the tree where all the sequences are identified is included as Supplementary material. Tree topology was drawn by using Dendroscope v.2.3 and the circular tree generated with FigTree v1.2.2.

detectable effects. In the presence of linoleic acid, there was an increase in the rate of oxygen consumption only in mitochondria from cells containing UCP1 or XP_503525 (Fig. 6). The linoleic acid-mediated increase in oxygen consumption was not observed in mitochondria from the cells containing the empty *p*YEDP vector, confirming the absence of a UCP in *S. cerevisiae*, and furthermore, that its mitochondrial carriers were not sensitive to low fatty acid concentrations [44,45,48]. When GDP was added, the linoleic-induced increase in oxygen consumption was fully reverted in mitochondria from the cells expressing either UCP1 or XP_503525. Similar results were obtained when the effect of linoleic acid and GDP were tested under passive swelling conditions with potassium acetate plus valinomycin. Proton permeability was sensitive to fatty acids and nucleotides only in mitochondria from UCP1- or XP_503525-expressing yeast (Fig. 7). Thus, the results clearly indicate that the XP_503525 protein behaves as an uncoupling protein. Additionally, the lack of response when XM_500457 is present rules out the possibility that this protein is a UCP and furthermore that the effects observed in *S. cerevisiae* mitochondria are simply due to the expression of any given foreign mitochondrial carrier.

It is well established that several mitochondrial carriers (e.g. ANT, dicarboxylate carrier, aspartate/glutamate carrier) can catalyze an increase in the membrane proton conductance in the presence of fatty acids [46]. However, this activity is insensitive to purine nucleotides (ANT could be an exception). In 2006, Borecky et al. [49] reported that the gene product of SCCCLR1076E12.g was a UCP (ScPUMP5). However, these authors tested the effect of linoleic acid on transport but not that of purine nucleotides. Subsequently, Palmieri et al. [50] demonstrated that PUMP5 was not a UCP but instead it was a dicarboxylate carrier. Here, it is shown that XP_500457 is a DIC but it is unable to induce a fatty acid uncoupling activity. In contrast, XP_503525 exhibited only a marginal dicarboxylate carrier activity while it was an active oxaloacetate carrier. In addition, XP_503525 was stimulated by fatty acids and inhibited by GDP as a *bona fide* UCP would.

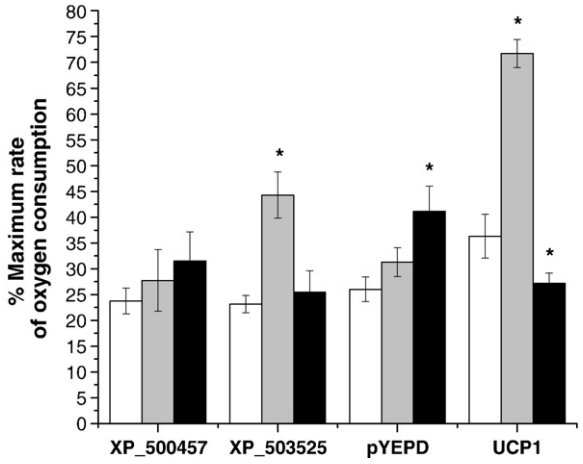


**Fig. 6.** Respiratory activity of isolated mitochondria from three *Saccharomyces cerevisiae* strains. Incubation mixture as in Fig. 1, except 3 mM NADH as a respiratory substrate. Rates are expressed as percentage of the respiratory activity in the presence of FCCP which was $421 \pm 28$ nmol$O_2$ (min mg protein)$^{-1}$. Each *Saccharomyces cerevisiae* strain contained a heterologous protein as indicated: XP_503525, XP_500457, UCP1 or the empty plasmid *p*YEDP. UCP1 was the positive control and the strains containing the *p*YEDP empty vector were the negative control. Additions were as follows: empty bars, State 4; gray bars, 48 μM linoleic acid; black bars, 48 μM linoleic acid plus 1 mM GDP. Data represent the mean values ± SEM of five independent experiments. *$P<0.05$ as compared to the control.

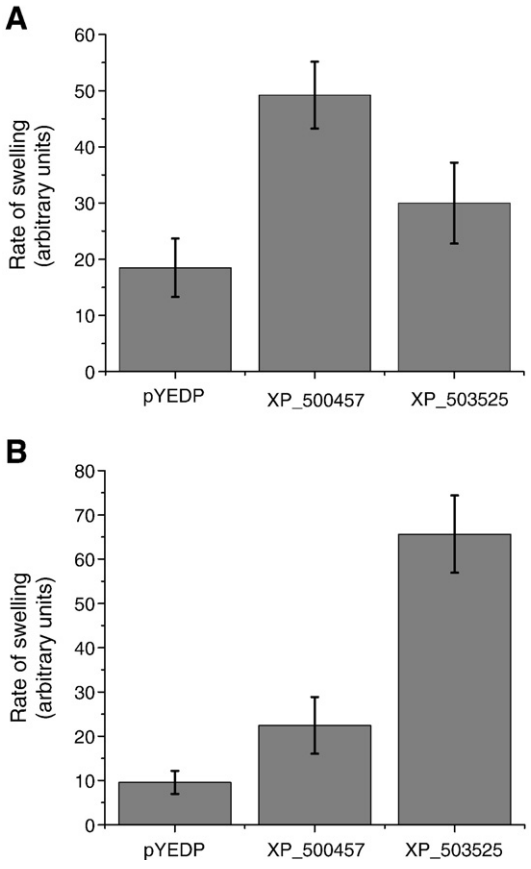


**Fig. 5.** Characterization of the transport activities of the XP_503525 and XP_500457 proteins. Both XP_503525 and XP_500457 were heterogously expressed in *Saccharomyces cerevisiae*. Their transport properties were evaluated in isolated mitochondria as follows: (A) succinate (DiC) transport. Mitochondria were resuspended in iso-osmotic medium: 0.125 M ammonium succinate, 1 μM antimycin, 10 mM Tris–malate pH 6.8 and the rate of swelling was followed spectrophotometrically at 540 nm in an Aminco/Olis DW2000 spectrophotometer. Swelling was induced by addition of 5 mM $K_2HPO_4$. (B) Oxaloacetate transport. This activity was evaluated as the sulfate-mediated mitochondrial swelling. Mitochondria were resuspended in iso-osmotic medium: 0.125 M ammonium sulfate, 1 μM antimycin, 10 mM Tris–malate pH 6.8 and the rate of swelling was measured at 540 nm. Data from three independent experiments ± SEM each performed in duplicate. *$P<0.05$ compared to the control (*p*YEDP).

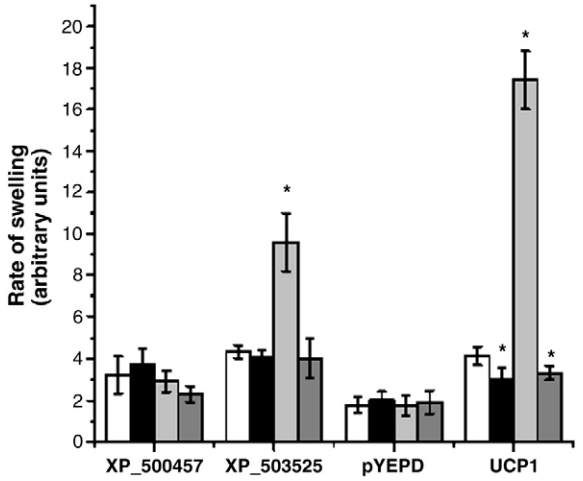


**Fig. 7.** Proton permeability of *Saccharomyces cerevisiae* mitochondria expressing different mitochondrial carriers. Incubation mixture: 0.2 M potassium acetate, 5 mM $K_2HPO_4$, 2 mM $MgCl_2$, 1 mg/mL BSA, 1 μM carboxyatractyloside, 10 μg/mL oligomycin, 1 μM antimycin and 1 mM Tris–malate, pH 6.8. Swelling was initiated by 1 μM valinomycin. Proton permeability was determined from the swelling data. Conditions were as follows: empty bars, basal rate; black bars, 1 mM GDP; light gray bars, 48 μM linoleic acid; dark gray bars, 48 μM linoleic acid plus 1 mM GDP. Data represent the mean values ± SEM of five independent experiments each performed in duplicate. *$P<0.05$ compared to basal swelling.

### 3.6. Lipid handling by Y. lipolytica and the role of its uncoupling protein

The physiological role of most members of the UCP family is still under debate. Undoubtedly, a thermoregulatory role in unicellular organisms can be ruled out since it is not possible to establish a thermal gradient between the cell and its environment [23]. Currently, there are many data consistent with the participation of the UCPs in the defense against oxidative stress [2,4,5,6,7,23,40], e.g. in the amoeboid *A. castellanii*, activation of a UCP decreases ROS and vice versa, UCP inhibition increases ROS [24]. Here, it is likely that the UCP activity results in an increase in proton conductance that leads to a higher respiratory rate and the concomitant decrease in ROS production. This UCP-mediated uncoupling would be particularly important under resting, non-phosphorylating, conditions. Indeed, a decrease in ADP/O has been observed in plants [51] and unicellular organisms such as *A. castellanii* or *D. discoideum* [20,52].

An interesting observation is that in unicellular organisms, the UCP activity generally coexists with an alternative oxidase (AOX). However, little is known about the interactions between these two proteins or about the physiological implications and regulation resulting from their interaction. Both activities seem to inhibit the mitochondrial production of ROS, although by different mechanisms. AOX decreases the production of ROS through the oxidation of ubiquinol, while the UCP-like activity decreases the $\Delta\Psi$ accelerating the respiratory chain. However, regulation seems to work in opposite ways, as AOX is activated by GDP and inhibited by fatty acids [53]. There is one case where it has been suggested that the joint presence of an alternative respiratory chain and a UCP results in increased adaptability to adverse environments: *C. albicans* seems to depend on both its UCP and its AOX to increase its resistance to ROS and enhance its invasiveness [18,54].

*Y. lipolytica* is an oleaginous yeast with a dynamic fatty acid metabolism [55]. *Y. lipolytica* stores large amounts of triglycerides in lipid bodies. While in the exponential phase lipids are predominantly used to build membranes, in the stationary phase the fatty acids are liberated from the lipid bodies and are used as a carbon source [56,57]. When lipid bodies are degraded during the stationary phase, the major fatty acids released are linoleic, oleic, stearic and palmitic [38]. In isolated mitochondria, it was found that these fatty acids were able to modulate the respiratory activity, probably through the interaction with a UCP (Fig. 1). Since the cytosolic concentration of fatty acids vary widely, it is possible that they may act as internal messengers. The major site for fatty acid β-oxidation in animal cells is the mitochondrion. In contrast, in plant and yeasts, β-oxidation generally takes place in peroxisomes [55,58,59]. Therefore, the activation of a UCP in *Y. lipolytica* would not accelerate fatty acid oxidation as it occurs with UCP1 in the mammalian brown adipose tissue. Instead, it would be linked to the decrease in ROS production and/or the reoxidation of NAD(P)H under the conditions of low ATP demand of the stationary phase. It would be of interest to establish if in *Y. lipolytica* the variations in UCP-like activity influence the production of industrially desirable organic acids.

### 3.7. Evolutionary considerations

The closest homologs of XP_503525 are a group of yeast carriers that only present a 55–60% identity with the *Yarrowia* carrier. The homolog in *S. cerevisiae* is the carrier protein NP_012802 that has been characterized as the mitochondrial transporter for oxaloacetate and sulfate [28]. The observed sequence divergence does not imply a different carrier function but it could be sufficient to explain a change in transport properties. To put it in perspective, the human UCP1 displays a 60% identity with its ortholog in *Cyprinus carpio* [60]. This comparison is of relevance since the phylogenetic analyses of the UCP family have revealed that the UCP1 from eutherian mammals has been subject of a strong structural divergence that has accompanied the development of brown adipose tissue as a thermogenic organ.

Thus, the carp UCP1 does not have a thermoregulatory function since its expression in the liver decreases when fish are exposed to cold, and furthermore, its functional characterization has demonstrated that the carp protein does not display the distinctive nucleotide-sensitive basal proton conductance found in the eutherian UCP1 [60,61]. Finally, it should be remembered that in *S. cerevisiae* there are no orthologs of the known members of the UCP family, from UCP1 to UCP5 and indeed, that there is no evidence for the presence of a UCP-like activity activated by fatty acids and inhibited by nucleotides [44,45].

Phylogenetic analyses should be the basis for the nomenclature of protein families. The position of XP_503525 in the reconstructed phylogeny of the mitochondrial carrier protein superfamily presented in Fig. 4 does not allow naming this gene product as an uncoupling protein. The phylogeny implies that it is an oxaloacetate carrier although the data presented demonstrate that it has evolved to sustain a nucleotide-sensitive uncoupling activity that is activated by fatty acids as *bona fide* UCPs do. Therefore, we can envisage that the oxaloacetate carrier from *S. cerevisiae* would have a single metabolite transport activity while as our data suggest that the *Y. lipolytica* XP_503525 protein may have evolved to allow a fatty acid-induced uncoupling activity.

## Acknowledgements

This work was partially supported by grants from the Spanish Ministry of Science and Innovation to ER (BFU2006-08182 and Consolider-Ingenio CSD2007-00020). Research in Mexico was supported by grants from DGAPA/UNAM IN217109 and CONACyT 79989 to SU. The AECI, from the Spanish Ministry of Foreign Affairs and Cooperation, provided a visiting fellowship to LALM. LALM is a CONACyT fellow/Biochemistry PhD student at UNAM. We thank Pilar Zaragoza and Ramón Méndez for their expert technical assistance.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.09.003.

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