

## Variability of Wax Ester Fermentation in Natural and Bleached *Euglena gracilis* Strains in Response to Oxygen and the Elongase Inhibitor Flufenacet

SARA TUCCI,<sup>a,1</sup> ROSTISLAV VACULA,<sup>b</sup> JURAJ KRAJCOVIC,<sup>b</sup> PETER PROKSCH<sup>c</sup> and WILLIAM MARTIN<sup>a</sup>

<sup>a</sup>Institute of Botany III, Heinrich-Heine University of Duesseldorf, Universitaetsstrasse 1, 40225 Duesseldorf, Germany, and

<sup>b</sup>Institute of Cell Biology, Faculty of Natural Sciences, Comenius University, 84215 Bratislava, Slovak Republic, and

<sup>c</sup>Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University of Duesseldorf, Universitaetsstrasse 1, 40225 Duesseldorf, Germany

**ABSTRACT.** *Euglena gracilis* is able to synthesize adenosine triphosphate under anaerobic conditions through a malonyl-independent fatty acid synthesis leading to wax ester fermentation. Mitochondrial fatty acid synthesis uses acetyl-CoA and propionyl-CoA as C2- and C3-donors for de novo synthesis of even- and odd-numbered fatty acids, respectively. *Euglena*'s wax ester fermentation has only been described in the *E. gracilis* strain 1224-5/25 Z. Here we investigate eight *E. gracilis* strains isolated in 1932–1958 from different localities in Europe and two bleached substrains of *E. gracilis* 1224-5/25, obtained by treatment with streptomycin and ofloxacin, and examine their anaerobic growth, wax ester fermentation, and wax ester composition. Under ambient oxygen levels, all strains accumulated wax esters in concentrations between 0.3% and 3.5% of the dry weight, but the strains revealed marked differences in wax ester accumulation with respect to anaerobic growth. Most fermenting strains tested showed increased wax ester synthesis under anaerobic conditions as well as the increased synthesis of odd-numbered fatty acids and alcohols suggesting an activation of the mitochondrial fatty acid biosynthesis pathway. Addition of the elongase inhibitor flufenacet to the growth medium specifically reduced the accumulation of odd-numbered fatty acids and alcohols and tended to increase the overall yield of anaerobic wax esters.

**Key Words.** Fatty acid methyl esters (FAMES), fatty alcohols (FALcs), gas chromatography (GC).

IN eukaryotes, aerobic respiration takes place in mitochondria, which for many species are the main sources of adenosine triphosphate (ATP) synthesis (McBride, Neuspiel, and Wasiak 2006). However, many eukaryotes inhabit oxygen poor (hypoxic) or oxygen-free (anaerobic) environments and, accordingly, possess alternative pathways leading to ATP production. Recent advances have shown that the pathways of oxygen-independent ATP synthesis in eukaryotes either directly or indirectly involve mitochondria (Embley and Martin 2006). Adenosine triphosphate can be synthesized directly within the organelle, as in the case of anaerobic mitochondria (Lantsman et al. 2008; Tielens et al. 2002) or hydrogenosomes, H<sub>2</sub>-producing forms of mitochondria (Müller 1997; van der Giezen 2009). Relict mitochondrion-derived organelles, mitosomes, do not themselves synthesize ATP but instead play a crucial role in providing functions, such as FeS cluster assembly for proteins involved in cytosolic ATP synthesis (Goldberg et al. 2008; Tovar et al. 2003; van der Giezen 2009). The occurrence across all major eukaryotic lineages of oxygen-independent ATP synthesis pathways associated with mitochondria (van der Giezen 2009) is not surprising given the comparatively recent, in terms of geological time, oxygenation of the oceans only some 580 million years ago (Canfield, Poulton, and Narbonne 2007; Dietrich, Tice, and Newmann 2006), almost a billion years after eukaryotes arose, such that the origin and early diversification of eukaryotes took place at a time when the oceans were anoxic and sulfidic (Mentel and Martin 2008).

Whereas many eukaryotes are specialized to aerobic or anaerobic habitats, some are facultatively anaerobic, able to survive, and generate ATP with the participation of mitochondrial pathways with or without the presence of oxygen. One such example is the alga *Chlamydomonas reinhardtii*, the mitochondria of which respire normally in the presence of oxygen, but which after 30 min of anaerobic, heterotrophic, dark conditions express a suite of enzymes and produce a spectrum of metabolic end products, including H<sub>2</sub>, acetate, and formate (Dubini et al. 2009; Mus et al. 2007), otherwise

typical of hydrogenosomes (Atteia et al. 2006). Anaerobic energy metabolism in *Euglena* differs from that in *Chlamydomonas* in that it employs some enzymes shared with hydrogenosomes, but generates very different end products. Several *Euglena* species can grow at very low oxygen concentrations (Buetow 1989a): *Euglena gracilis* can survive up to 6 mo of hypoxia when grown in the dark with culturing on lactate (Carre, Bomsel, and Calvayrac 1988). In the presence of oxygen, the pyruvate stemming from glycolysis is transported into the mitochondrion and undergoes oxidative phosphorylation. The synthesized acetyl-CoA enters a modified Krebs cycle with a succinate-semialdehyde shunt. Oxygen is the final acceptor for the electrons resulting from the glucose breakdown and most of the ATP, produced via oxidative phosphorylation *gracilis*, is able to perform a unique wax ester fermentation, in which the fatty acid synthesis serves as electron sink when the wax esters are synthesized from its polysaccharide paramylon ( $\beta$ -1-3-glucan) (Inui et al. 1984).

In the absence of oxygen, the pyruvate stemming from glycolysis in *Euglena* is reduced to acetyl-CoA by pyruvate:NADP<sup>+</sup> oxidoreductase (PNO), an oxygen-sensitive enzyme (Inui et al. 1987; Rotte et al. 2001) that also was recently found in the anaerobic protist *Blastocystis* (Lantsman et al. 2008). The resulting acetyl-CoA is used as both primer and C2 donor, with NADH as electron donor for the mitochondrial malonyl-CoA-independent fatty acid biosynthesis. A portion of the synthesized fatty acids is reduced to alcohol, esterified with the fatty acids, and stored in the cytosol as waxes. On return to aerobiosis the wax esters are rapidly degraded and paramylon granules are resynthesized (Inui et al. 1982). However, most of the studies on wax ester fermentation and on their composition were carried out to date only with a streptomycin (Sm)-bleached mutant of *E. gracilis* strain Z or with the green *E. gracilis* strain T (Inui et al. 1982; Schneider and Betz 1985). In order to study the variability with respect to anaerobic metabolism in *E. gracilis* species, we analyzed the wax ester fraction of eight *E. gracilis* strains isolated between 1932 and 1958 from different localities in Europe and two bleached substrains of *E. gracilis* 1224-5/25 obtained by treatment with Sm and ofloxacin (Of). In this work we report the influence of very low oxygen concentrations on the anaerobic metabolism and wax ester fermentation of these different strains. In additional experiments with the elongase inhibitor flufenacet (Trenkamp, Martin, and Tietjen 2004) we also show its effect on

Corresponding Author: S. Tucci, Department of General Pediatrics, University Children's Hospital, Moorenstraße 5, D-40225 Duesseldorf, Germany—Telephone number: +49 211 811 7685; e-mail: sara.tucci@uni-duesseldorf.de

<sup>1</sup>Present Address: Department of General Pediatrics, University Children's Hospital, Moorenstrasse 5, 40225 Duesseldorf, Germany.

the synthesis and composition of the wax ester fraction, underscoring physiological and biochemical differences between *E. gracilis* strains.

## MATERIALS AND METHODS

**Chemicals.** All chemicals used in this work were purchased from J. T. Backer (Griesheim, Germany), Biozym (Oldendorf, Germany), Merck (Darmstadt, Germany), Riedel de Haën (Seelze, Germany), Roche (Penzberg, Germany), and Roth (Karlsruhe, Germany). Flufenacet pestanal, Sm, and Of were purchased from Sigma-Aldrich (Schnellendorf, Germany).

**Medium and growth conditions.** *Euglena gracilis* strains 1224-5/3, -/4, -/10, -/13, -/19, -/23, -/27, -/25 strain Z, were obtained from Sammlung von Algenkulturen (Göttingen, Germany). The cells were cultivated with a BIOSTAT B 10L fermenter (Braun Biothec, Göttingen, Germany) in a plant growth chamber (Sanyo MLR-350, Loughborough, UK) as described in Hoffmeister et al. (2004). The cultivation was performed on a defined medium described in Ogbonna, Tomiyama, and Tanaka (1998) and Yamane et al. (2001) modified as follows: for 1L medium we used 12 g glucose, 0.8 g  $\text{KH}_2\text{PO}_4$ , 1.5 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g  $\text{CaCO}_3$ , 0.0144 g  $\text{H}_3\text{BO}_3$ , 2.5 mg vitamin B<sub>1</sub>, 20 µg vitamin B<sub>12</sub>, 1 ml trace elements solution, and 1 ml Fe solution. The trace elements solution contained 4.4 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.16 g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.3 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.32 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.38 g  $\text{CoSO}_4 \cdot 5\text{H}_2\text{O}$  in 100 ml distilled water. The iron solution contained 1.14 g  $(\text{NH}_4)_2\text{SO}_4\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  and 1 g EDTA/100 ml distilled water. Cultures were grown at constant temperature of 28 °C and pH 2.8 in a plant growth chamber (Sanyo MLR-350). Vitamins and glucose were added by sterile technique to the medium after sterilization.

Aerobic cultures were gassed with 2 l/min air while anaerobic cultures were gassed with 2 l/min nitrogen and grown in the dark. Cultures were harvested 6 d after the inoculation and centrifuged at 2,800 g for 5 min. The harvested cells were frozen at –20 °C or immediately used for lipid extraction. Flufenacet was added to the anaerobic cultures in a final concentration of 100 µM.

**Bleaching of *Euglena gracilis*.** Two *E. gracilis* bleached mutants (W1ZOfl and W1ZSmL) were induced by an Of and Sm treatment, respectively. Briefly, three Erlenmeyer flasks with 20 ml defined Koren–Hutner medium (Koren and Hutner 1967) were inoculated with 50,000 cells/ml (final concentration) of *E. gracilis* 1224-5/25 strain Z cultures. One flask was used as a control while the other two flasks were supplemented with 200 µg/ml Of and 500 µg/ml Sm, respectively. The cultures were incubated overnight at 28 °C. The next day between 150 and 300 cells were plated on defined Koren–Hutner medium plates with 7.5% (w/v) agar and incubated at 28 °C over 10 d. White colonies of Of and Sm mutants were picked and grown in modified Hutner's medium [19.8 g glucose; 0.04 mM  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.01 mM  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.009 mM  $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ ; 0.1 mM  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ; 0.6 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 mM KCl; 2 mM D<sub>2</sub>-malic acid; 0.1% (w/v)  $(\text{NH}_4)_2\text{HPO}_4$ ] at constant temperature of 28 °C and pH 3.5.

**Lipid extraction from *Euglena gracilis* cells.** Extraction of the total lipid fraction from *E. gracilis* cells was performed with a method from Folch, Lees, and Sloane-Stanley (1957) modified as follows. At the end of the growth period, the cells were collected by centrifugation at 4,500 g for 5 min. The collected cells were boiled in hot methanol and sonicated twice for 5 min. The cell residue was covered with  $\text{CHCl}_3/\text{MeOH}$  2:1 (v/v; 25 ml/g cells) and removed by filtration in a separatory funnel. The organic phase was extracted with one-fourth volume of a 0.88% (w/v) KCl solution. After phase separation the organic phase was extracted with  $\text{MeOH}/\text{H}_2\text{O}$  1:1 (v/v). The total lipid extract was then

evaporated under reduced pressure (Heidolph, Schwabach, Germany) at 40 °C.

**Purification of the wax ester fraction.** The lipid extract was fractionated on a silica gel column (F<sub>254</sub> Kieselgel 60, Merck), eluting the different lipid classes with solvents of increasing polarity as described in Nevenzel, Rodgker, and Mead (1965). The lipid composition of the collected fractions was analyzed on thin layer chromatography (Kieselgel 60, Merck, 0.25 mm) and the resulting bands compared with commercially available standards.

**Gas chromatographic (GC) analysis of fatty acid methyl esters (FAMES) and fatty alcohols (FALCs).** The wax ester fraction was transesterified overnight with 2 ml of 3% (v/v) concentrated  $\text{H}_2\text{SO}_4$  in dry methanol and the reaction carried out at 80 °C. After cooling, the reaction mixture was diluted with 6 ml of double-distilled water and the FAMES and FALCs mixture was extracted 3 times with *n*-hexane by centrifugation at 2,800 g for 2 min. The FAMES and FALCs were then separated by thin-layer chromatography on Silica gel plates (20 × 20 cm, 0.25 mm, Kieselgel 60 F<sub>254</sub>, Merck) with petroleum ether/diethyl ether (PE/DE) 8:2 (v/v) according to Kattner and Fricke (1986). Bands corresponding to FAMES and FALCs were identified with commercially available standards, scraped off after visualizing with 0.2% (v/v) 2',7'-dichlorofluorescein in ethanol and submitted to GC analysis. The analysis was performed with an Agilent 6850 series GC coated with a HP-5% (v/v) phenyl-methyl siloxane capillary column (30.0 m × 250 µm × 0.25 mm, Supelco, Vienna, Austria). Temperature programming from 120 °C for 8 min then 4 °C/min to 220 °C and a final hold for 8 min at 220 °C was used. Nitrogen was used as carrier gas.

**Northern blot analysis.** Total RNA from *E. gracilis* cells was purified with the TRIzol reagent (Invitrogen, Karlsruhe, Germany) as recommended by the manufacturer. RNA was transferred to HybondN nylon membrane (GE Healthcare, München, Germany) and hybridized with the radioactively labeled coding sequences of PNO, *trans*-2-enoyl-CoA reductase (TER) or actin. Signals were detected on imaging plates (barium-fluor halogen crystal) with a FLA3000 Fluorescence scanner version 1.8E and quantified with the Image Gauge software (version 3.0) (Fuji Photo film Co., Düsseldorf, Germany).

## RESULTS

### Wax ester content of the different *Euglena gracilis* species.

All *Euglena* strains tested accumulated wax esters in different amounts and proportion of fresh weight when cultivated at ambient oxygen levels (Table 1). The highest concentration was detected in the bleached mutants 1224-5/25 W1ZOfl and W1ZSmfL whose wax ester fraction represented 55.2% and 59.6% of the total lipid amount and 8.9% and 7.8% of the dry weight, respectively. All other strains accumulated wax esters in concentration between 0.3% and 3.5% of the dry weight.

As already reported for *E. gracilis* 1224-5/25 strain Z (Tucci, Proksch, and Martin 2006), the absence of oxygen induced a striking effect on the cultures and on their wax ester content. Under strictly anaerobic conditions, several of the tested cultures died, including both bleached mutants, but those strains that survived anaerobiosis accumulated about 10-fold more wax esters than in the presence of oxygen (Table 1). In contrast to the corresponding aerobically grown cells, *E. gracilis* strain -/13 showed a 20-fold enrichment of wax esters in the absence of oxygen, which represented >31% of the dry weight of the cells.

**The effect of oxygen and flufenacet on the fatty acid and alcohol composition.** Flufenacet belongs to the class of K3 herbicides and inhibits higher plant elongases but not FAS complexes in either *Arabidopsis* (in the chloroplast) or yeast

Table 1. Wax ester content in different strains of *Euglena gracilis*.

SAG strain	Aerobic			Anaerobic			Anaerobic flufenacet		
	FW (mg/g)	WE/DW	WE/lip	FW (mg/g)	WE/DW	WE/lip	FW (mg/g)	WE/DW	WE/lip
<i>E. gracilis</i> 1224-5/3	1.1	0.5	4.3	14.3	5.7	33.3	59.4	26.1	70.1
<i>E. gracilis</i> 1224-5/4	0.6	0.3	3.2	10.5	4.2	28.8	—	—	—
<i>E. gracilis</i> 1224-5/10	5.5	2.7	3.2	—	—	—	—	—	—
<i>E. gracilis</i> 1224-5/13	3.5	1.6	12.4	78.4	31.4	62.1	114.0	57.0	77.4
<i>E. gracilis</i> 1224-5/15	7.1	3.5	27.6	—	—	—	—	—	—
<i>E. gracilis</i> 1224-5/19	1.0	1.3	2.9	8.4	3.4	20.8	15.1	6.3	44.0
<i>E. gracilis</i> 1224-5/23	4.1	1.9	3.8	20.8	8.3	34.4	31.3	14.0	53.8
<i>E. gracilis</i> 1224-5/27	2.6	1.3	4.0	—	—	—	—	—	—
<i>E. gracilis</i> 1224-5/25 W1ZOfl	17.8	8.9	55.2	—	—	—	—	—	—
<i>E. gracilis</i> 1224-5/25 W1ZSmL	15.7	7.8	59.6	—	—	—	—	—	—
<i>E. gracilis</i> 1224-5/25 <sup>a</sup>	2.3	0.9	6	25.4	10.2	48.5	42.9	19.7	59.6

The cultures were grown under aerobic and anaerobic conditions. The wax ester content is expressed in mg/g fresh weight (FW), in percent of the total lipid fraction (WE/lip), and in percent of dry weight (DW). “—” indicate that the strain did not grow anaerobically. Cultures were gassed with 2 l/min air (aerobic) or with 2 l/min N<sub>2</sub> (anaerobic). Flufenacet was added to anaerobically grown cultures to a final concentration of 100 μM.

<sup>a</sup>The data about the wax ester content in *E. gracilis* 1224-5/25 are obtained from Tucci et al. (2006).

(Trenkamp et al. 2004). The mitochondrial fatty acid synthesis of *E. gracilis* -/25 strain Z occurs, as well as the elongation system, via CoA esters instead of ACP proteins (Hoffmeister et al. 2005). Addition of 100 μM of the elongase inhibitor flufenacet to anaerobic cultures enhanced the synthesis of wax esters, which accumulated in all the strains at higher concentrations (Table 1). However, strain -/4 did not survive the treatment with the herbicide. On the other hand, the proportion of waxes in strain -/13 increased up to 57% of the dry weight, comparable to the highest values previously reported in the literature for this species (Buetow 1989b).

The specific composition of the fatty acid and FALc constituents of the wax ester fraction was determined for all examined strains. The basic fatty acid and FALc profiles of aerobically grown strains -/10, -/15, and -/27 showed a very similar distribution (Fig. 1). The FAMES consisted mostly of the even number chains myristic (C14:0) and palmitic acid (C16:0), whereas in strain -/15 a discrete amount of C13:0 was detected. Unsaturated fatty acids were present in the wax esters fraction at very low concentration. Strains 1224-5/25 W1ZOfl and W1ZSmL bleached mutants showed nearly identical FAMES and FALcs profiles. In both substrains the synthesis of fatty acids and alcohols was clearly shifted toward shorter, saturated chain. The FAMES consisted mostly of C12:0, C13:0, and C14:0, while in the FALcs fraction C15:0 was also accumulated as one the major compounds.

The FAMES and FALcs profiles in the presence of oxygen of the facultatively anaerobic strains -/3, -/4, -/13, -/19, and -/23 were comparable with those of the aerobically grown strains (cf. Fig. 2A, B). In contrast, the absence of oxygen had a great influence on the fatty acids and FALcs compositions of the different strains. Whereas with substantial differences among the strains, growth at strictly anaerobic conditions led to an accumulation of odd-number chain C13:0 acids and alcohols (cf. Fig. 2B, C), while the proportion of C16 and C18 fatty acids and alcohols in dark-synthesized wax esters decreased.

The combination of the two factors, anaerobiosis and the treatment with the herbicide flufenacet, resulted in a reduction of the odd-numbered constituents, although the total wax ester synthesis was not inhibited (Table 1). The concentration of all fatty acids and alcohols >C18 was dramatically reduced (cf. Fig. 2D, E) as was the proportion of odd-chain constituents (C13:0 and C15:0) in the wax esters.

**Northern blot analysis of different *Euglena* strains.** Northern hybridization revealed differences in the expression at RNA level of the TER and PNO involved in the anaerobic wax ester

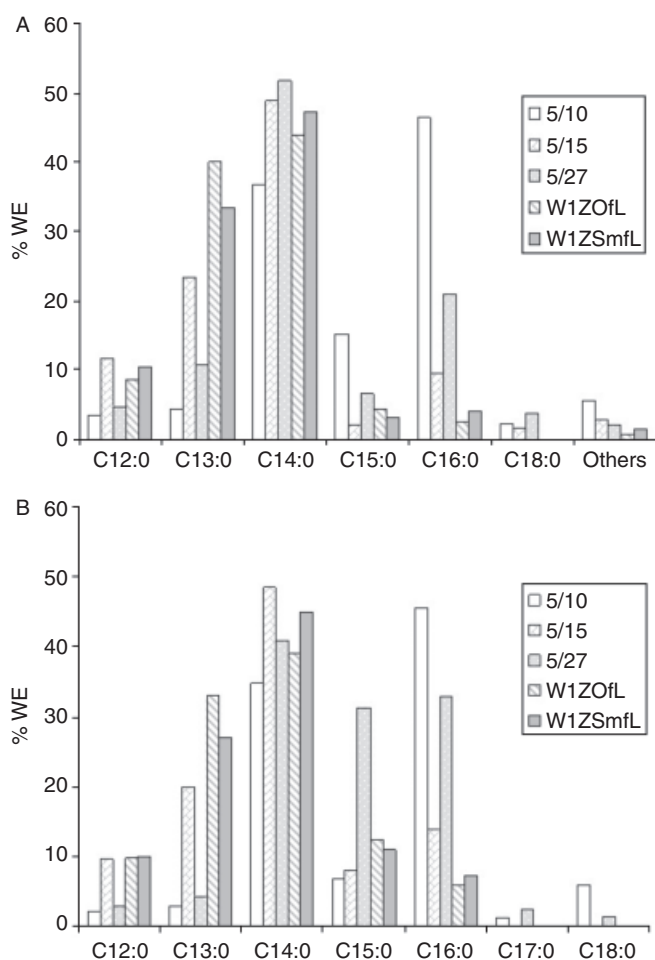


Fig. 1. Fatty acid methyl esters (FAMES) (A) and fatty alcohols (FALcs) (B) composition of the wax ester fraction in the different *Euglena gracilis* strains. The bars represent the amount of the fatty acids and alcohols in the wax ester fraction of *E. gracilis* strains -/10, -/15, -/27, -/25 W1ZOfl and -/25 W1ZSmL grown under aerobic conditions. In the FAMES graph the bar for others includes all unsaturated fatty acids present in the wax ester fraction.

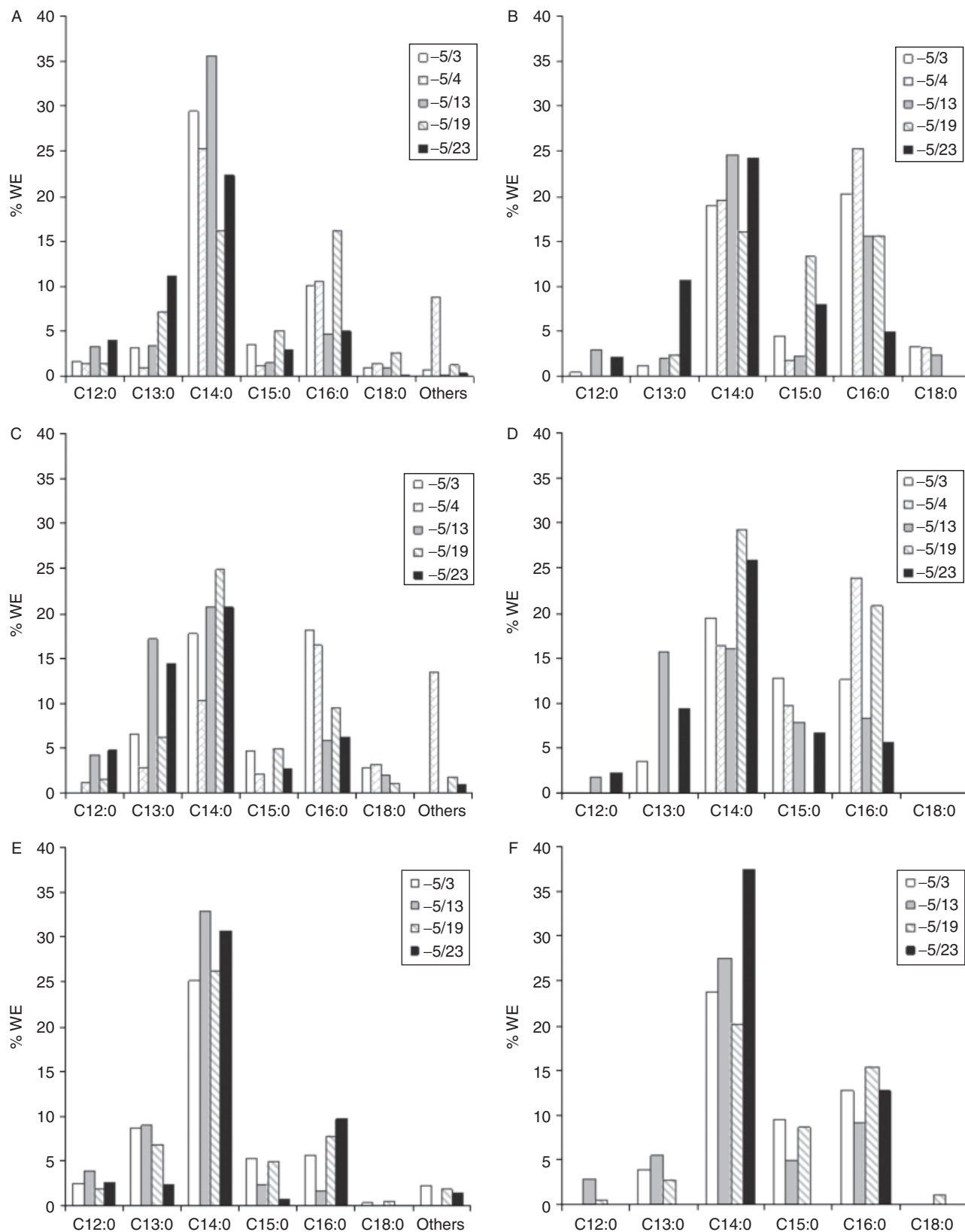


Fig. 2. Fatty acid methyl esters (FAMES) (A, C, and E) and FALCs (B, D, and F) composition of the wax ester fraction in *Euglena gracilis* strains. The bars represent the amount of the fatty acids and alcohols in the wax ester fraction of *E. gracilis* strains -/3, -/4 -/13, -/19, and -/23 grown under aerobic (A and B), anaerobic (C and D), and anaerobic conditions (E and F) in the presence of flufenacet. In the FAMES graph the bar for others includes all unsaturated fatty acids present in the wax ester fraction. Flufenacet was added to anaerobically grown cultures (E and F) to a final concentration of 100  $\mu$ M.

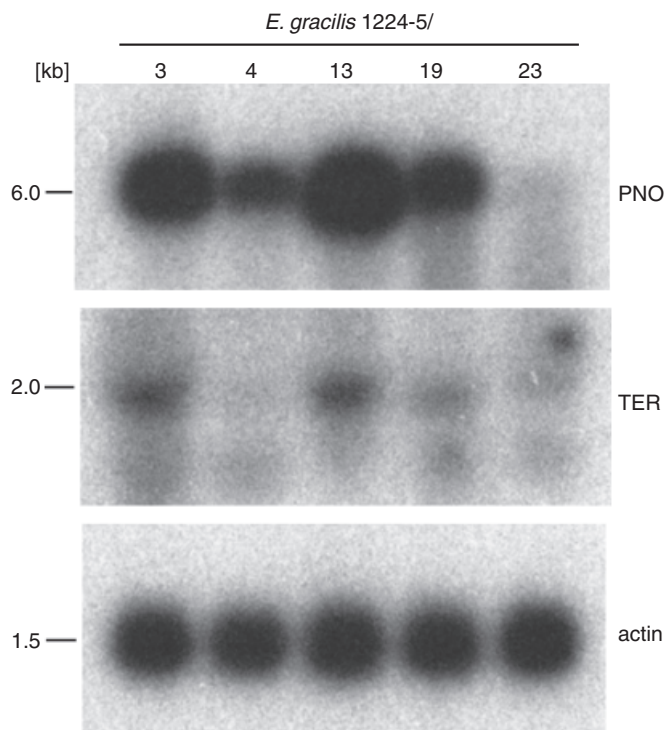


Fig. 3. Northern blot analysis of RNA isolated from different strains of *Euglena gracilis*. Total RNA was extracted from *Euglena gracilis* strains 1224-5/3, -/4, -/13, -/19, and -/27 grown under aerobic conditions. The extracted RNA was probed with radioactively labeled hybridization probes for the pyruvate:NADP<sup>+</sup> oxidoreductase (PNO) and *trans*-2-enoyl-CoA reductase (TER) sequences. Five micrograms RNA of each *Euglena* strain were loaded onto the gel.

fermentation of *E. gracilis* strain Z (Hoffmeister et al. 2005; Rotte et al. 2001) (Fig. 3). Both PNO and TER were expressed and detectable at mRNA level in all four analyzed strains. In contrast to the other strains, only strains -/3 and -/13 showed higher expression for both PNO with 2.4- and 2-fold induction, and TER with 3.6- and 2.4-fold induction, respectively (Fig. 3).

## DISCUSSION

The shift of *Euglena* cells from aerobic to anaerobic conditions induces the malonyl-CoA independent synthesis of wax esters using the reserve polysaccharide paramylon (Inui et al. 1982). Mitochondrial fatty acid synthesis uses acetyl-CoA and propionyl-CoA as C2- and C3-donors, for de novo synthesis of even- and odd-numbered fatty acids, respectively. This ability to survive lower oxygen tension has been reported for at least several *E. gracilis* species (Buetow 1989a). To compare the resistance to anoxia and the ability to perform wax ester fermentation in some of them, nine different *Euglena* strains were cultivated under different conditions and their wax ester fractions were analyzed. As already reported for *E. gracilis* 1224-5/25 Z (Tucci et al. 2006), all strains accumulated wax esters by aerobic cultivation but in different amounts and proportions, as the wax ester concentrations ranged between 0.3% and 8.9% of the dry weight.

The ability of *E. gracilis* -/25 strain Z to survive anaerobiosis up to 6 mo at 5°C on a non-fermentable carbon source was described by Carre et al. (1988). During these conditions ca 70% of *E. gracilis* dry weight was represented by wax esters (Buetow 1989b). However, in this work we demonstrated that

only the *Euglena* strain -/13 reached ~ 60% of the dry weight. Of particular note were the wax ester concentration and composition of the bleached *E. gracilis* WIZOfL and WIZSmfL. In agreement with earlier work (Inui et al. 1984), a Sm bleached mutant of *E. gracilis* -/25 accumulated more shorter and saturated chains with a clear prevalence of C13:0 and C14:0 acids and alcohols. The paucity of unsaturated acids in the wax ester fraction of the bleached mutants during our work confirms the chloroplasts damage and the loss in enzymatic activity including that of the FAS type II. Very surprisingly, we observed that in contrast to all data reported to date (Inui et al. 1982, 1983, 1984), bleached strains *E. gracilis* WIZOfL and WIZSmfL were unable to survive when they were brought to anaerobiosis. The very high wax ester content in bleached *Euglena* in ambient oxygen together with the finding that they are incapable of performing wax ester fermentation under anaerobic conditions also suggests mitochondrial damage due to the bleaching treatment and a consequent redirection of reducing equivalents to maintain redox balance.

Comparison of the wax ester composition in anaerobically grown strains revealed distinct differences in the fatty acid and alcohol profiles. The proportion of even-numbered constituents was reduced and the fatty acid and alcohol patterns were shifted toward shorter and odd-numbered chains. The presence of these components suggests the involvement of the [FRD(RQ)]-propionyl-CoA route in *Euglena* (Fig. 4). The synthesis of propionyl-CoA, which occurs via the methyl-malonyl-CoA pathway (Nagai, Otha, and Saito 1971; Schneider and Betz 1985), requires the reduction of fumarate to succinate with the participation of rhodoquinone (RQ) (Hoffmeister et al. 2004; Takamiya et al. 1993; Tielens et al. 2002; Van Hellemond and Tielens 1994). The reduction of fumarate is a NADH-consuming reaction, essential to maintain the redox balance in anaerobically functioning mitochondria (Tielens et al. 2002; Tielens and Van Hellemond 1998; Van Hellemond and Tielens 1994; Van Hellemond et al. 2003). The increase of odd-numbered fatty acids and alcohols in the wax ester fraction of anaerobically grown cultures indicates the participation of the PNO-dependent mitochondrial fatty acid biosynthesis system, because the formation of odd-chains by  $\alpha$ -oxidation can be excluded in fermenting *Euglena* cells (Schneider, Borkowski, and Betz 1984).

Anaerobic growth and treatment with flufenacet showed that odd chain fatty acids and alcohols were reduced in the wax ester fraction compared with growth without flufenacet, probably due to the inhibition of the condensing step in the mitochondrial fatty acid synthesis. However, the total wax ester production was not inhibited and the accumulation of even-numbered chain fatty acids and alcohols with C14:0 and C16:0 as the major compounds became particularly prominent. This suggests an inhibition of the mitochondrial fatty acid synthesis in *Euglena*, which occurs, as well as elongation system, via CoA esters instead of ACP-proteins (Hoffmeister et al. 2005), consistent with the finding that flufenacet does not inhibit FAS complexes in either *Arabidopsis* (in the chloroplast) or yeast (Trenkamp et al. 2004) but higher plant elongases instead. The simplest interpretation for the lack of odd-numbered carbon constituents in response to flufenacet without loss of wax ester accumulation is that the mitochondrial fatty acid synthesis was inhibited and that electron flux was shunted to the cytosolic or chloroplast fatty acid synthesis pathway to maintain redox balance. Alternatively, it is possible, but unlikely in our view, that propionyl-CoA-dependent fatty acid synthesis was specifically inhibited in mitochondria. This is unlikely because of the mode of action of flufenacet, which is thought to involve covalent binding of the elongase active site (Trenkamp et al. 2004). *Euglena gracilis* -/4 did not grow at all in the presence of flufenacet. Additionally, Northern blot analysis showed that the mRNA expression of either PNO or TER did not correlate with the

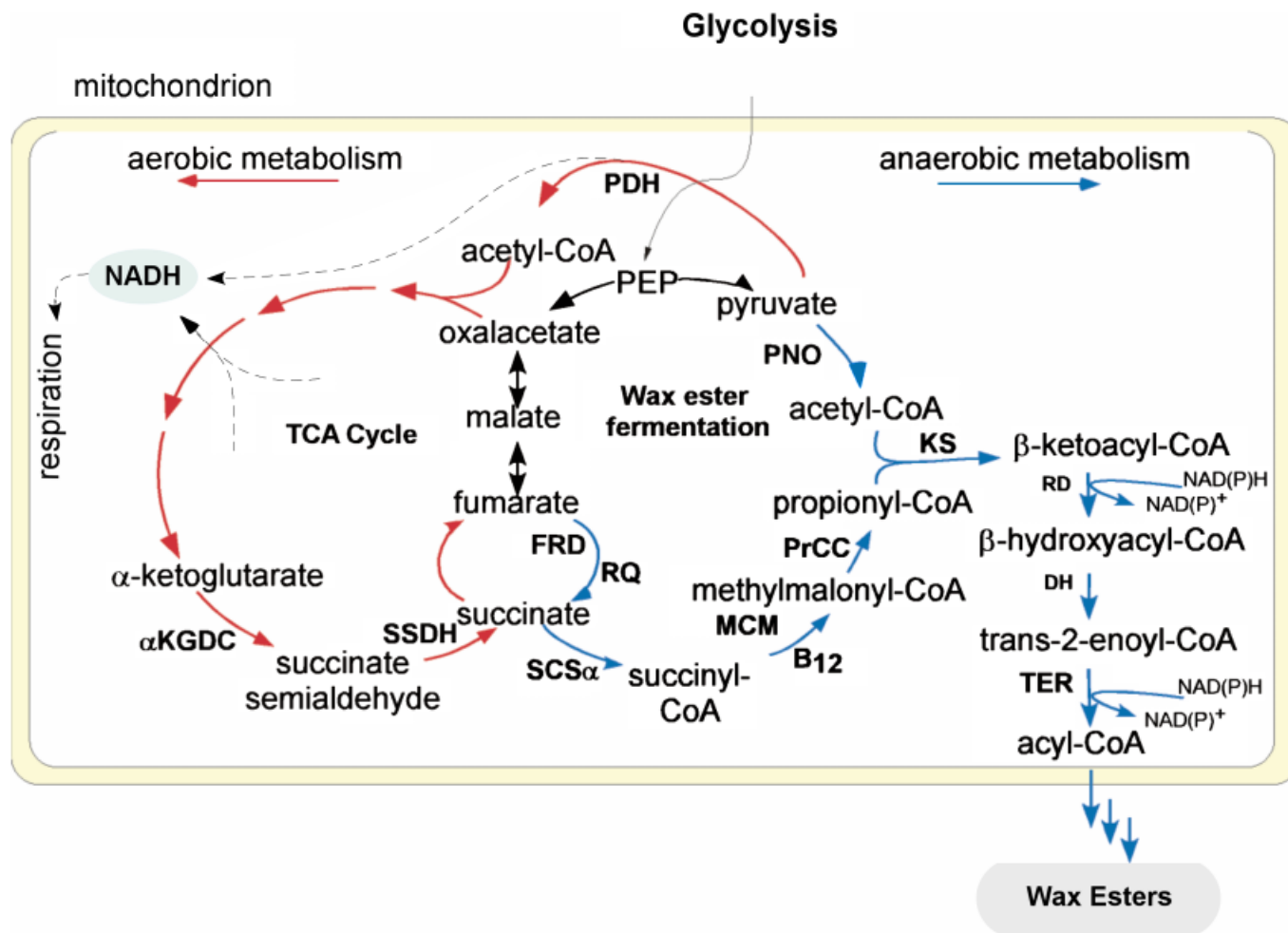


Fig. 4. Working model for the energy metabolism in the facultatively anaerobic mitochondrion of *Euglena gracilis*.  $\alpha$ -KGDC,  $\alpha$ -ketoglutarate decarboxylase (Buetow 1989a); SSDH, succinate semialdehyde dehydrogenase (Buetow 1989a); FRD, fumarate reductase; RQ, rodoquinone (Hoffmeister et al. 2004); SCS $\alpha$ , succinyl-CoA  $\alpha$ -synthetase; MCM, methylmalonyl mutase; PrCC, propionyl-CoA carboxylase; PDH, pyruvate dehydrogenase (Hoffmeister et al. 2004); PEP, phosphoenolpyruvate; PNO, pyruvate:NADP<sup>+</sup> oxidoreductase (Rotte et al. 2001); KS,  $\beta$ -ketosynthase; RD,  $\beta$ -ketoreductase; DH,  $\beta$ -hydroxyacyl-dehydrogenase; TER, *trans*-2-enoyl-CoA reductase (Hoffmeister et al. 2005). This metabolic model was modified accordingly to Schneider and Betz (1985) and Tielens et al. (2002).

ability of different *Euglena* strains to grow at lower oxygen tension.

*Euglena gracilis* -5/25 strain Z possesses a single reticulate mitochondrion biochemically prepared for a prompt switch to anaerobic conditions. Other *E. gracilis* strains have been reported to survive lower oxygen tension (Buetow 1989b), but whether they also possess such a facultatively anaerobic organelle and perform wax ester fermentation had not been tested. *Euglena gracilis* belongs to the class of euglenids that show very interesting biodiversity (Breglia, Slamovits, and Leander 2007; Leander, Esson, and Breglia 2007). In this study we demonstrate that there is also more variability and diversity with respect to anaerobic energy metabolism among *E. gracilis* strains held in culture collections than previously suspected. There is currently great interest in algal biofuel production (Beer et al. 2009), but *Euglena* is currently not amenable to genetic manipulation, although it might be a rich source of relevant genes. Although we do not currently suggest *Euglena* wax esters as a general alternative to biofuel production involving, for example, H<sub>2</sub>-synthesis in the *Chlamydomonas* system (Dubini et al. 2009; Mus et al. 2007), we do note that the dark-green dried cell mass of anaerobically grown

strains consisting of >10% dry weight of wax esters burned vigorously when ignited with a match.

#### ACKNOWLEDGMENTS

We thank Margarete Stracke for excellent technical support. This work was funded by Deutsche Forschungsgemeinschaft (DFG; SFB-TR1 project C3) and by grants from the Ministry of Education of the Slovak Republic (VEGA 1/0416/09, to J.K. and VEGA 1/0118/08, to R.V.).

#### LITERATURE CITED

- Atteia, A., van Lis, R., Gelius-Dietrich, G., Adrait, A., Garin, J., Joyard, J., Rolland, N. & Martin, W. 2006. Pyruvate: formate lyase and a novel route of eukaryotic ATP-synthesis in anaerobic *Chlamydomonas* mitochondria. *J. Biol. Chem.*, **281**:9909–9918.
- Beer, L. L., Boyd, E. S., Peters, J. W. & Posewitz, M. C. 2009. Engineering algae for biohydrogen and biofuel production. *Curr. Opin. Biotechnol.*, **20**:264–271.

- Breglia, S. A., Slamovits, C. H. & Leander, B. S. 2007. Phylogeny of phagotrophic euglenids (Euglenozoa) as inferred from hsp90 gene sequences. *J. Eukaryot. Microbiol.*, **54**:86–92.
- Buetow, D. E. 1989a. Morphology and ultrastructure of *Euglena*. In: Buetow, D. E. (ed.), *The Biology of Euglena*. Academic Press, San Diego, CA. **1**:109–184.
- Buetow, D. E. 1989b. The mitochondrion. In: Buetow, D. E. (ed.), *The Biology of Euglena, Subcellular Biochemistry and Molecular Biology*. Academic Press, San Diego, CA. **IV**:247–314.
- Canfield, D. E., Poulton, S. W. & Narbonne, G. M. 2007. Late-Neoproterozoic deep-ocean oxygenation and the rise of animal life. *Science*, **315**:92–95.
- Carre, I., Bomsel, J. L. & Calvayrac, R. 1988. Decay of cytochromes and appearance of cyanide-sensitive electron transfer pathway in *Euglena gracilis* grown in anoxia. *Plant Sci.*, **54**:193–202.
- Dietrich, L. E. P., Tice, M. M. & Newmann, D. K. 2006. The co-evolution of life and Earth. *Biology*, **16**:R395–R400.
- Dubini, A., Mus, F., Seibert, M., Grossman, A. R. & Posewitz, M. C. 2009. Flexibility in anaerobic metabolism as revealed in a mutant of *Chlamydomonas reinhardtii* lacking hydrogenase activity. *J. Biol. Chem.*, **284**:7201–7213.
- Embley, T. M. & Martin, W. 2006. Eukaryotic evolution, changes and challenges. *Nature*, **440**:623–630.
- Folch, J. M., Lees, M. & Sloane-Stanley, G. H. 1957. A simple method for the isolation and purification of total lipid from animal tissues. *J. Biol. Chem.*, **226**:497–509.
- Goldberg, A. V., Molik, S., Tsaousis, A. D., Neumann, K., Kuhnke, G., Delbac, F., Vivares, C. P., Hirt, R. P., Lill, R. & Embley, T. M. 2008. Localization and functionality of microsporidian iron-sulphur cluster assembly proteins. *Nature*, **452**:624–628.
- Hoffmeister, M., Piotrowski, M., Nowitzki, U. & Martin, W. 2005. Mitochondrial *trans*-2-enoyl-CoA reductase of wax ester fermentation from *Euglena gracilis* defines a new family of enzymes involved in lipid synthesis. *J. Biol. Chem.*, **280**:4329–4338.
- Hoffmeister, M., van der Klei, A., Rotte, C., van Grinsven, K. W. A., van Hellemond, J. J., Henze, K., Tielens, A. G. M. & Martin, W. 2004. *Euglena gracilis* rhodoquinone: ubiquinone ratio and mitochondrial proteome differ under aerobic and anaerobic conditions. *J. Biol. Chem.*, **279**:22422–22429.
- Inui, H., Miyatake, K., Nakano, Y. & Kitaoka, S. 1982. Wax ester fermentation in *Euglena gracilis*. *FEBS Lett.*, **150**:89–93.
- Inui, H., Miyatake, K., Nakano, Y. & Kitaoka, S. 1983. Production and composition of wax ester by fermentation of *Euglena gracilis*. *Agric. Biol. Chem.*, **47**:2669–2671.
- Inui, H., Miyatake, K., Nakano, Y. & Kitaoka, S. 1984. Fatty acid synthesis in mitochondria of *Euglena gracilis*. *Eur. J. Biochem.*, **142**:121–126.
- Inui, H., Ono, K., Miyatake, K., Nakano, Y. & Kitaoka, S. 1987. Purification and characterization of pyruvate:NADP<sup>+</sup> oxidoreductase in *Euglena gracilis*. *J. Biol. Chem.*, **262**:9130–9135.
- Kattner, G. & Fricke, H. S. G. 1986. Simple gas-liquid chromatographic method for the simultaneous determination of fatty acids and alcohols in wax esters of marine organisms. *J. Chromatog.*, **361**:263–268.
- Lantsman, Y., Tan, K. S., Morada, M. & Yarlett, N. 2008. Biochemical characterization of mitochondrial-like organelle from *Blastocystis* sp. subtype 7. *Microbiol. SGM*, **145**:2757–2766.
- Leander, B. S., Esson, H. J. & Breglia, S. A. 2007. Macroevolution of complex cytoskeletal systems in euglenids. *BioEssays*, **29**:987–1000.
- McBride, H. M., Neuspiel, M. & Wasiak, S. 2006. Mitochondria: more than just a powerhouse. *Curr. Biol.*, **16**:551–560.
- Mentel, M. & Martin, W. 2008. Energy metabolism among eukaryotic anaerobes in light of Proterozoic ocean chemistry. *Phil. Trans. R. Soc. B*, **363**:2717–2729.
- Müller, M. 1997. Evolutionary origins of trichomonad hydrogenosomes. *Parasitol. Today*, **13**:166–167.
- Mus, F., Dubini, A., Seibert, M., Posewitz, M. C. & Grossman, A. R. 2007. Anaerobic acclimation in *Chlamydomonas reinhardtii* anoxic gene expression, hydrogenase induction, and metabolic pathways. *J. Biol. Chem.*, **282**:25475–25486.
- Nagai, J., Otha, T. & Saito, E. 1971. Incorporation of propionate into wax esters by etiolated *Euglena*. *Biochem. Biophys. Res. Comm.*, **42**:523–529.
- Nevenzel, J. C., Rodgker, W. & Mead, J. F. 1965. The lipids of *Ruvettus pretiosus* muscle and liver. *Biochemistry*, **4**:1589–1594.
- Ogbonna, J. C., Tomiyama, S. & Tanaka, H. 1998. Heterotrophic cultivation of *Euglena gracilis* Z for efficient production of  $\alpha$ -tocopherol. *J. Appl. Phycol.*, **10**:67–74.
- Rotte, C., Stejskal, F., Zhu, G., Keithly, J. S. & Martin, W. 2001. Pyruvate:NADP<sup>+</sup> oxidoreductase from the mitochondrion of *Euglena gracilis* and from the apicomplexan *Cryptosporidium parvum*: a biochemical relic linking pyruvate metabolism in mitochondriate and amitochondriate protists. *Mol. Biol. Evol.*, **18**:710–720.
- Schneider, T. & Betz, A. 1985. Waxmonoester fermentation in *Euglena gracilis* T. Factors favouring the synthesis of odd-numbered fatty acids and alcohols. *Planta*, **166**:67–73.
- Schneider, T., Borkowski, C. & Betz, A. 1984. Wax ester formation in *Euglena gracilis* during anaerobiosis and photoheterotrophic growth. In: Sybesma, C. (ed.), *Advances in Photosynthesis Research*. Martinus Nijhoff, The Hague. **III**:445–448.
- Takamiya, S., Kita, K., Wang, H., Weinstein, P. P., Hiraishi, A., Oya, H. & Aoki, T. 1993. Developmental changes in the respiratory chain of *Ascaris* mitochondria. *Biochim. Biophys. Acta*, **1141**:65–74.
- Tielens, A. G. M. & Van Hellemond, J. J. 1998. The electron transport chain in anaerobically functioning eukaryotes. *Biochim. Biophys. Acta*, **1365**:71–78.
- Tielens, A. G. M., Rotte, C., Van Hellemond, J. J. & Martin, W. 2002. Mitochondria as we don't know them. *Trends Biochem. Sci.*, **27**:545–592.
- Tovar, J., León-Avila, G., Sánchez, L. B., Sutak, R., Tachezy, J., van der Giezen, M., Hernández, M., Müller, M. & Lucocq, J. M. 2003. Mitochondrial remnant organelles of *Giardia* function in iron-sulphur protein maturation. *Nature*, **426**:172–176.
- Trenkamp, S., Martin, W. & Tietjen, K. 2004. Specific and differential inhibition of very long-chain fatty acid elongases from *Arabidopsis thaliana* by different herbicides. *Proc. Natl. Acad. Sci. USA*, **101**:11903–11908.
- Tucci, S., Proksch, P. & Martin, W. 2006. Fatty acid biosynthesis in mitochondria of *Euglena gracilis*. In: Benning, C. & Ohlrogge, J. (ed.), *Advances in Plant Lipid Research: Proceedings of the 17th International Symposium on Plant Lipids*, East Lansing, Michigan, July 2006. Michigan State University Press, p. 133–136.
- Van der Giezen, M. 2009. Hydrogenosomes and mitosomes: conservation and evolution of functions. *J. Eukaryot. Microbiol.*, **56**:221–231.
- Van Hellemond, J. J. & Tielens, A. G. M. 1994. Expression and functional properties of fumarate reductase. *Biochem. J.*, **304**:321–331.
- Van Hellemond, J. J., van der Klei, A., Van Weelden, S. W. H. & Tielens, A. G. M. 2003. Biochemical and evolutionary aspects of anaerobically functioning mitochondria. *Phil. Trans. R. Soc. Lond.*, **358**:205–215.
- Yamane, Y., Utsunomiya, T., Watanabe, M. & Sasaki, K. 2001. Biomass production in mixotrophic culture of *Euglena gracilis* under acidic condition and its growth energetics. *Biotech. Lett.*, **23**:1223–1228.

Received: 08/07/09, 10/06/09; accepted: 10/08/09