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Anaerobic respiration coupled with mitochondrial fatty acid synthesis in wax ester fermentation by *Euglena gracilis*

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In *Euglena gracilis*, wax ester fermentation produces ATP during anaerobiosis. Here, we report that anaerobic wax ester production is suppressed when the mitochondrial electron transport chain complex I is inhibited by rotenone, whereas it is increased by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The ADP/ATP ratio in anaerobic cells is elevated by treatment with either rotenone or CCCP. Gene silencing experiments indicate that acyl-CoA dehydrogenase, electron transfer flavoprotein (ETF), and rhodoquinone (RQ) participate in wax ester production. These results suggest that fatty acids are synthesized in mitochondria by the reversal of β-oxidation, where *trans*-2-enoyl-CoA is reduced mainly by acyl-CoA dehydrogenase using the electrons provided by NADH via the electron transport chain complex I, RQ, and ETF, and that ATP production is highly supported by anaerobic respiration utilizing *trans*-2-enoyl-CoA as a terminal electron acceptor.

Keywords: anaerobic respiration; *Euglena gracilis*; mitochondrial electron transfer system; mitochondrial fatty acid synthesis; wax ester fermentation

Most eukaryotes, when under anaerobic conditions, obtain ATP by fermentation, where glucose is catabolized to pyruvate through the glycolytic pathway. This yields a net gain of two ATP molecules from substrate-level phosphorylation with the concomitant formation of two molecules of NADH per molecule of glucose [1,2]. Pyruvate is further metabolized to reoxidize NADH, which is necessary to sustain metabolic flux through glycolysis, and a variety of anaerobic end products, such as lactate and ethanol, are formed. ATP can also be produced by anaerobic respiration, where NADH is oxidized by transferring electrons through the mitochondrial electron transport chain to a terminal electron acceptor other than O_2 and ATP is then synthesized by utilizing the proton motive force developed by the electron transport [1–3]. In certain eukaryotes, fumarate of endogenous origin is used as a terminal electron acceptor, resulting in the formation of succinate.

Abbreviations
CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ETF, electron transfer flavoprotein; RQ, rhodoquinone; UQ, ubiquinone.

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Euglena gracilis, a photosynthetic unicellular protist, has a unique anaerobic energy generating system called wax ester fermentation, in which wax esters are synthesized as end products from paramylon, a storage polysaccharide (β-1,3-glucan) [4,5]. Anaerobically synthesized wax esters are coupled with saturated fatty acids and alcohols, mainly myristic acid and myristyl alcohol, and accumulated in cells [6,7]. During wax ester fermentation, fatty acids are synthesized using acetyl-CoA as both primer and C_2 donor in the mitochondria, essentially by the reversal of β-oxidation [8]. It had long been believed that NAD(P)H-dependent *trans*-2-enoyl-CoA reductase (acyl-CoA: $NAD(P)^+$ *trans*-2-oxidoreductase) functions in the fatty acid synthesis, instead of acyl-CoA dehydrogenase linked to the electron transfer flavoprotein (ETF) (acyl-CoA:ETF 2,3-oxidoreductase) that is utilized in the β-oxidation [8,9]. However, details of mitochondrial fatty acid synthesis, in particular the reduction in *trans*-2-enoyl-CoA, remain to be clarified. In addition, the physiological significance of why *Euglena* cells produce wax esters as an anaerobic end product instead of the more common lactate or ethanol is not well understood.

In the present paper, we report that the production of wax esters is suppressed in anaerobic *Euglena* cells when the mitochondrial electron transport chain complex I is inhibited by rotenone, whereas an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), induces an increase in anaerobic wax ester production. On the basis of results obtained by gene silencing experiments, we suggest that *trans*-2-enoyl-CoA is reduced mainly by acyl-CoA dehydrogenase in mitochondrial fatty acid synthesis, and that electrons for this reduction are provided by NADH via complex I, rhodoquinone (RQ), and ETF. In addition, we also suggest that ATP is synthesized by utilizing the proton motive force developed from the electron transport through complex I.

Materials and methods

Organism and culture

Euglena gracilis SM-ZK, a nonphotosynthetic mutant strain [10], was cultured in Koren–Hutner medium containing glucose as the major carbon source [11] with aeration at 27 °C for 3 or 4 days until the late logarithmic or early stationary phase of growth. Anaerobic treatment was performed as follows: an aliquot (2 mL) of the aerobic cell culture was dispensed into 5 mL plastic tube and bubbled with argon gas for 1 min. Rotenone (10 mM) or CCCP (2 mM) dissolved in methanol was added to a final concentration of 50 μM or 10 μM, respectively. Tubes were then tightly capped and allowed to stand under anaerobic conditions for 24 h. Cells in the culture were counted with a CDA-1000 particle counter (Sysmex, Kobe, Japan).

RNA interference

Double strand RNA (dsRNA) was synthesized and purified using a MEGAscript RNAi kit (Thermo Fisher Scientific, Waltham, MA, USA), and total RNA extracted from *Euglena* cells was reverse transcribed with SuperScript III reverse transcriptase (Thermo Fisher Scientific) according to a method described previously [7]. Template DNA containing T7 promoter at both ends was amplified from *Euglena* cDNA by PCR using the primers listed in Table 1. Sequences of mRNA encoding target genes were obtained using a TBLASTN search against a *E. gracilis*

Table 1. Oligonucleotide primers for the synthesis of dsRNA used in RNA interference experiments.

Name of dsRNA	Target gene		Sequence[a]
ETF-dsRNA-1	*etfalpha*	Forward	ctaatacgactcactatagggagaTTCTGCAGAATATGCAAATTTG
		Reverse	ctaatacgactcactatagggagaTTCCGGTCTGTCCAACCTGT
ETF-dsRNA-2	*etfbeta*	Forward	ctaatacgactcactatagggagaTTGATGAGATCGCTGTTGAAC
		Reverse	ctaatacgactcactatagggagaTTCAATCGGAGATCACAGGACAG
RQUA-dsRNA	*rquA*	Forward	ctaatacgactcactatagggagaTTGGGGATGCATGCCTGATGGAGAAC
		Reverse	ctaatacgactcactatagggagaTTGAACTGCTTTCGCAGCTCCGCGTC
ACD1-dsRNA	*acd1*	Forward	ctaatacgactcactatagggagaTTGGACTCTGGACACAGTTAAGACTGA
		Reverse	ctaatacgactcactatagggagaATCCCATCGTGTGGCTTGCCAATCAG
TER-dsRNA-1	*ter*	Forward	ctaatacgactcactatagggagaTGCCTGTGCGTGGCAACGGTA
		Reverse	ctaatacgactcactatagggagaAAGCCACGAATCTTGGGCTGGA
TER-dsRNA-2	*ter*	Forward	ctaatacgactcactatagggagaGGTATTGTTGGCGACTGGATCCAA
		Reverse	ctaatacgactcactatagggagaAGAAACGCCACGGTGTTGTACCAGC

[a] Lower case letters show T7 promoter sequence.

RNA-Seq database (DRA accession number: SRP060591) [12]. These genes included ETF alpha (*etfalpha*; accession ID GDJR01012930.1) and beta (*etfbeta*; GDJR0101 3070.1) subunit genes, RQ biosynthesis gene A (*rquA*; GDJR01043002.1), and acyl-CoA dehydrogenase 1 gene (*acd1*; GDJR01042416.1). In addition, previously reported cDNA sequence data were used in NAD(P)H-dependent *trans*-2-enoyl-CoA reductase (*ter*; AY741582) [9].

Euglena cells suspended in phosphate-buffered saline (4×10^6 cells/400 μL) in a 0.2 cm gap cuvette were electroporated with 30 μg of dsRNA using an ECM630 (BTX, Holliston, MA, USA) at 0.5 kV and 200 μF. After electroporation, the cells were cultured in Koren–Hutner medium under aerobic conditions for 3 or 4 days. The extent of silencing was examined by semiquantitative RT-PCR (Table S1).

Analyses of wax esters

As described previously [7], lipids were extracted from *Euglena* cells and directly subjected to gas–liquid chromatography to determine the wax ester content. The lipid extract dissolved in hexane was separated on a silica gel column (Silica gel 60, Merck, Darmstadt, Germany; 6 × 75 mm) equilibrated with hexane, and wax esters were eluted with diethylether-hexane (1 : 99, by vol.). After saponification, the constituent fatty acids and alcohols of wax esters were analyzed by gas–liquid chromatography [7].

Determination of ADP/ATP ratio

Into cell suspension, 70% perchloric acid was added at a final concentration of 3.5%, homogenized by sonication and neutralized with 3 M K_2CO_3 at 4 °C. After centrifugation for the removal of precipitate, the ratio of ADP/ATP in the supernatant was determined using a commercial kit (ADP/ATP Ratio Assay Kit (Bioluminescent), Abcam, Cambridge, UK).

Quinone analysis

Quinone content was determined using an HPLC-electrochemical detection system according to the method described by Shiobara *et al.* [13]. Cells incubated under anaerobic conditions for 24 h were harvested by centrifugation, and 2-propanol was added (20 μL·mg^{-1} wet weight of cells) to extract quinones and quinols. In total, 1 μL of 120 mM $FeCl_3$ solution dissolved in 2-propanol was added to 100 μL of the extract to oxidize quinols prior to analysis [14], and this was used as a sample for measurement. Rhodoquinone-9 (RQ_9) was prepared from *Ascaris suum* [15] and was used as a standard for HPLC analysis in addition to ubiquinone-10 (UQ_{10}), which was purchased from Wako Chemicals (Osaka, Japan).

Results

Anaerobic wax ester production in the presence of rotenone and CCCP

When aerobically grown *Euglena* cells were transferred to anaerobic conditions, wax ester fermentation took place and amounts of wax esters (approximately 40 μg/10^6 cells) were produced during anaerobic incubation for 24 h. To explore whether the mitochondrial electron transport chain participates in anaerobic wax ester production, rotenone was added at 50 μM to inhibit mitochondrial complex I. In the presence of rotenone, the level of anaerobically produced wax esters reduced to <50% of that in control cells (Fig. 1). This result suggests that mitochondrial complex I is involved in wax ester fermentation. In contrast, an uncoupler, CCCP (10 μM), stimulated anaerobic wax ester production, and the amount of wax esters accumulated increased 1.7-fold compared to the control.

When the constituent fatty acids and alcohols of wax esters produced by anaerobic *Euglena* cells were analyzed after saponification, odd-numbered fatty acids and alcohols were detected in large quantities (40% and 35% of total, respectively), as has been reported previously [6,7]. As shown in Fig. 2, when CCCP was added during anaerobic incubation, the proportions of odd-numbered fatty acids and alcohols decreased both to <15%. In addition, the average carbon chain lengths of the constituent fatty acids and alcohols became slightly longer in the presence of either rotenone or CCCP. Propionyl-CoA is used as a primer for the synthesis of odd-numbered fatty acids

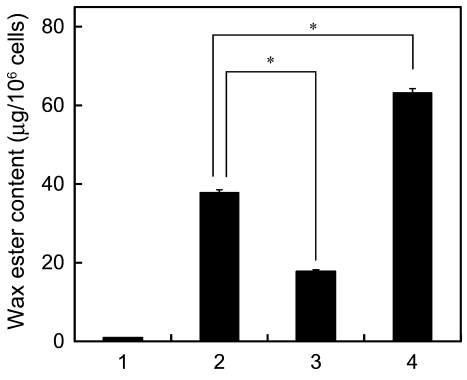

Fig. 1. Effects of rotenone and CCCP on anaerobic production of wax esters. Cells cultured aerobically for 4 days were transferred to anaerobic conditions, and wax ester content was determined before (bar 1) or after (bars 2, 3, 4) anaerobic incubation for 24 h in the absence (bar 2) or presence of rotenone (bar 3) or CCCP (bar 4). Data are presented as means ± SD ($n = 3$). *$P < 0.05$.

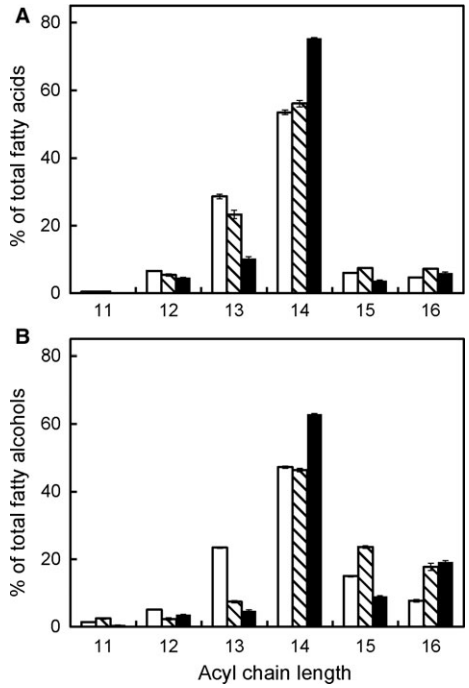

Fig. 2. Fatty acid and alcohol moieties composed of anaerobically produced wax esters in the presence of rotenone and CCCP. Cells cultured aerobically for 4 days were incubated under anaerobic conditions for 24 h in the absence (open bars) or presence of rotenone (shaded bars) or CCCP (solid bars). Lipids were extracted from the cells, wax esters purified from the lipid extract were hydrolyzed, and the fatty acids (A) and alcohols (B) were analyzed by gas–liquid chromatography. Data are presented as means ± SD ($n = 3$).

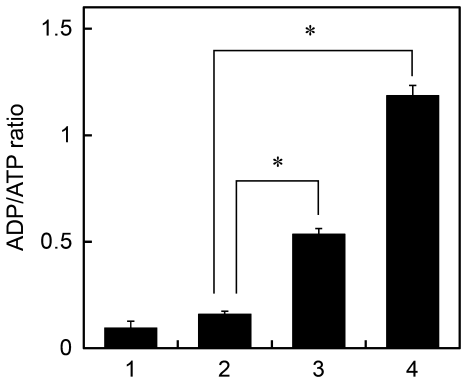

Fig. 3. The ADP/ATP ratio in the cells incubated under anaerobic conditions in the presence of rotenone or CCCP. Cells cultured aerobically for 4 days were transferred to anaerobic conditions, and ADP/ATP ratio in the cells was determined before (bar 1) or after (bars 2, 3, 4) anaerobic incubation for 24 h in the absence (bar 2) or presence of rotenone (bar 3) or CCCP (bar 4). Data are presented as means ± SD ($n = 3$). *$P < 0.05$.

instead of acetyl-CoA [16]. In *E. gracilis*, propionyl-CoA has been reported to be synthesized from phosphoenolpyruvate through oxaloacetate, fumarate, succinate, and methylmalonyl-CoA, and fumarate reductase is thought to be involved in the conversion from fumarate to succinate in this pathway [17]. Hence, it is expected that propionyl-CoA synthesis is influenced by rotenone and CCCP because mitochondrial complex I participates in the electron transfer from NADH to fumarate during the fumarate reductase reaction. However, it remains unclear why CCCP (but not rotenone) induced large decreases in the proportions of odd-numbered fatty acids and alcohols in wax esters produced in anaerobic *Euglena* cells.

ADP/ATP ratio in the anaerobic cells in the presence of rotenone and CCCP

The ratio of ADP/ATP in *Euglena* cells did not change significantly under anaerobic incubation for 24 h (Fig. 3), confirming that ATP can be produced by wax ester fermentation at a sufficient level in these cells. However, the ADP/ATP ratio greatly increased in cells exposed to anaerobic conditions in the presence of rotenone. A further increase in the ADP/ATP ratio was observed in the presence of CCCP. These results suggest that, in anaerobic *Euglena* cells, ATP is synthesized by mitochondrial complex V (ATP synthase) using a proton motive force developed by the electron flow through complex I.

Participation of ETF, RQ, and acyl-CoA dehydrogenase in anaerobic wax ester production

To further clarify the function of the mitochondrial electron transport chain in wax ester fermentation, a gene silencing experiment was performed that targeted ETF, which links acyl-CoA dehydrogenase to the electron transport chain. Gene silencing of either alpha or beta subunit of ETF, which was confirmed by semi-quantitative RT-PCR (Fig. S1A), did not show any significant effects on the growth of *Euglena* cells under aerobic conditions (data not shown). However, the production of wax esters was significantly suppressed in both of these gene-silenced cell lines when they were incubated anaerobically (Fig. 4A). These results suggest that ETF, probably together with acyl-CoA dehydrogenase, participates in mitochondrial fatty acid synthesis in anaerobic *Euglena* cells.

It is well known that RQ, which has a more negative redox potential than UQ, is used to transfer electrons from mitochondrial complex I to fumarate reductase

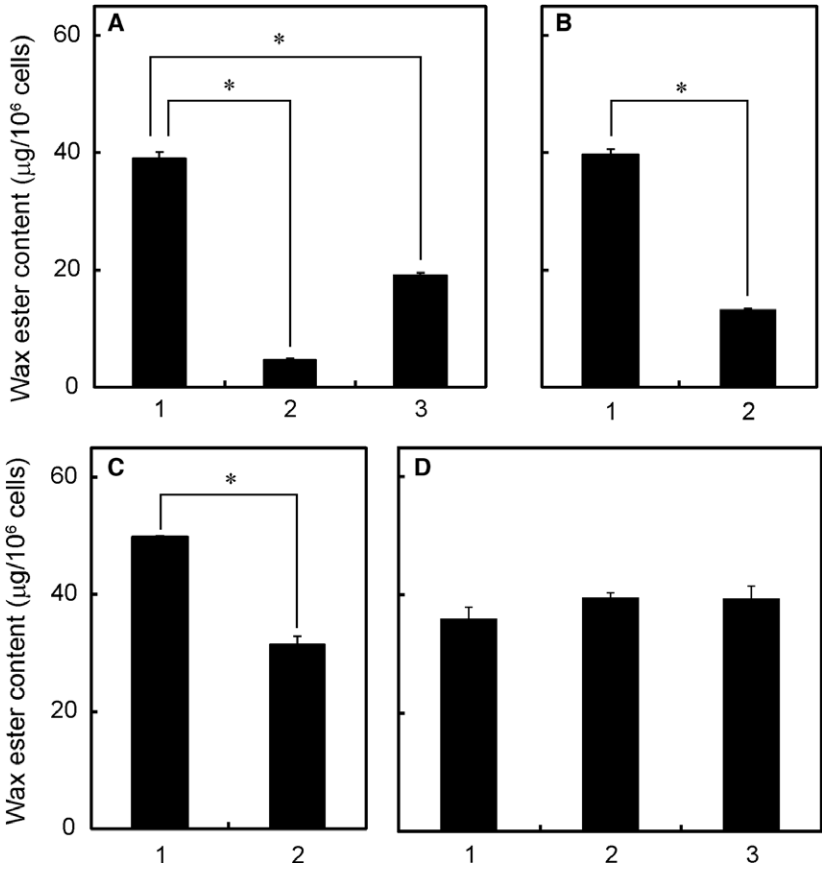

Fig. 4. Participation of ETF, RQ, and acyl-CoA dehydrogenase in anaerobic wax ester synthesis. A: ETF alpha (*etfalpha*) or beta (*etfbeta*) subunit gene was silenced by introducing the corresponding dsRNA (ETF-dsRNA-1 and ETF-dsRNA-2, respectively) into cells. The *etfalpha*- (bar 2) or *etfbeta*- (bar 3) silenced or control (bar 1) cells were cultured aerobically for 4 days and then incubated under anaerobic conditions for 24 h, and wax ester content was determined. B: The RQ biosynthesis gene A (*rquA*) was silenced by introducing the corresponding dsRNA (RQUA-dsRNA) into cells. The *rquA*-silenced (bar 2) or control (bar 1) cells were cultured under aerobic conditions for 4 days, incubated under anaerobic conditions for 24 h, and wax ester content was determined. C: The acyl-CoA dehydrogenase gene (*acd1*) was silenced by introducing the corresponding dsRNA (ACD1-dsRNA) into cells. The *acd1*-silenced (bar 2) or control (bar 1) cells were cultured under aerobic conditions for 3 days, incubated under anaerobic conditions for 24 h, and wax ester content was determined. D: The *trans*-2-enoyl-CoA reductase gene (*ter*) was silenced by introducing the corresponding dsRNA (TER-dsRNA-1 or TER-dsRNA-2) into cells. The *ter*-silenced cells with TER-dsRNA-1 (bar 2) or with TER-dsRNA-2 (bar 3) or control cells (bar 1) were cultured under aerobic conditions for 3 days, incubated under anaerobic conditions for 24 h, and wax ester content was determined. Data are presented as means ± SD ($n = 3$). *$P < 0.05$.

during anaerobic respiration [1–3,17]. In addition, it is also known that *Euglena* cells contain RQ_9 together with UQ_9 [18]. Previously, a gene (*rquA*) encoding putative methyltransferase involved in the synthesis of RQ from UQ was identified in *Rhodospirillum rubrum* [19], and its ortholog was found in *Euglena* RNA-Seq database [12]. When *Euglena rquA* was silenced, total RQ_9 content (sum of quinone and quinol forms) was greatly reduced, whereas total UQ_9 content was slightly increased (Table 2). This is the first direct evidence showing that the eukaryotic rhodobacter homologs of *rquA* actually participate in the synthesis of RQ. The anaerobic production of wax esters in *rquA*-silenced cells was attenuated to one-third of the control cells (Fig. 4B). These results suggest that RQ_9 (rather than UQ_9) mainly functions to mediate electron transfer

Table 2. Ubiquinone-9 (UQ_9) and rhodoquinone-9 (RQ_9) contents in the *rquA*-silenced cells. Data are presented as means ± SD ($n = 3$).

	Total UQ_9 content	Total RQ_9 content
	(pmol/10^6 cells)	
Control	86.7 ± 3.44	51.6 ± 3.44
rquA-silenced	104 ± 2.42	1.10 ± 0.364

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between complex I and ETF in mitochondrial fatty acid synthesis in anaerobic *Euglena* cells.

The *Euglena* RNA-Seq database [12] suggests that there are at least seven ETF-linked acyl-CoA dehydrogenase orthologs. One of these (named *acd1*), which has a mitochondrial targeting sequence and is expressed the most abundantly, was silenced by introducing its dsRNA in *Euglena* cells to confirm that acyl-CoA dehydrogenase is involved in mitochondrial fatty acid synthesis. As shown in Fig. 4C, anaerobic wax ester production in *acd1*-silenced cells was lowered to 60% than that in the control. In contrast, when NAD(P)H-dependent *trans*-2-enoyl-CoA reductase that can utilize crotonyl-CoA and *trans*-2-hexenoyl-CoA [9] was knocked down by gene silencing, anaerobic wax ester production was not affected (Fig. 4D) although the enzyme protein, which was examined by western blotting, disappeared almost completely in the gene-silenced cells (Fig. S2). Recently, *trans*-2-enoyl-CoA reductase has been shown to be important in chloroplast development under aerobic conditions rather than in anaerobic wax ester synthesis (T. Tomiyama, K. Goto, Y. Tanaka, T. Maruta, T. Ogawa, Y. Sawa, T. Ito, T. Ishikawa, unpublished data), although this enzyme is reportedly localized in the mitochondria [9].

Discussion

The data presented here show that acyl-CoA dehydrogenase mainly functions to reduce *trans*-2-enoyl-CoA during mitochondrial fatty acid synthesis in *E. gracilis* under anaerobic conditions and that electrons for reduction are provided by NADH via complex I, RQ and ETF (Fig. 5). These data also strongly suggest that during wax ester fermentation, *Euglena* cells produce ATP by anaerobic respiration coupled with mitochondrial fatty acid synthesis, in addition to substrate-level phosphorylation in the cytosolic glycolytic pathway. Therefore, more amounts of ATP can be obtained via wax ester fermentation compared with that via common lactate or ethanol fermentation.

The anaerobic respiration occurring in *E. gracilis* seems to resemble that reported in *Ascaris* (giant roundworm) [2,20,21]. In *Ascaris* under anaerobic conditions, acetyl-CoA and propionyl-CoA (or two molecules of propionyl-CoA) are condensed and subsequently reduced in mitochondria, and 2-methylbutanoate (or 2-methylpentanoate) is synthesized as an end product. In this process, ATP is produced by anaerobic respiration linked to the reduction in 2-methyl branched-chain enoyl-CoA, in which electrons are provided by NADH via mitochondrial complex I, RQ, and ETF. However, in contrast to *Ascaris* mitochondria in which the anaerobic end products, 2-methylbutanoate and 2-methylpentanoate, are short-chain fatty acids, in *Euglena* mitochondria, long-chain fatty acids, mainly myristic acid, are synthesized. Therefore, to the best of our knowledge, this is the first report showing that anaerobic respiration occurs coupled with long-chain fatty acid synthesis by the reversal of β-oxidation.

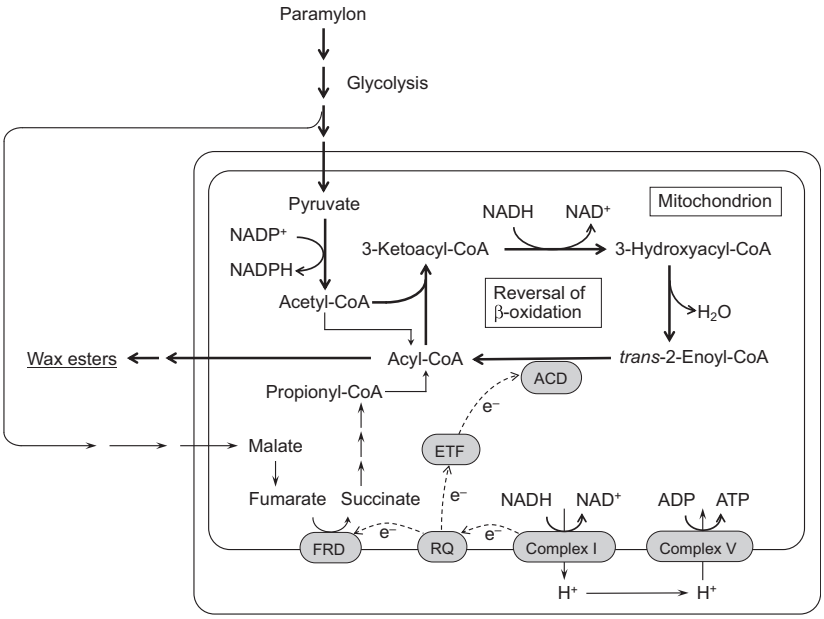

Fig. 5. Mitochondrial fatty acid synthesis by the reversal of β-oxidation linking with ATP synthesis by anaerobic respiration in *Euglena gracilis*. Abbreviations: ACD, acyl-CoA dehydrogenase; ETF, electron transfer flavoprotein; FRD, fumarate reductase; RQ, rhodoquinone.

Algal biofuel is considered to be an attractive alternative to fossil fuels. Biofuel produced from *Euglena* wax esters exhibits good cold flow properties with high oxidative stability and is proposed to be suitable to use as a drop-in jet fuel [5,7]. For industrial applications, however, it will be necessary to improve the productivity of wax esters from anaerobic *Euglena* cells. In this context, the results obtained in the present study will be valuable; in particular, mitochondrial uncoupling reagents, such as CCCP, could be useful tools for improving productivity. Anaerobic *Euglena* cells produce wax esters from paramylon to obtain ATP; therefore, the rate of wax ester production should be controlled by the amount of ATP required in cells. CCCP acts as an ionophore, causes a decrease in proton motive force developed in the mitochondrial inner membrane, and reduces ATP synthesis by anaerobic respiration, in contrast to rotenone, which directly inhibits mitochondrial complex I. Therefore, mitochondrial fatty acid synthesis would not be suppressed even in the presence of CCCP because electron flow through complex I is not inhibited. To compensate for a decrease in ATP synthesized by anaerobic respiration, glycolysis may accelerate to increase ATP synthesis by substrate-level phosphorylation. Consequently, the anaerobic production of wax esters should be greatly accelerated in the presence of CCCP, providing a possible route toward unraveling the potential of this organism as a renewable energy resource.

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Author contributions

All members conceived and designed the experiments, and discussed the results for the completion of the manuscript. MN, HA, AN, and TO performed the experiments and analyzed the data. KS performed quinone analysis. MN and HI wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Semiquantitative RT-PCR analysis to determine the degree of gene silencing.

Fig S2. Immunodetection of *trans*-2-enoyl-CoA reductase protein. C, without dsRNA; 1, TER-dsRNA-1 introduced; 2, TER-dsRNA-2 introduced.

Table S1. Oligonucleotide primers and amplification cycle numbers for semiquantitative RT-PCR.