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Divergence of the mitochondrial electron transport chains from the green alga *Chlamydomonas reinhardtii* and its colorless close relative *Polytomella* sp.

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Abstract

Compelling evidence exists that the colorless algae of the genus *Polytomella* arose from a green *Chlamydomonas*-like ancestor by losing its functional photosynthetic apparatus. Due to the close relationship between the colorless and the green chlorophyte, *Polytomella* sp. appeared as a useful indicative framework for structural studies of *Chlamydomonas reinhardtii* mitochondria. However, comparative studies reported here unexpectedly revealed significant differences between the mitochondrial respiratory systems of the two algae. Two-dimensional blue native/SDS-PAGE of isolated mitochondria indicated that cytochrome-containing respiratory complexes III and IV in the two chlorophytes contrast in size, subunit composition and relative abundance. Complex IV in *Polytomella* is smaller than its counterpart in *C. reinhardtii* and occurs in two forms that differ presumably in the presence of subunit COXIII. The cytochrome *c* and the iron–sulfur Rieske protein of both chlorophytes revealed structural differences on the amino acid sequence level. Under comparable culture conditions, the colorless alga exhibits lower levels of cytochrome *c* and complex IV but a higher respiratory activity than the green alga. Cytochrome *c* levels were also found to be differently regulated by the growth conditions in both algae. The divergence between the respiratory systems in the two related chlorophytes can be viewed as a consequence of the loss of photosynthetic activity and/or of the adaptation to the environment via the acquisition of a more flexible, heterotrophic metabolism. Our understanding of mitochondrial function and evolution is expected to be greatly enhanced via further parallel studies of photosynthetic/non-photosynthetic algae, for which this study forms an incentive.

Keywords: Blue native PAGE; Chlorophyte; Heterotrophic lifestyle; Mitochondrial cytochrome; Photosynthesis; Respiratory chain

1. Introduction

Unicellular colorless algae are found the world over in a myriad of soils and aqueous habitats. Original descriptions of colorless species can be found as far back as the late 19th century. A few colorless algae have long been described as non-photosynthetic equivalents of photosynthetic algae since they share morphological and reproduction characteristics with green algae and possess plastid-related organelles that contain starch granules. The algae from the genus *Prototheca* for example were proposed to be relatives of the green algae *Chlorella* [1] and the species *Polytoma* and *Polytomella* are regarded as colorless species of the order Volvocales [2]. It has been hypothesized that some colorless algae derived from photosynthetic algal ancestors by losing a functional photosynthetic apparatus [3]. The evolutionary link between some colorless and green species has been

Abbreviations: AOX, alternative oxidase; BN-PAGE, blue native polyacrylamide gel electrophoresis; Cyt, cytochrome; ISP, iron–sulfur protein; MPP, mitochondrial processing peptidase; OXPHOS, oxidative phosphorylation; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride

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confirmed by phylogenetic sequence analysis of nuclear small-subunit (SSU) rRNA genes [4,5], mitochondrial DNA or remnant plastid DNA. For instance, fragmentation and scrambling of the mitochondrial ribosomal RNAs occur similarly in the colorless alga *Polytomella parva* [6] and in the green algae *Chlamydomonas reinhardtii* and *C. eugametos* [7,8]. Also, both *Polytomella* and *Chlamydomonas* species have a reduced size mitochondrial DNA which codes for a limited set of respiratory proteins [9,10].

The events that have led to the supposed loss of photosynthesis and the emergence of the colorless algae bear a speculative character in part due to the limited number of studied colorless species. Among the different scenarios invoked are: (i) adaptation to a long-term heterotrophic lifestyle; (ii) appearance of mutations that have irreversibly affected photosynthetic ability or (iii) acquisition of foreign genes by lateral gene transfer.

The loss of the chloroplast is expected to have had an impact on the mitochondrion as a whole. Indeed, mitochondria are crucial for optimal chloroplast metabolism: In photosynthetic organisms, these organelles possess several specific enzymes which play a key role under light conditions in balancing the cellular energy and redox status (for a review, see [11]). So far, comparative studies dedicated to the mitochondrial energy metabolism of the colorless algae and their green counterparts are scarce. At present, the duo of photosynthetic/non-photosynthetic algae best suited for mitochondrial comparative studies is represented by *C. reinhardtii* and *Polytomella* sp. Recent technical advances have allowed comprehensive studies on *C. reinhardtii* mitochondria: purification of mitochondria [12], micro-analytic methods to characterize mitochondrial proteins [13–15] and the sequencing of the *C. reinhardtii* nuclear genome [16,17]. Fact however remains that the study of the mitochondria of the green alga (and of photosynthetic organisms in general) is severely hampered by the presence of a chloroplast. *Polytomella*, which lacks chloroplasts, has proven useful to investigate certain aspects of the structure of respiratory complexes in the chlorophytes. Indeed, the oxidative phosphorylation (OXPHOS) systems in *C. reinhardtii* and *Polytomella* sp. share several unusual structural features: (i) a heterodimeric COX II subunit of complex IV (COXII) [14,18]; (ii) a decreased hydrophobicity of the nucleus-encoded COX III subunit of complex IV that could facilitate import into the mitochondrion [19]; and (iii) subunits α and β of complex V exhibit extensions on the N- and C-terminus, respectively [20–22]. The discovery of these shared unusual traits has strengthened the assumption that *Polytomella* species have evolved from a *Chlamydomonas*-like ancestor. Yet, significant differences between the mitochondrial electron transport chains of *C. reinhardtii* and *Polytomella* have been observed too. Cytochrome *b* (cyt *b*) of *Polytomella* complex III exhibits an α-absorption at 567 nm [23] which is 3–4 nm red-shifted compared to cyt *b* of *C. reinhardtii* [24,25] and of mammals and plants [26,27]. Also, in contrast to *C. reinhardtii* [12,28], *Polytomella* sp. lacks a typical SHAM-sensitive alternative oxidase (AOX) [29]. The origin of AOX is uncertain but its occurrence in a number of prokaryotes is suggestive of an endosymbiotic acquisition [30]. The absence of an AOX in *Polytomella* sp. may be related to the loss of photosynthetic metabolism or could be a consequence of newly acquired metabolic features or regulatory mechanisms. Indeed, unlike *C. reinhardtii*, *Polytomella* sp. is able to grow under a wide range of pH and in the presence of various carbon sources [31].

Here, we extend the investigation on the mitochondrial respiratory chains of the algae *C. reinhardtii* and *Polytomella* sp. Our results revealed unexpected differences in the structure and composition of the cyanide-sensitive respiratory chain of the evolutionary related algae. This interesting divergence provides grounds to further research efforts that aim to assess the impact on mitochondria of on one hand the loss of photosynthetic activity and on the other the consequences of newly acquired metabolic features or regulatory mechanisms.

2. Materials and methods

2.1. Algal strains, growth conditions, and mitochondria isolation

C. reinhardtii cell wall-less mutant strain 84CW15 was grown at 25–26 °C in continuous light and agitation on TAP medium [32] supplemented with 1% (w/v) sorbitol. *Polytomella* sp. (198.80, E.G. Pringsheim) was grown aerobically at room temperature on MAP medium which consists of 30 mM sodium acetate, 35 mM Mes, 1 mM potassium phosphate (pH 7.4), 7.4 mM NH_4Cl, 0.3 mM $CaCl_2$, 0.5 mM $MgSO_4$; trace elements and vitamins were added as in [31]. The pH of MAP medium was adjusted to 5.7–5.9 with KOH. Mitochondria were isolated from *C. reinhardtii* and *Polytomella* sp. cells in their exponential growth phase [12,31]. Due to the significant chlorophyll contamination, the mitochondria of *C. reinhardtii* were further purified on a Percoll gradient [12]. Protein concentration in isolated mitochondria was determined using the modified Lowry procedure [33] after protein precipitation with methanol/chloroform [34].

2.2. Protein electrophoresis and analysis

For BN-PAGE, mitochondrial proteins were solubilized in the presence of *n*-dodecyl-β-maltoside according to [14,15]. Blue native gels consisted of a separating gel (5–12% [w/v] acrylamide) and a stacking gel (4% [w/v] acrylamide) [14]. For second-dimension analysis, BN-PAGE lanes or excised gel pieces corresponding to the protein complexes of interest were applied onto either Glycine- or Tricine-SDS-PAGE. In order to achieve the separation of *Polytomella* subunits COXIIA and COXIIB

on Tricine-gels, 8 M urea was added to the resolving gel. 2D-gels were stained for proteins either with Coomassie brilliant blue or with silver or for cytochromes with the TMBZ/H_2O_2 method [35]. Apparent molecular masses were estimated using prestained molecular mass markers (Benchmark, Invitrogen). Electroblotting of proteins onto a nitrocellulose membrane was carried out as described [22]. Signals were visualized using the enhanced chemiluminescence system (SuperSignal® West Pico Kit, Pierce). The antisera used were raised against specific peptides of *Polytomella* sp. subunits COXIIA and COXIIB.

2.3. Isolation of Polytomella sp. cDNAs

A *Polytomella* sp. λZAPII cDNA library was screened for cytochrome *c* (cyt *c*) and iron–sulfur Rieske protein (ISP) using as heterologous probes the open-reading frame of the *C. reinhardtii* cyt *c* and ISP cDNAs. Sequencing services were provided by MWG Biotech using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems). The nucleotide sequences discussed in this paper were deposited in the DDBJ/EMBL/GeneBank nucleotide sequence database under the accession numbers AJ416378 (*Polytomella* sp. cyt *c* cDNA sequence) and AJ619758 (*Polytomella* sp. ISP cDNA sequence). Molecular mass and p*I* were calculated using the Compute pI/MW tool at www.expasy.org/tools/pi_tool.html.

2.4. Optical spectroscopy

Spectra were recorded at room temperature using a DW-2a UV/VIS SLM Aminco spectrophotometer modified with the OLIS DW2 conversion and the OLIS software (On-line Instrument System Inc.). Mitochondria were resuspended in 50 mM potassium phosphate buffer, pH 7.4 and sonicated 2×15 s before use. The reduction of mitochondrial cytochromes was done as described in the figure legend; spectra were recorded approximately 10 min after the addition of the reductants. For each batch of mitochondria, 5 spectra were recorded and averaged. The spectra shown are representative of 3 (*C. reinhardtii*) and 4 (*Polytomella* sp.) independent preparations of mitochondria. Cytochrome contents were determined from ascorbate-reduced minus air-oxidized spectra or dithionite-reduced minus air-oxidized spectra, using the following millimolar extinction coefficients: $\Delta_{550-540\ nm}$=14.5 $mM^{-1}cm^{-1}$ (cyt c+c_1); $\Delta_{563-575\ nm}$=28 $mM^{-1}cm^{-1}$ (*C. reinhardtii* cyt *b*); $\Delta_{567-575\ nm}$=28 $mM^{-1}cm^{-1}$ (*Polytomella* sp. cyt *b*) and $\Delta_{608-625\ nm}$=20 $mM^{-1}cm^{-1}$ (hemes a+a_3) [36].

2.5. Oxygen uptake measurements

Oxygen uptake by isolated mitochondria was monitored at 27 °C with a Clark-type oxygen electrode in 10 mM potassium phosphate, 0.1% fatty acid free BSA, 250 mM sorbitol, 10 mM KCl and 5 mM $MgCl_2$ (pH 7.2) [12]. The concentrations of the respiratory substrate and inhibitors are indicated in the legend of Table 2.

3. Results and discussion

3.1. Comparative BN-PAGE analysis of C. reinhardtii and Polytomella mitochondria

In this work, BN-PAGE was used to compare the mitochondrial complexes of the photosynthetic alga *C. reinhardtii* and its non-photosynthetic close relative *Polytomella* sp. Intact mitochondria were isolated from *C. reinhardtii* cells grown in the presence of light and acetate (mixotrophic conditions), and from *Polytomella* sp. cells grown on acetate as sole carbon source. The application of solubilized mitochondria to BN-PAGE allowed the separation of the major algal OXPHOS complexes which were identified as previously described [14,15]. The profile of the mitochondrial protein complexes of acetate-grown *Polytomella* cells on blue native gels (Fig. 1) was found to be similar to that of cells grown on ethanol at pH 6.0, reported earlier [15]. The dominant mitochondrial protein bands correspond to complex V (approximately 1700 kDa), complex I (1000 kDa), complex III (450 kDa) and a bifunctional aldehyde/alcohol dehydrogenase (ADHE) (200 kDa) (Fig. 1). Complex IV did not appear as a major band on BN-PAGE (Fig. 1) but was detected in the 150–180 kDa range by immunoblotting, using a specific antibody raised against COXIIA subunit (not shown).

BN-PAGE analysis revealed that the major OXPHOS complexes of *Polytomella* sp. migrate slightly faster than

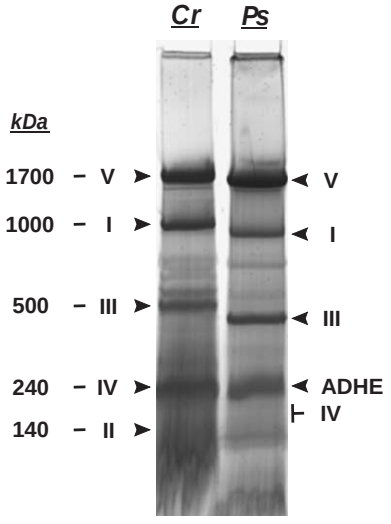

Fig. 1. BN-PAGE separation of *C. reinhardtii* and *Polytomella* sp. mitochondrial proteins. The total mitochondrial proteins (400 μg) from *C. reinhardtii* (Cr) and *Polytomella* (Ps) were solubilized in the presence of β-dodecyl-*n*-maltoside. Estimated molecular masses and identities of protein complexes are indicated. I–V refer to the OXPHOS complexes. ADHE indicates the position of the bifunctional aldehyde/alcohol dehydrogenase in *Polytomella* sp.

those of *C. reinhardtii* (Fig. 1). The same holds true for the OXPHOS complexes of bovine mitochondria as compared to those of *C. reinhardtii* [14]. Protein mobility on BN-PAGE is mainly determined by the level of bound Coomassie blue G dye, which is fairly proportional to the size of the protein complex. The different blue native electrophoretic mobility of the *Polytomella* and *C. reinhardtii* OXPHOS complexes thus indicates their distinct molecular mass but may in addition signify structural differences, when these affect the binding of Coomassie blue. BN-PAGE analysis also showed differences in the relative band intensity of the OXPHOS complexes in the two algae.

Blue native gel lanes containing mitochondrial proteins from both sources were applied on Glycine-SDS-PAGE. Coomassie blue-stained 2D gels showed clear differences in the relative levels of OXPHOS complexes in the two algae. With respect to *C. reinhardtii*, the mitochondria of *Polytomella* seem to exhibit lower complex I levels and higher complex V levels (Fig. 2A,B). 2D gels also revealed that in contrast to *C. reinhardtii*, complex IV subunits in *Polytomella* sp. were poorly visible (Fig. 2A,B). Immunoblot analysis of *Polytomella* 2D SDS-PAGE patterns, using specific antisera, showed that subunits COXIIA and COXIIB are present as a doublet (Fig. 2C). It follows that complex IV is resolved on BN-PAGE into two major forms, *L* (175 kDa) and *S* (150 kDa). This situation contrasts with that in *C. reinhardtii*, in which complex IV is present on BN-PAGE as a major monomeric form and a minor dimeric form [14]. Multiple forms of complex IV are commonly observed on BN-PAGE; besides monomers, complex IV is found associated in homodimers and in supercomplexes with other respiratory complexes [37].

3.2. Complex III in C. reinhardtii and Polytomella sp.: composition, origin and evolution

Complexes III from *C. reinhardtii* and *Polytomella* are resolved on BN-PAGE as homodimers of approximately 500 kDa and of 450 kDa, respectively (Fig. 1). In order to have insights into the distinct electrophoretic behavior of the algal complexes III, pieces of BN-PAGE containing the complexes of interest were analyzed on Glycine 2D SDS-PAGE (Fig. 3). In the 45–55 kDa range, where the core proteins are commonly found, *Polytomella* sp. complex III shows two subunits of 55 and 46 kDa (Fig. 3) corresponding to core 1 and core 2, respectively [38]. In contrast, *C. reinhardtii* complex III exhibits four bands of 53, 48, 47 and 46 kDa present in dissimilar stoichiometry (Fig. 3, [14]). Only the largest of these proteins is recognized by an antiserum raised against *Neurospora crassa* core 1 [14]. The presence of substoichiometric bands in *C. reinhardtii* complex III might suggest that the proteins of 47 and 46 kDa result from a limited proteolysis of the 48 kDa protein. Nevertheless, two facts argue against this: (i) the same protein pattern has been observed when *C. reinhardtii* complex III was purified by anion-exchange chromatography [24,39] and (ii) the ISP known to be subject to proteolytic cleavage [40] was not affected. The occurrence

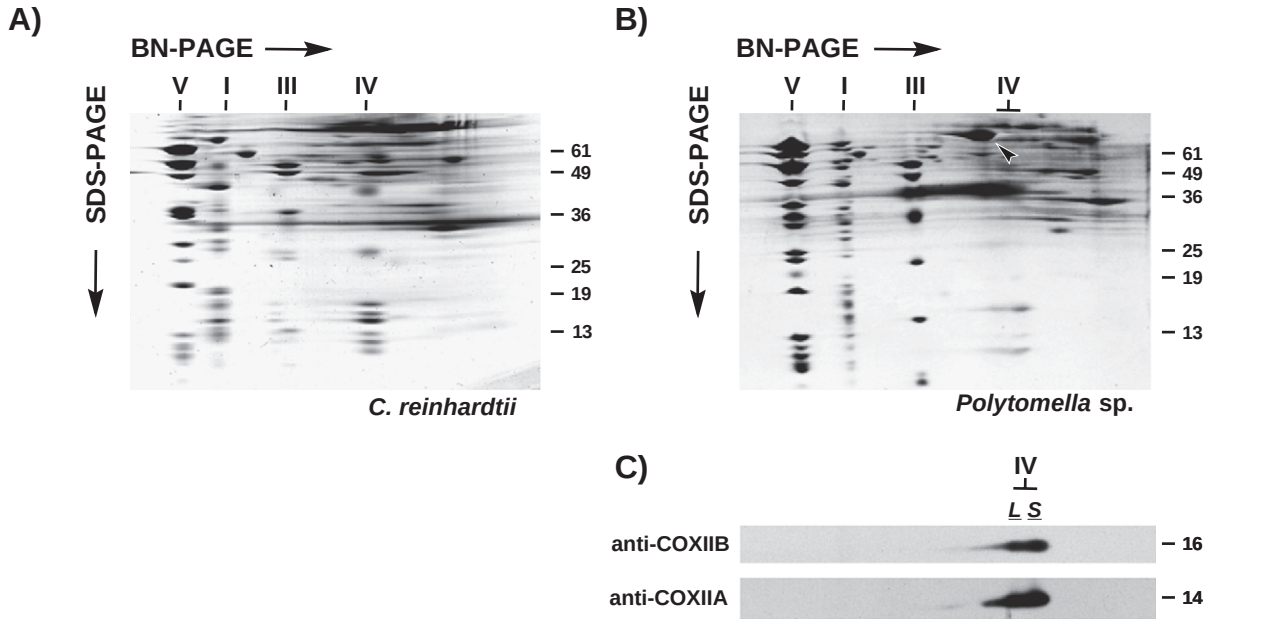

Fig. 2. Two-dimensional resolution of the mitochondrial protein complexes from *C. reinhardtii* and *Polytomella* sp. (A, B) BN-PAGE lanes loaded with 1 mg of total mitochondrial proteins were placed horizontally on a 13.5% acrylamide Glycine SDS-gel for subsequent resolution of protein complexes into their respective subunits. 2D SDS-polyacrylamide gels were stained with Coomassie Brilliant blue R250. (C) Mitochondrial proteins from *Polytomella* sp. separated on 2D BN/Tricine SDS-PAGE in the presence of urea were transferred onto nitrocellulose membrane and probed with specific antibodies raised against *Polytomella* subunits COXIIA and COXIIB. *L* and *S* refer to the large and small forms of *Polytomella* sp. complex IV. The numbers of the right of the three panels indicate the molecular mass of the standard proteins (in kDa).

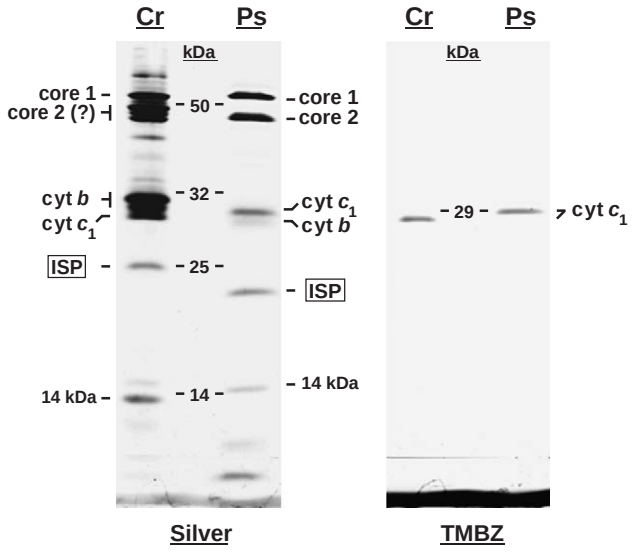

Fig. 3. Subunit composition of complex III from *Polytomella* sp. and *C. reinhardtii*. 2D resolution of chlorophyte complex III after BN-PAGE on a 12% polyacrylamide Glycine SDS-PAGE. Size of standard proteins is given in the middle of the panels. Subunit identification is based on previous works [14,38,39]. Left panel, silver-stained SDS-PAGE; right panel, TMBZ stained gel.

of multiple bands in the core region might also indicate that the core proteins in *C. reinhardtii* are encoded by several genes, as it is the case in photosynthetic eukaryotes [41,42]. In plants, the core proteins form the mitochondrial processing peptidase (MPP), which specifically cleaves off the target sequence of precursor proteins upon their import into the mitochondria (core1, β-MPP; core 2, α-MPP) [43]. In mammals and yeasts however, the β- and α-MPP subunits form a soluble matrix-located complex with the core proteins proteolytically inactive. In *Polytomella*, complex III lacks MPP activity [44] whereas in *C. reinhardtii* this activity seems, at least in part, to be soluble [45]. This is interesting since *C. reinhardtii* core 1 protein (53 kDa) (JGI, Chlamydomonas v2.0/C_1290016) was projected to be proteolytically active; the protein assigned as core 2 (48 kDa) (C_490077) however lacks the consensus sequence for α-MPP subunits [14]. Additional searches of the *C. reinhardtii* genome (available at http://genome.jgi-psf.org/chlre2/chlre2.home.html) and EST databases using the core 2 (α-MPP) sequence of potato (P29677) yielded a gene that encodes a precursor protein of approximately 53 kDa (C_10187); the predicted protein shows 42% identity to plant α-MPP subunits and possesses an almost complete α-MPP consensus sequence (not shown). This protein may play part in the abovementioned soluble MPP activity but could also represent a (substoichiometric) band near the position of core 2 on SDS-PAGE. As for core 1, recent *C. reinhardtii* genome searches did not provide evidence for any isomers or other mitochondrial β-MPP proteins. Protein sequencing of the bands at the core 2 position in *C. reinhardtii* complex III and further proteomic studies with mitochondria will be required to clarify the situation. Anyhow, in view of the divergence between the green and the colorless alga, our data put forward the differences in the evolution of the core proteins in two closely-related chlorophytes.

In the 30 kDa region where the cytochromes of complex III usually migrate on SDS-PAGE, three bands could be distinguished in *C. reinhardtii* for only two bands in *Polytomella* sp. (Fig. 3). SDS-PAGE stained for hemes allowed the unequivocal assignment of the algal cyt c_1 (Fig. 3, right panel). The poorly visible protein band below cyt c_1 in *Polytomella* corresponds to cyt *b* (Fig. 3; [38]). In *C. reinhardtii,* it is not known which of the two bands found above cyt c_1 represents cyt *b*; in any case, *Polytomella* cyt *b* appears to have a higher mobility than that of *C. reinhardtii* (Fig. 3). Amino acid sequences of cyt *b* of both algae predict very similar molecular masses but indicate two amino acid substitutions (A36S, W114Y) in the close environment of the heme b_H of *Polytomella* sp. [25]. These substitutions are nevertheless unlikely to account for the distinct cyt *b* behavior on SDS-PAGE. Also the iron–sulfur Rieske protein (ISP) of the colorless alga runs faster on SDS-PAGE than its counterpart in the green alga (Fig. 3). The *Polytomella* ISP is encoded as a precursor protein of 253 amino acids that exhibits 64% sequence identity to its equivalent in *C. reinhardtii*, with the highest identity in the C-terminal region containing the conserved residues required for the binding of the [2Fe–2S] clusters (Fig. 4A). Upon import of the nucleus-encoded ISP precursor into the mitochondria, its targeting sequence is cleaved off by the MPP [46]. The residues that surround the cleavage site in *C. reinhardtii* ISP [39] are also found in *Polytomella* ISP (Fig. 4A), suggesting a conserved processing site. On this assumption, the mature *Polytomella* ISP would be smaller, attributable to a gap of 11 residues at the N-terminus (Fig. 4A). The shorter N-terminus of *Polytomella* ISP is not expected to have a strong effect on the protein electron transfer properties since it is located far-off from the structural features important for the ISP function: the "motifs I and II" and the "pro-loop" [47]. The crystal structure of bovine heart bc_1 complex [48,49] indicates that the soluble N-terminal domain of the ISP is positioned away from the heme b_H. A link between the distinct features of the ISP and cyt *b* in *Polytomella* sp. can thus not readily be envisioned. Taken together, the differing apparent molecular masses of cyt *b* and ISP in the two algae may be a factor in the mobility of complex III on BN-PAGE.

Multiple sequence alignment of mature ISPs from various sources shows that the matrix-located N-terminal domain of the mature ISP is shorter in non-photosynthetic organisms (Fig. 4B). Alignments made with the Clustal W 1.82 [50] and T-coffee [51] software were similar and define a first, short conserved domain at the N-terminal end of the protein followed by a gap of 12–14 residues in non-photosynthetic organisms (Fig. 4B). A longer N-terminal domain in photosynthetic eukaryotes raises the issues of the localization and the potential role of this

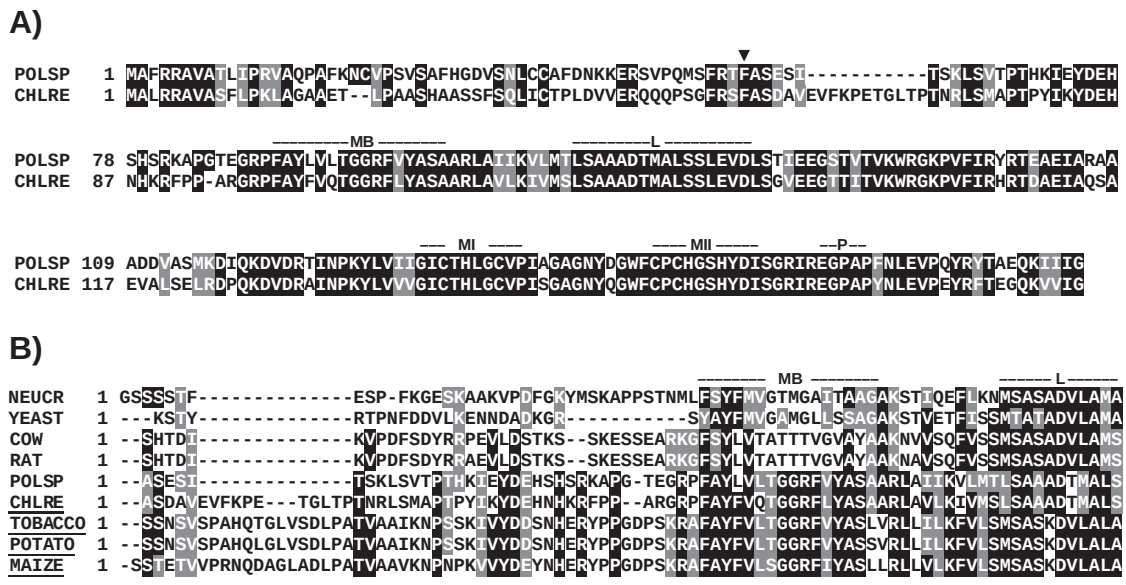

Fig. 4. Sequence alignment of mitochondrial iron–sulfur Rieske (ISP) proteins. (A) Sequence alignment of mitochondrial ISP precursors from *Polytomella* (POLSP) and *C. reinhardtii* (CHLRE). ▼ indicates the cleavage site in *C. reinhardtii* ISP precursor [39]. The [2Fe–2S]-cluster binding domains (Motif I and Motif II) and the polyproline loop (P) are indicated above the sequences [47]; L, predicted linker sequence that connects the transmembrane anchor (MB) to the extrinsic carboxy-terminal domain. Molecular masses estimated for the predicted mature apoproteins are 21.6 kDa for *Polytomella* sp. and 22.7 kDa for *C. reinhardtii*. (B) Multiple sequence alignment of the N-terminal domain of mitochondrial ISP from non-photosynthetic and photosynthetic (underlined) organisms. The sequences used for comparison are: POLSP, *Polytomella* sp. (AJ619758), CHLRE, *C. reinhardtii* (CAC86460), YEAST, *S. cerevisiae* (A29318), NEUCR, *N. crassa* (P07056), tobacco (P49729), potato (P37841), maize (A41607), cow (AAA30515) and rat (A32296). Sequence alignments were generated with CLUSTALW 1.82 (available at www.ebi.ac.uk/ClustalW). Identical residues are surrounded by black, conservative replacements are surrounded by grey.

additional domain within the complex. In mammals, the ISP precursor is processed by the matrix-located MPP whereas in plants, the core proteins fulfill this function [43,44]. Viewing the crystal structure of bovine heart bc_1 [48], the N-terminal domain of the ISP reaches into the interior of the core 1 protein. A longer N-terminus of mature plant ISP might thus be required for an effective processing of the ISP precursor by MPP integrated into complex III. It would be informative to test the processing efficiency of plant complex III toward non-plant ISP and *C. reinhardtii* ISP, which exhibits an N-terminal domain distinct from that of plant ISP (Fig. 4B).

On the assumption that the redox proteins in the chlorophyte complexes III share a common ancestral origin, the sequence data suggest that specific molecular events (mutation and/or deletion) have occurred in the ISP and cyt *b* genes after the separation of the colorless lineage from the green ancestor. Whereas the extended N-terminus of the ISP and the core protein heterogeneity may be seen as photosynthetic traits that were lost in *Polytomella*, the two amino acid substitutions (A36S, W114Y) in the cyt *b* are more difficult to appraise. In fact, both substitutions were found hitherto only in the colorless alga *P. parva* (AAL65271) whereas the green alga *Chlamydomonas eugametos* (AAC39350) exhibits only the first one. It could be imagined that the substitutions in the colorless alga cyt *b* are more specific adaptations to the different mechanistic and/or structural environment of complex III that have arisen after the acquisition of alternative metabolic pathways.

3.3. Oligomerization and stability of complex IV in *C. reinhardtii* and *Polytomella* sp.

Where *C. reinhardtii* complex IV runs mainly as a monomer with a small dimeric portion on BN-PAGE, *Polytomella* complex IV exhibits two monomeric forms of approximately 175 kDa (*L*) and 150 kDa (*S*) (Fig. 2). The algal complexes IV were further analyzed by the application of the corresponding blue native gel bands on Tricine-SDS-PAGE. *C. reinhardtii* complex IV was resolved into 10 distinct subunits (Fig. 5), most of which have been identified by N-terminal sequencing [14]. *Polytomella* complex IV (*L*-form) was resolved into eight distinct subunits (Fig. 5), of which those that compose the core of the respiratory complex, i.e. subunits COXI, COXIIA, COXIIB, and COXIII, have been unambiguously identified [18,19]. The molecular mass of the core is calculated to be of 100 kDa for both algae. Thus, the difference in apparent molecular mass observed on BN-PAGE between the *L*-form of *Polytomella* sp. complex IV and *C. reinhardtii* complex IV monomer (Fig. 1) may be ascribed to fewer and/or smaller subunits, as judged by the subunit patterns obtained on 2D gels (Fig. 5).

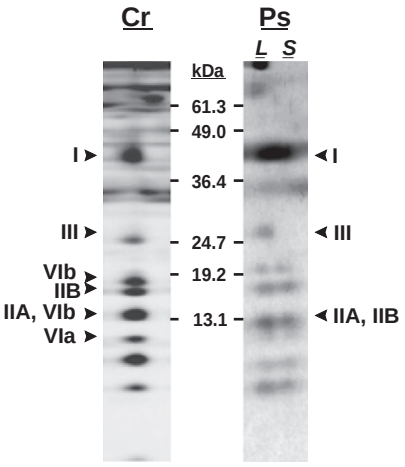

Fig. 5. Subunit composition of complex IV from *C. reinhardtii* and *Polytomella* sp. *C. reinhardtii* (Cr) and *Polytomella* sp. (Ps) complex IV separated on BN-PAGE were resolved on Tricine SDS-PAGE. Due to the low abundance of complex IV in mitochondria from *Polytomella* cells grown on acetate (Fig. 1), complex IV from the colorless alga was extracted from a BN-PAGE run with mitochondria isolated from cells grown on ethanol at pH 6.0 [15]. Subunit identification is based on previous works [14,18,19]. The size of standard proteins (in kDa) is given in the middle of the panel. *L* and *S* refer to the large and small forms of *Polytomella* sp. complex IV.

As compared to the *L*-form, the *S*-form of *Polytomella* complex IV lacks a 25 kDa protein whose absence could solely explain the difference in electrophoretic mobility on BN-PAGE. Yet, it cannot be ruled out that the *L*- and *S*-forms differ in other low molecular mass subunits that are not visible on SDS-PAGE or in cofactors that were lost during electrophoresis. On account of its size and migration on SDS-PAGE, the 25 kDa protein that is missing in the *S*-form of complex IV is predicted to be core subunit COXIII. Of note, two forms of complex IV (of 220 and 300 kDa) have also been observed with digitonin-solubilized mitochondria from bean (*Phaseolus vulgaris*); these forms however differ in the presence of one of the so-called "supernumerary" subunits, the COXVIb-type subunit [52]. As a membrane-bound subunit found at the junction between monomers in the dimeric form of the complex [53], COXIII is known to dissociate from the bacterial and mammalian complex by high salt and detergent concentrations [54,55]. The solubilization of mitochondrial protein complexes with dodecyl maltoside (or digitonin) for BN-PAGE is considered gentle and is not expected to lead to the dissociation of subunit COXIII from complex IV. Moreover, the unusual features of subunits COXIII (reduced hydrophobicity) [19] and COXII (heterodimeric nature) [18] cannot alone account for the abnormal behavior of *Polytomella* complex IV on BN-PAGE as they are shared by both *Polytomella* and *C. reinhardtii*. The different behavior of the chlorophyte complexes IV on BN-PAGE may thus arise from subunits in the close environment of the core of the complex or from a distinct lipid environment in the mitochondrial inner membrane. In that respect, high levels of mitochondrial α-hexadecadienoic acid have been reported in *Polytomella* [29]. It is known that variations in the lipid environment but also in other parameters such as pH and surface charge can induce conformational changes [56]. Different *in vivo* conditions in the two algae that relate to these parameters could cause structural differences that translate into a different stability (and activity) of the complex.

It is unclear whether the *L*- and *S*-forms of *Polytomella* complex IV observed on BN-PAGE result from inappropriate solubilization or BN-PAGE conditions or denote a heterogeneity in the mitochondrial membrane. The latter option is supported by the fair reproducibility of the proportion between the two forms; also, their occurrence increases on BN-PAGE when applied mitochondria were isolated from cells grown under different conditions (pH, carbon source) under which complex IV levels are higher ([15], unpublished results). If the different oligomeric forms of *Polytomella* complex IV do occur in vivo, it would be of great interest to determine their physiological relevance.

3.4. Structural divergence of cytochrome c_{550} in the chlorophytes

In mitochondria, cyt c_{550} is the electron transfer partner of complexes III and IV. A cDNA that encodes *Polytomella* sp. cyt c_{550} was isolated and sequenced (AJ416378). The 643-bp cDNA contained an open reading frame encoding a 112 amino acid protein with a predicted molecular mass of 11.8 kDa and a p*I* of 9.4. *Polytomella* sp. and *C. reinhardtii* cyt *c* share 85% identity; the identity includes the deviations from the general pattern of cyt *c* structure reported earlier [57] for residues 19 (T19V), 39 (Q/K39V) and 78 (T78N) (Fig. 6; horse heart cyt *c* numbering). Multiple sequence alignment indicates that *Polytomella* cyt *c* sequence differs from all eukaryote cyt *c* sequences available to date in the substitution of three highly conserved tyrosine residues (48, 74 and 97) by phenylalanine residues (●, Fig. 6). Of all three substitutions, the Y48F is probably the most interesting. First, the Y48F substitution is rare; it has hitherto been found in no other eukaryotic cyt *c* and in cytochrome c_2 of only two bacteria, the α-proteobacteria *Novosphingobium aromaticivorans* (ZP_00095539) and *Rhodopseudomonas palustris* (ZP_00008515). Second, residue Y48 forms a hydrogen bond to heme propionate-7 [58]. A Y48F variant form of *Saccharomyces cerevisiae* cyt *c*, obtained by site-directed mutagenesis, exhibits a decreased redox potential value (−27 mV) [58]. Based on this data, the redox potential of *Polytomella* sp. cyt *c* is predicted to be moderately lower than that of *C. reinhardtii*, determined earlier to be of +280 mV [24]. A lower redox potential may not have a major effect on the electron transport, but can be indicative of the adaptation of the cyt *c* to its physiological partners in the respiratory chain. Alternatively, a lower redox potential could indicate an adjustment of the cyt *c* to other/novel physiological partners yet to be identified.

Fig. 6. Amino acid sequence alignment of eukaryotic apocytochrome c_{550}. ▼, residues that are found only in *C. reinhardtii* and *Polytomella* sp. cytochrome *c* sequences; ●, highly conserved tyrosine residues that are substituted by phenylalanine residues in *Polytomella* sp. The sequences used for comparison are of POLSP, *Polytomella* sp. (PSP416378), CHLRE, *C. reinhardtii* (AAA33084); SPOLE, spinach (CCSP); YEAST, *S. cerevisiae* (CAA24605); ARATH, *Arabidopsis thaliana* (AAB72175); PLAFA, *Plasmodium falciparum* (AAN36650). The multiple sequence alignment was generated with CLUSTAL W 1.82.

3.5. Mitochondrial electron transfer chains and respiration in the acetate-grown chlorophytes

Typical dithionite-reduced minus air-oxidized difference spectra of the mitochondria from both algae are shown in Fig. 7. The α-region of the difference spectrum recorded with *C. reinhardtii* mitochondria exhibited absorption peaks at 550 and 608 nm and a shoulder at 563 nm. Based on previous spectroscopic data [24,59,60], the absorption peaks were assigned to mitochondrial cyt *c* (c_{550} and c_1 of complex III) and cyt *a* of complex IV, respectively, and the shoulder to cyt *b* of complex III. The difference spectrum of *Polytomella* sp. mitochondria exhibited three prominent absorption peaks at 553, 567 and 608 nm corresponding respectively to the absorption peaks of cytochromes *c*, *b* and *a* of the cyanide-sensitive respiratory pathway [18,23]. In both algae, the absorption peak of complex IV is shifted 3–5 nm to the red as compared to the corresponding absorption peaks in mammals [61] and plants [62,63]. The shift suggests an atypical environment for the hemes *a* in the chlorophytes and might be due to an unusual subunit structure, e.g. the heterodimeric nature of subunit COXII.

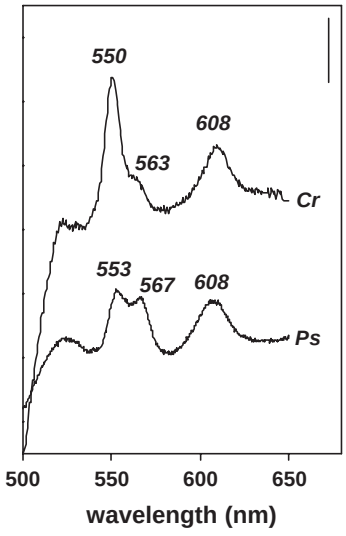

Fig. 7. Dithionite-reduced *minus* air-oxidized spectra of *C. reinhardtii* and *Polytomella* sp. mitochondria. Mitochondria were isolated from *C. reinhardtii* cells grown under mixotrophic conditions (Cr) and from *Polytomella* sp. cells grown on acetate at pH 6.0 (Ps). The mitochondria were resuspended in 50 mM potassium phosphate (pH 7.4) at a protein concentration of 3.0 mg/ml for *C. reinhardtii* and of 6.0 mg/ml for *Polytomella* sp. Reduction was achieved by adding a few grains of dithionite to the sample. Bar represents 0.02 absorbance unit.

Mitochondrial heme *b* contents calculated from the dithionite-reduced minus air-oxidized spectra were estimated to be of 0.14 nmol *b*/mg proteins in the green alga and of 0.12 nmol *b*/mg proteins in the colorless alga (Table 1). Heme *b* absorption in mitochondria results from the absorption of heme *b* of complex II (561 nm) and of b_L+b_H of complex III. In the case of *Polytomella* sp. mitochondria, the heme *b* absorption was calculated using the α-peak at 567 nm where the contribution of complex II heme *b* is likely to be low. Thus, the spectroscopic data point to similar amounts of complex III in the algae growing on acetate as carbon source. Ascorbate-reduced minus air-oxidized spectra were recorded (not shown) to determine the mitochondrial heme *a* and *c* contents in the chlorophytes. Contents in heme *a* in *Polytomella* were half those in *C. reinhardtii* (Table 1). On 2D BN/SDS-PAGE, *Polytomella* complex IV subunits were indeed poorly visible (Fig. 2), suggesting a similar trend. Nonetheless, a certain degree of complex IV dissociation upon the solubilization of the mitochondria cannot be ruled out. Spectroscopic data also indicated that the ratio cyt c_{550}/complex IV is 2.5- to 3.0-fold higher in the photosynthetic alga than in its non-photosynthetic counterpart. Loss of cyt *c* is unlikely since

Table 1
Heme contents of mitochondria isolated from *C. reinhardtii* and *Polytomella* sp.

	C. reinhardtii	*Polytomella* sp.
aa_3 (nmol mg^{-1})	0.24	0.11
b (nmol mg^{-1})	0.14	0.12
c (nmol mg^{-1})	1.02	0.17

Heme contents were estimated from the difference spectra using the absorption coefficients and wavelength pairs reported in Materials and methods. The values reported (in nmol/mg protein) are the means of 3 measurements for *C. reinhardtii* and 4 measurements for *Polytomella* sp. mitochondria.

Table 2
Succinate-sustained oxygen consumption by mitochondria isolated from *C. reinhardtii* and *Polytomella* cells following the addition of different inhibitors

	C. reinhardtii		*Polytomella* sp.	
	Activity[a]	% Inhibition	Activity[a]	% Inhibition
No inhibitor	80.0 (±5.5)	–	100.0 (±4.5)	–
KCN	28.0 (±2.5)	65.0	2.8 (±0.5)	97.2
AA	30.0 (±1.2)	62.5	3.0 (±0.2)	97.0
KCN+SHAM	2.0 (±0.1)	97.5	2.5 (±0.3)	97.5

[a] O_2 uptake by isolated mitochondria is expressed in nmolO_2/min./mg protein. Each value reported is the mean of 3 to 4 measurements. The respiratory substrate used was succinate (10 mM). The respiratory inhibitors used were: AA, Antimycin A (1 μM); KCN (1 mM), SHAM, salicylhydroxamic acid (2 mM). Protein concentrations were of 0.25 mg protein/ml assay buffer for *Polytomella* sp. mitochondria and of 0.3 mg protein/ml assay buffer for *C. reinhardtii* mitochondria.

the isolated mitochondria used in this work had respiratory control ratios ranging from 1.9 to 2.1 using glutamate/malate as respiratory substrate (not shown), consistent with their functional and structural intactness. Despite their significantly lower levels of cyt *c* and complex IV (Table 1), mitochondria isolated from the colorless alga exhibited succinate-sustained respiratory rates that were 20% higher than those from the green alga (Table 2). As shown by inhibitor studies, respiration on succinate in *Polytomella* involves only the cytochrome pathway (complexes III, IV, cyt *c*), whereas in *C. reinhardtii*, electrons from succinate oxidation can be routed to both the cyanide-sensitive and cyanide-insensitive pathways (Table 2). Since respiratory complexes possess a considerable compensation capacity [64], the observed difference in respiratory rates likely reflects a different structural organization or control of respiration in the algae rather than distinct capacities of the individual respiratory chain components. As judged by its relative higher oxygen uptake, the colorless alga seemingly acquired a more efficient respiratory electron transport chain that could represent adjustments related to, for example, the lack of AOX. In this respect, the mitochondrial lipid content may be of importance since it is known to be distinct in the two algae [29]. Lipids can affect not only the function of the protein complexes [56] but also their interactions: In yeast for example, the phospholipid cardiolipin is necessary for the association of complexes III and IV into supercomplexes [65]. The interactions between complex III, cyt *c* and complex IV could very well be an important determinant of the electron transport efficiency in the algae.

3.6. Growth conditions and steady-state fluctuations in mitochondrial cytochrome levels

This work provides a comparison of mitochondria isolated from the related algae grown on acetate and at similar pH. The distinct respiratory systems in the two algae may reflect their roles in divergent metabolic contexts in vivo. This is clearly illustrated by the differential accumulation of cyt *c* in the algae and by the fact that *Polytomella* can grow under a wide range of acidic pH and on various carbon sources, while *C. reinhardtii* cannot. In *Polytomella* sp., cyt *c* levels increase sharply with decreasing pH [31] whereas in *C. reinhardtii*, cyt *c* is upregulated in mixotrophic conditions as compared to autotrophic [66] and heterotrophic conditions (AA, RvL, unpublished results). The OXPHOS complexes in *Polytomella* sp. accumulated at lower pH or with growth on ethanol as opposed to growth on acetate, where the accumulation of cyt *c* was found to be 2–3 fold higher relative to that of the complexes [15]. However, the comparison of the visible spectra of isolated mitochondria from ethanol-grown cells at different pH [31] or grown on acetate (this work) indicated that the relative amounts of complexes III and IV did not change significantly (not shown). It seems therefore that cyt *c* in chlorophytes may have alternative functions to transporting electrons between complex III and IV. In mammals for example, cyt *c* is known to mediate extramitochondrial NADH oxidation [67] and the activity of sulfite oxidase [68]. Similar pathways may exist in the unicellular algae that could be regulated by environmental factors such as pH, light and carbon source. No data relating the cyt *c* content in the green alga with the content in respiratory complexes under variable environmental circumstances are available as yet.

3.7. Outlook

Polytomella sp. and *C. reinhardtii* are closely related species that share several highly unusual features of their mitochondrial proteins. However, experimental findings suggested differences in their mitochondria, such as the failure of *Polytomella* mitochondria to enter a 20% Percoll solution and the higher amounts of detergent required for their proper solubilization. Further studies showed a number of divergent structural features in the mitochondrial respiratory chains of both algae. On one hand, *Polytomella* sp. does not share with *C. reinhardtii* features that could be referred to as typical for mitochondria in photosynthetic organisms, such as the presence of an AOX [29], a longer N-terminal soluble domain of the ISP (this work), and tentatively, isoforms of core proteins (this work). Also, the major OXPHOS complexes of *Polytomella* exhibit lower apparent molecular masses on BN-PAGE than those of *C. reinhardtii* and plants [14,52]. One could speculate that smaller complexes in *Polytomella* sp. result from the loss or modification of several subunits important for the function and/or regulation of mitochondrial electron transport as part of photosynthetic metabolism. On the other hand, the mitochondria of the colorless alga *Polytomella* sp. exhibit characteristics which are so far unique in eukaryotes, such as an atypical cyt *b* absorption spectrum [23], an amino acid substitution in the proximity of the heme in cyt *c* (this work), and high steady-state levels of a mitochondrial bifunctional aldehyde/alcohol dehydrogenase under aerobic

conditions [15]. Altogether, these structural differences anticipate a complex evolution of the mitochondrial proteome in the chlorophytes. Evidence for the acquisition of foreign genes after the divergence of the colorless lineage has still to be reported. Therefore, the differences in algal mitochondrial protein sequences reported to date are more likely to result from gene rearrangements in both nuclear and mitochondrial compartments, and could be viewed as adjustments in a mitochondrial respiratory chain that shifted from a photosynthetic to a non-photosynthetic cell-context.

Further appraisal of the mitochondrial proteome of the algae as well as the sequencing of the nuclear genome of the colorless alga are desirable: It seems fit to re-investigate the colorless algae with modern genomic and proteomic tools. Broadening the range of colorless/green algae under scrutiny will expand the currently very limited knowledge on the interrelated processes of photosynthesis and mitochondrial respiration; establishing potential relationships between new metabolic features and the loss of photosynthesis will shed more light on the flexibility and evolution of respiratory chains.

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