

# Identification of prokaryotic homologues indicates an endosymbiotic origin for the alternative oxidases of mitochondria (AOX) and chloroplasts (PTOX)

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## Abstract

The alternative oxidase is a ubiquinol oxidase that has been found to date in the mitochondrial respiratory chain of plants, some fungi and protists. Because of its sparse distribution among eukaryotic lineages and because of its diversity in regulatory mechanisms, the origin of AOX has been a mystery, particularly since no prokaryotic homologues have previously been identified. Here we report the identification of a gene encoding a clear homologue of the mitochondrial alternative oxidase in an  $\alpha$ -proteobacterium, and the identification of three cyanobacterial genes that encode clear homologues of the plastid-specific alternative oxidase of plants and algae. These findings suggest that the eukaryotic nuclear genes for the alternative oxidases of mitochondria and chloroplasts were acquired via endosymbiotic gene transfer from the eubacterial ancestors of these two organelles, respectively.

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## 1. Introduction

Mitochondrial respiration in higher animals is strongly inhibited by cyanide, which blocks the cytochrome-mediated electron transport to oxygen at the level of complex IV (cyt *c* oxidase). In addition to typical mitochondrial cyanide-sensitive respiration, most higher plants, some fungi and some protozoa have long been known to possess an alternative oxidase (AOX), which is inhibited specifically by salicylhydroxamic acid (SHAM) (Schonbaum et al., 1971). First discovered in plant thermogenic tissues, AOX is an additional terminal oxidase; electron flux to AOX branches from the classical electron-transport chain at the level of the ubiquinone pool leading to the direct reduction of oxygen to

water (reviewed in Vanlerberghe and McIntosh, 1997). AOX-catalyzed O<sub>2</sub> reduction is not coupled to proton pumping and hence not to oxidative phosphorylation; the energy is released as heat. Apart from its role in thermogenic tissues, the function of this “energy-wasting” AOX in plants was long a puzzle, but is now considered to allow the plant to adapt its mitochondrial respiratory efficiency to ensure that the energy charge, and hence the growth rate of the plant, remains stable under variable environmental conditions (Macfarlane et al., 2002; Moore et al., 2002). An alternative function has been suggested in which AOX plays a role in the oxidative defence mechanism (Purvis, 1997; Möller, 2001).

Recently, it has been suggested that AOX is evolutionarily related to other di-iron proteins. The binuclear center, coordinated by two histidines and four glutamate residues, has been modelled within a four helix-bundle (Siedow and Umbach, 2000; Berthold et al., 2000). As di-iron proteins can activate molecular oxygen, it was suggested that AOX-mediated O<sub>2</sub>-scavenging could reduce

*Abbreviations:* AOX, mitochondrial alternative oxidase; PTOX, plastid alternative oxidase.

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the generation of reactive oxygen species, which could have been an early function of alternative oxidases (Gomes et al., 2001).

AOX is a low molecular weight mitochondrial protein (approximately 34 kDa) that is encoded in the nucleus. The enzyme is translated as a precursor protein with a mitochondrial targeting sequence that is removed during import into the mitochondrion.

In plant mitochondria, the cyanide-resistant oxidase is present as a disulfide-linked dimer protein. The activity of the oxidase *in vitro* is clearly regulated by a redox-sensitive intersubunit disulfide bond and also by  $\alpha$ -keto acids, pyruvate in particular (Umbach et al., 1994). Using site-directed mutagenesis, it was shown that a highly conserved cysteine residue located at the N-terminus of the plant protein is the site for both pyruvate activation and disulfide bond formation (Finnegan et al., 1997; Rhoads et al., 1998).

A cyanide-resistant oxidase has also been identified in the mitochondria of the unicellular photosynthetic alga *Chlamydomonas reinhardtii* (Dinant et al., 2001). In contrast to the situation in higher plants, *C. reinhardtii* AOX does not exhibit at its N-terminus the cysteine residue thought to participate in the regulatory dimerization of the plant enzyme and in the pyruvate activation (Dinant et al., 2001). The expression of the algal AOX seems to be influenced by the source of nitrogen, being greatly enhanced by nitrate and down-regulated by ammonium (Baurain et al., 2003).

Long thought to be specific to higher plants, AOX is now turning up also in many non-photosynthetic eukaryotes. It plays an important role in maintaining redox balance in blood stream-forms of trypanosomes (Chaudhuri et al., 1995). Cyanide-resistant respiration is also common among various lineages of fungi (Joseph-Horne et al., 2000). In contrast to plants, mitochondrial AOX activity in fungi is present as a monomer, and is not stimulated by pyruvate (Umbach and Siedow, 2000; Joseph-Horne et al., 2000) but is up-regulated by a high matrix ATP/ADP ratio (Joseph-Horne et al., 2000).

Recently, a SHAM-sensitive oxidase associated to thylakoid membranes was also identified in photosynthetic organisms. This plastid alternative oxidase (PTOX) is a component of an electron transport chain associated to carotenoid biosynthesis as well as the oxidase of the chlororespiratory pathway (Wu et al., 1999; Carol et al., 1999). PTOX oxidase is encoded by a nuclear gene, synthesized as a precursor protein containing an N-terminal extension which is proteolytically cleaved off after import into the chloroplast (Carol et al., 1999). So far, PTOX genes have been found in higher plants, in the chlorophyte *C. reinhardtii* (Courmac et al., 2000) and in the chlorarachniophyte *Bigeloviella natans* (Archibald et al., 2003).

PTOX and AOX sequences are related and both exhibit at their C-terminus the iron-binding motifs typical of Type II di-iron carboxylate proteins (Carol and Kuntz, 2001). Because of the sparse distribution among eukaryotic lineages and the different regulatory mechanisms, the origin of the

SHAM-sensitive alternative oxidases has been a mystery, particularly since no prokaryotic homologues have been identified. Here we investigate the evolutionary origin of mitochondrial and plastid AOX from the standpoint of genome sequences.

## 2. Materials and methods

Alternative oxidase homologues were identified by BLAST searches of sequenced genomes at the TIGR website (<http://www.tigr.org/tdb/>) and at GenBank (<http://www.ncbi.nlm.nih.gov/>). Homologues were retrieved and aligned using ClustalW (Thompson et al., 1994). Protein LogDet distances (Lockhart et al., 1994) were calculated with the LDDist program available at <http://artedi.ebc.uu.se/molev/software/LDDist.html>. Protein sequences were used instead of nucleotides because third positions are always saturated in comparisons of archaeobacteria, eubacteria and eukaryotes. NeighborNet networks of sequence similarity were constructed with NNet (Bryant and Moulton, 2002) and visualized with Splitstree (Huson, 1998).

## 3. Results and discussion

### 3.1. A mitochondrial-type aox gene in the $\alpha$ -proteobacterium *Novosphingobium aromaticivorans*

Database searching detected one single clear homologue of mitochondrial AOX among available prokaryotic sequence data, a hypothetical protein encoded within a large contiguously assembled sequence (contig) from the genome sequencing project of the  $\alpha$ -proteobacterium *N. aromaticivorans* (accession no. NZ\_AAAV01000000). *N. aromaticivorans* is a strictly aerobic, aromatic hydrocarbon-degrading bacterium isolated from coastal plain sediments.

The  $\alpha$ -proteobacterial AOX protein has a predicted molecular mass of 26 kDa and therefore is significantly shorter than the fungal and plant mitochondrial AOX. The schematic representation in Fig. 1 shows that the predicted *N. aromaticivorans* AOX exhibits high similarity with the C-terminal two-thirds of mitochondrial AOX sequences which accommodates the active site (Siedow and Umbach, 2000). In particular, the spacing of the ligands of the iron atoms (E and H residues) is conserved between the  $\alpha$ -proteobacterial and the mitochondrial enzymes (Fig. 1). *Novosphingobium* predicted AOX sequence displays the highest identity with plant homologues (52–55% identity) (Table 1). Highest sequence similarity is found in the regions that surround the iron ligands and that are predicted to form  $\alpha$ -helices.

Interestingly, the bacterial enzyme sequence lacks the N-terminal domain of the mature mitochondrial AOX that is highly variable between eukaryotic groups (plants vs. fungi) (Siedow and Umbach, 2000) and therefore seems to be

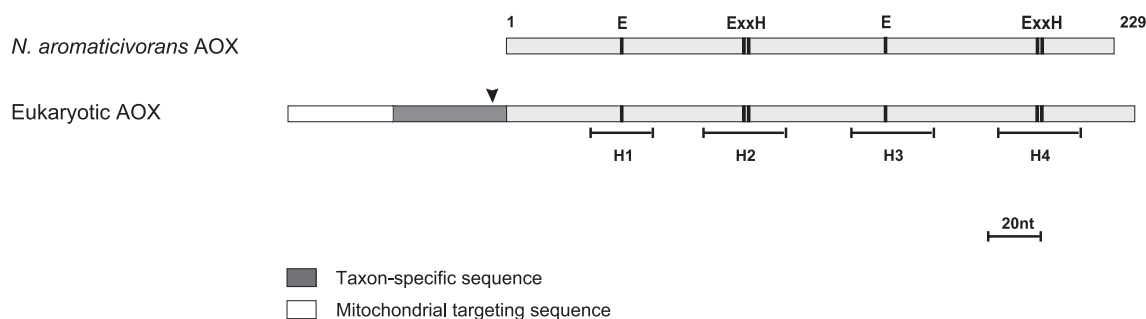


Fig. 1. Schematic representation of *N. aromaticivorans* putative AOX and mitochondrial AOX. Motifs (ExxH) and glutamate residues (E) that are proposed to be the Fe-ligands are indicated; ▼, regulatory cysteine residue in higher plants; H1–4, indicates the position of the predicted  $\alpha$ -helices in the eukaryotic enzyme.

taxon-specific. This domain, located in the matrix, is important for regulation of the plant oxidase activity (cysteine residue).

Even distant homologues were not detected among any other prokaryotic genomes (except few cyanobacteria, see below). The absence of *aox* genes in sequenced prokaryotes, including the  $\alpha$ -proteobacteria *Agrobacterium tumefaciens* (2.8 Mb), *Bradyrhizobium japonicum* (9.1 Mb), *Caulobacter crescentus* (4 Mb), *Mesorhizobium loti* (7 Mb), *Rickettsia conorii* (1.3 Mb), *Rickettsia prowazekii* (1.1 Mb) and *Sinorhizobium meliloti* (3.7 Mb), indicates that this gene has a very sparse distribution across sequenced bacterial genomes.

The current patterns of sequence similarity among mitochondrial AOX and their single prokaryotic homologue are shown in Fig. 2A. The precise placement of the  $\alpha$ -proteobacterial homologue as the root in Fig. 2A is highly uncertain. It does not correspond to the placement of the root among eukaryotes suggested recently by Stechmann and Cavalier-Smith (2002), who presented gene fusion data indicating that the root of eukaryotic phylogeny lies on or close to the branch separating animals and fungi from all other eukaryotes. However, in the network of LogDet sequence distances for AOX, the higher plant and algal sequences are separated both by the root and by homologues from *Dictyostelium* and trypanosomes. Although no AOX sequences are currently available from animals, SHAM-inhibitable AOX activity has been reported in mitochondria

of the lugworm *Arenicola marina*, a marine invertebrate that inhabits a sulfide-rich, anaerobic environment (Völkel and Grieshaber, 1997). It is, however, not known whether the lugworm activity is attributable to an enzyme homologous to typical eukaryotic AOX. When additional AOX sequences become available, it can be expected that the patterns of similarity currently observed in Fig. 2A will change somewhat, in particular the position of the  $\alpha$ -proteobacterial root.

### 3.2. A plastid-type *aox* gene is present in cyanobacteria

Database searching revealed the existence of a gene encoding a putative PTOX in three cyanobacteria: the filamentous nitrogen-fixing bacterium *Nostoc* sp. PCC 7120 (BAB73795), the marine unicellular *Synechococcus* sp. WH 8102 (ZP\_00115232) and *Prochlorococcus marinus* subsp. *pastoris* str. CCMP1986 (CAE18795). The amino-acid identity between putative bacterial PTOX and known PTOX is shown in Table 2. While *Synechococcus* PTOX sequence shares low similarity with all eukaryotic sequences available so far (<25% amino acid identity), the sequences of *Nostoc* and *Prochlorococcus* predicted enzymes are highly similar to plant PTOX (average of 50% identity).

The cyanobacterial PTOX homologues are, at their N-terminus, shorter than the eukaryotic PTOX sequences, which is in part explained by the lack of an organelle targeting sequence. Fig. 3 shows a multiple sequence alignment of cyanobacterial predicted PTOXs with the C-terminal part of known PTOXs. The bacterial sequences exhibit the conserved glutamate and histidine residues. However, *Prochlorococcus* PTOX lacks a region between the predicted helices III and IV leading to a shorter spacing of the Fe-ligands.

It should be stressed that AOX and PTOX are distantly related members of a common protein family, as database searching reveals. However, pairwise amino acid identity in comparisons of AOX vs. PTOX sequences is very low, in the range of 25%. For example, mitochondrial AOX and chloroplast PTOX sequences of *Arabidopsis* share 26% amino acid identity in the pairwise comparison. Similarly, the *Nostoc* PTOX and *Novosphingobium* AOX homologues share 22.8% identity. By contrast, the *Novosphingobium*

Table 1

Amino acid identity (in %) between *Novosphingobium* predicted AOX sequence and mitochondrial AOX sequences from different sources

	1	2	3	4	5	6
1. <i>NOVSP</i>	100	-	-	-	-	-
2. <i>ARATH</i>	52	100	-	-	-	-
3. <i>ORYSA</i>	51	62	100	-	-	-
4. <i>CHLRE</i>	38	28	32	100	-	-
5. <i>NEUCR</i>	36	27	32	35	100	-
6. <i>PICAN</i>	37	22	24	33	40	100

NOVSP, *N. aromaticivorans* (NZ-AAAV01000000); ARATH, *A. thaliana* (BAA22625); ORYSA, *O. sativa* (BAB71944); CHLRE, *C. reinhardtii* (AAG33633); NEUCR, *Neurospora crassa* (EAA32850); PICAN, *Pichia anomala* (S17517).

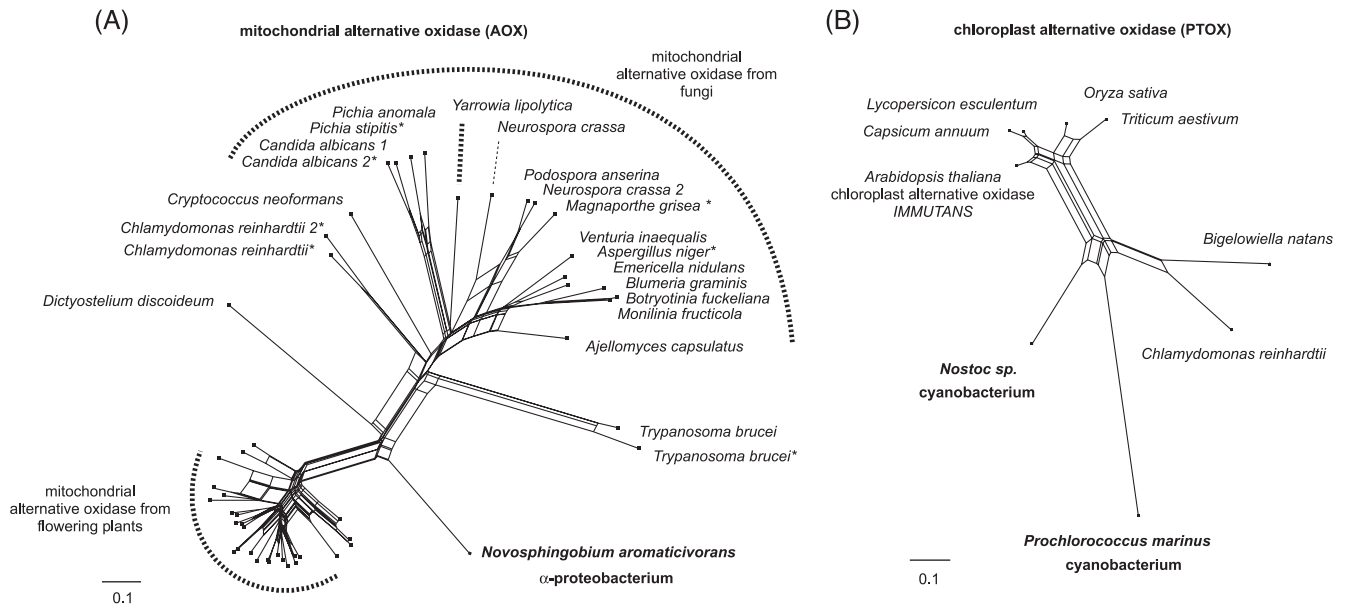


Fig. 2. Phylogenetic networks for mitochondrial AOX (A) and chloroplast PTOX (B). Asterisks indicate sequences with biochemically characterized function. AOX and PTOX sequences are distantly related and very probably share a common ancestor (see text and Fig. 3).

AOX shares on average 50% amino acid identity with AOX sequences from eukaryotes (Fig. 2A) and the *Nostoc* PTOX homologue shares on average 50% amino acid identity with PTOX sequences from eukaryotes (Fig. 2B). Thus, although AOX and PTOX are clearly related, they are very difficult to incorporate into a single alignment, as indicated by the 16 residues conserved between the two groups shown in Fig. 3, and in our view they are too distantly related to justify a joint phylogenetic analysis.

The absence of an alternative oxidase-type gene in other cyanobacterial sequenced genomes, including *Synechocystis* sp. PCC 6803 (3.6 Mb) and *Thermosynechococcus elongatus* str. BP-1 (2.6 Mb) is intriguing and underscores the need for further sampling of cyanobacterial genomes in order to get a better picture of the number and nature of genes that cyanobacteria donated to the plant lineage (Martin et al., 2002).

Table 2  
Amino acid identity (in %) between cyanobacterial predicted PTOX sequences and PTOX sequences from different photosynthetic organisms

	1	2	3	4	5	6	7
1. NOSTOC	100	-	-	-	-	-	-
2. PROMA	38	100	-	-	-	-	-
3. SYNECH	21	27	100	-	-	-	-
4. ARATH	53	39	21	100	-	-	-
5. ORYSA	55	38	19	60	100	-	-
6. CHLRE	49	39	21	31	36	100	-
7. BIGNA	45	29	23	38	36	48	100

NOSTOC, *Nostoc* PCC 7120 (BAB73795); PROMA, *P. marinus* subsp. *pastoris* str. CCMP1986 (CAE18795); SYNECH, *Synechococcus* sp. WH8102 (ZP\_00115232); ARATH, *A. thaliana* (CAA06190); ORYSA, *O. sativa* (AAC35554); CHLRE, *C. reinhardtii* (AAM12876); BIGNA, *B. natans* (AAP79178).

The current patterns of sequence similarity among PTOX and their single prokaryotic homologues are shown in Fig. 2B. The branch length of the cyanobacterial homologues indicates that it is a clear homologue of the nuclear-encoded chloroplast enzyme.

### 3.3. Origin of the mitochondrial and plastid SHAM-sensitive alternative oxidases

The presence of a homologue of the *aox* gene in an  $\alpha$ -proteobacterium, the ancestor of mitochondria (Gray et al., 1999), suggests that this eukaryotic nuclear gene has been inherited from the ancestor of mitochondria and was later lost in the mitochondria of most organisms. In light of the fact that only one proteobacterial AOX homologue has so far been detected, the alternative explanation of lateral gene transfer of the *aox* gene from a eukaryote (or more specifically a plant) to the *Novosphingobium* lineage cannot be excluded at this time. Although, as strong evidence for eukaryote to prokaryote lateral gene transfer events is missing, we believe this scenario to be unlikely.

The recently discovered plastid *ptox* gene turns out to be also present in cyanobacteria, strongly favouring the view that this gene, too, was inherited from the cyanobacterial ancestor of chloroplasts.

Recent data suggest that the *aox* gene can be lost from the genome when its product is no longer needed, that is under loss of photosynthetic activity/through loss of a functional chloroplast. In *Polytomella*, a close relative of *C. reinhardtii* that lacks functional chloroplasts, no evidence for mitochondrial AOX activity was observed (Reyes-Prieto et al., 2002).



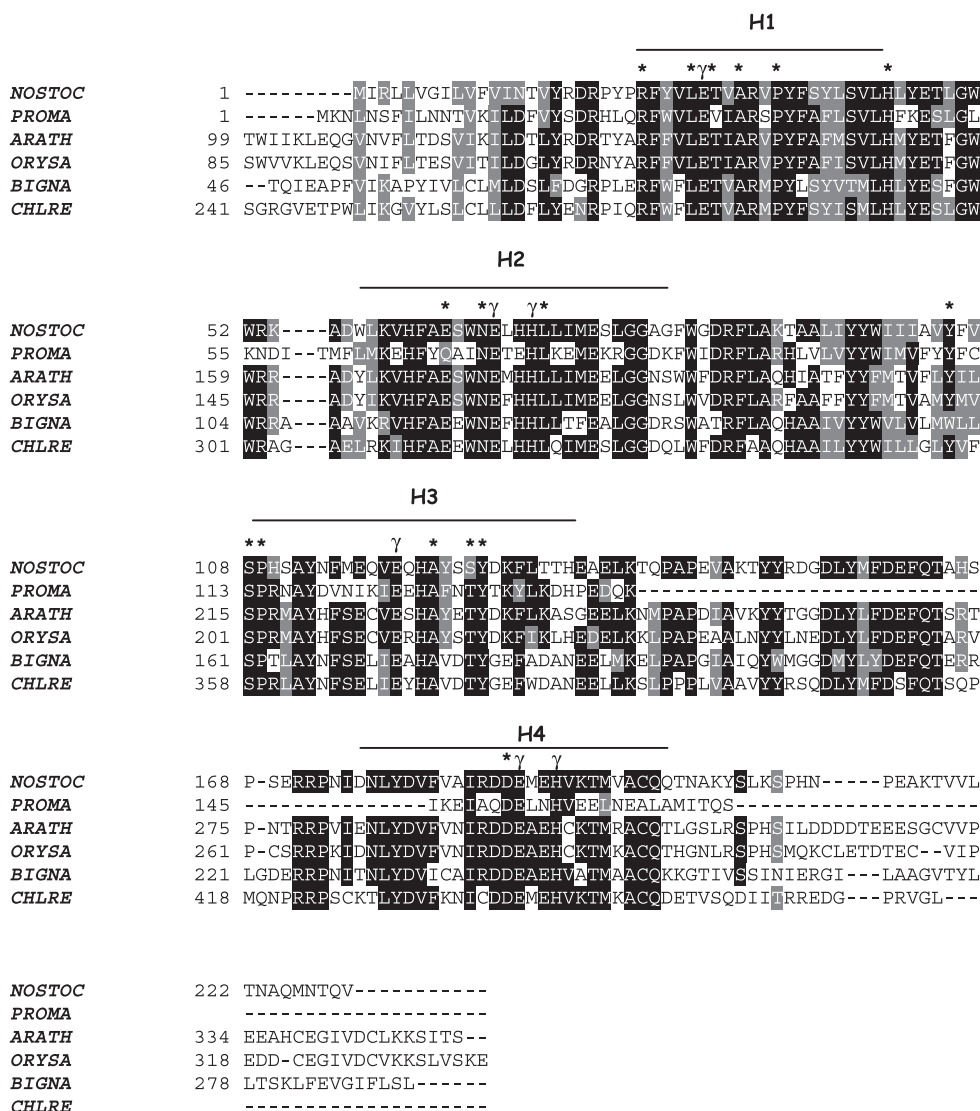


Fig. 3. Alignment of the predicted cyanobacterial PTOX-like sequences with the C-terminus of PTOX sequences from various photosynthetic eukaryotes (BOXSHADE program). The accession numbers are as follows: NOSTOC, *Nostoc* PCC 7120 (BAB73795); SYNECH, *Synechococcus* sp. WH8102 (ZP\_00115232); PROMA, *P. marinus* subsp. *pastoris* str. CCMP1986 (CAE18795); ARATH, *Arabidopsis thaliana* (CAA06190); ORYSA, *Oryza sativa* (AAC35554); CHLRE, *C. reinhardtii* (AAM12876) and BIGNA, *B. natans* (AAP79178). Black background corresponds to residues completely conserved between analyzed sequences; grey background corresponds to conservative substitutions.  $\gamma$ , residues that may bind the di-iron active center; \*, residues conserved in both mitochondrial AOX and PTOX; H1–H4 denote putative  $\alpha$ -helices.

The number of prokaryotes in which a gene for AOX was discovered, is extremely limited. This underscores the need for continued microbial genome sequencing in order to better understand the origins of chloroplasts and mitochondria. The current sample of  $\alpha$ -proteobacteria is still strongly biased towards medically or agriculturally relevant species. Since mitochondria have not only aerobic, but also anaerobic biochemical pathways (Tielens et al., 2002), complete genome sequences among relatives of the genus *Rhodospirillum* will be of particular interest, because their anaerobic physiology involves the production of succinate, propionate, acetate, formate, CO<sub>2</sub> and H<sub>2</sub> (Imhoff and Trüper, 1992), strikingly similar to the spectrum of end products found

among anaerobic mitochondria (Tielens et al., 2002; Müller, 2003).

Note: After completion of this study, a paper appeared that reports the presence of AOX in *N. aromacitivorans* and shows evidence for the oxidase activity of this protein (Stenmark and Nordlund, 2003).

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