

Bioenergetic Evolution in Proteobacteria and Mitochondria

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Abstract

Mitochondria are the energy-producing organelles of our cells and derive from bacterial ancestors that became endosymbionts of microorganisms from a different lineage, together with which they formed eukaryotic cells. For a long time it has remained unclear from which bacteria mitochondria actually evolved, even if these organisms in all likelihood originated from the α lineage of proteobacteria. A recent article (Degli Esposti M, et al. 2014. Evolution of mitochondria reconstructed from the energy metabolism of living bacteria. *PLoS One* 9:e96566) has presented novel evidence indicating that methylotrophic bacteria could be among the closest living relatives of mitochondrial ancestors. Methylotrophs are ubiquitous bacteria that live on single carbon sources such as methanol and methane; in the latter case they are called methanotrophs. In this review, I examine their possible ancestry to mitochondria within a survey of the common features that can be found in the central and terminal bioenergetic systems of proteobacteria and mitochondria. I also discuss previously overlooked information on methanotrophic bacteria, in particular their intracytoplasmic membranes resembling mitochondrial cristae and their capacity of establishing endosymbiotic relationships with invertebrate animals and archaic plants. This information appears to sustain the new idea that mitochondrial ancestors could be related to extant methanotrophic proteobacteria, a possibility that the genomes of methanotrophic endosymbionts will hopefully clarify.

Key words: mitochondria, bioenergetics, endosymbiosis, methylotrophs, methanotrophs.

Introduction

That mitochondrial organelles derive from ancestral bacteria (Sagan 1967; Margulis 1996) are now widely accepted, but the question of their precise origin remains open (Gray 2012). The endosymbiotic transition that transformed these ancestral bacteria (or proto-mitochondria) into current mitochondria has been fundamental in the evolution of life (Lane and Martin 2010). According to the current consensus, this event occurred only once, at least 1.5 billion years ago, and was followed by divergent evolutionary paths leading to altered forms of the organelles adapted to anaerobic lifestyles (Müller et al. 2012; Mentel et al. 2014). Consistent evidence has indicated that proto-mitochondria emerged from the α lineage of proteobacteria (Andersson et al. 1998, 2003; Williams et al. 2007; Gray 2012; Müller et al. 2012). This α lineage occupies a central position in “phylum” proteobacteria, because it originated after the separation of the predominantly anaerobic δ and ε branches but before the split of the (predominantly) facultatively anaerobic γ and β branches (Battistuzzi et al. 2004).

Proto-mitochondria evolved soon after, when current oxygen levels were established in the atmosphere, emerged

land, and the photic zone of Proterozoic oceans, while the rest of the oceans remained fundamentally anoxic (Johnston et al. 2009; Müller et al. 2012). Because at this time all life forms were restricted to the oceans, proto-mitochondria evolved in one of the following ecological environments: (1) photic surface of the ocean; (2) oxic–anoxic interface in oceanic or other aquatic regions; and (3) medium deep ocean, including hydrothermal vents and cold seeps. In environment (1), the ancestors of mitochondria might have been equipped with the protective pigments of purple nonsulfur photosynthetic bacteria such as *Rhodospirillum* (Esser et al. 2004) or were already adapted to the oligotrophic pelagic lifestyle of the bacteria which are dominant in current seas, for example, *Pelagibacter* (Thrash et al. 2011). In environment (2), the ancestors of mitochondria should have been facultatively anaerobic, as in the case of extant aquatic bacteria that are free-living (e.g., *Magnetococcus*; Schübbe et al. 2009) or predatory (e.g., *Micavibrio*; Davidov et al. 2006). Finally, in environment (3), the ancestors of mitochondria must have had additional metabolic capacities to survive under oligotrophic conditions and eventually exploit, via chemiosynthesis, local areas enriched in chemical nutrients such as H_2S , nitrate, reduced

metals, and methane. Currently, such metabolic pathways are present in α - and γ -proteobacteria that have become endocellular symbionts of deep-sea invertebrates inhabiting hydrothermal vents, cold seeps, and fallen wood (Dubilier et al. 1999; Blazejak et al. 2006; DeChaine and Cavanaugh 2006; Cavanaugh et al. 2013), or of peat bog mosses (Dedysh 2011; Kip et al. 2011). From a theoretical standpoint, a wide metabolic versatility enabling adaptation to all the above environments would produce maximal probability for being close to the mitochondrial ancestors. At the moment, such a versatility seems to be present in a few families of cultivated α - and γ -proteobacteria and also some uncultivated organisms discovered in metagenomic surveys (Wrighton et al. 2012). However, the requirement for metabolic versatility is not a strict condition for defining the ancestors of mitochondria, which might have been confined to only one of the above-mentioned habitats.

Another factor that deserves consideration is bacterial adaptation to endocellular symbiosis. Proto-mitochondria that had evolved in Proterozoic environments must have reduced their metabolic versatility during the transition to symbiotic lifestyle. Although we can only guess the details of this process, we can deduce its contours by comparison with various examples of metabolic adaptation to symbiosis or parasitism that have been documented for extant microorganisms (Vannini et al. 2007; Moran et al. 2009; Manzano-Marín et al. 2010; Sachs et al. 2011; Caffrey et al. 2012). In general, the stability of the host environment reduces evolutionary pressure on organisms that have undergone the transition from free-living to endocellular symbionts, resulting in genome reduction and erosion affecting energy metabolism also (Moran et al. 2009; Caffrey et al. 2012). The original metabolic capacity of mitochondrial ancestors has thus been lost (Huynen et al. 2013; Degli Esposti et al. 2014), contrary to popular reconstructions which depict their bioenergetic capacity as equivalent to that of mammalian mitochondria (Emelyanov 2001; Gabaldón and Huynen 2003). Recently, the progressive gene loss leading to the current bioenergetic capacity of mitochondria has been reconstructed by using a systematic genomic analysis of extant α -proteobacteria and protists' mitochondria (Degli Esposti et al. 2014). The analysis led to the novel indication that a group of proteobacteria, the methylotrophs, may contain the extant relatives of proto-mitochondria (Degli Esposti et al. 2014). Interestingly, some of these methylotrophs such as *Methylocystis* possess a metabolic versatility suitable for all environments (1)–(3) mentioned above (Dam et al. 2012). They are also related to the methanotrophic endosymbionts of *Spagnum* mosses dominating peat bogs and northern wetlands (Raghoebarsing et al. 2005; Dedysh 2011; Kip et al. 2011; Belova et al. 2013).

The work of Degli Esposti et al. (2014) has excluded from mitochondrial ancestry many bacteria that had been previously proposed as mitochondrial relatives or ancestors, for

example, *Rhodospirillum rubrum* (Esser et al. 2004; Abhishek et al. 2011; Thiergart et al. 2012) and *Rickettsia* (Andersson et al. 1998; Sicheritz-Pontén et al. 1998; Williams et al. 2007; Abhishek et al. 2011; Ferla et al. 2013). Therefore, this work will inevitably generate controversy in the fields of molecular evolution, microbiology, and bioenergetics. Herein, I will examine major issues that may help resolving such controversy within an overview of the common bioenergetic features of proteobacteria and mitochondria.

Common Bioenergetic Features of Proteobacteria and Mitochondria

Mammalian mitochondria constitute the best known model for the energy metabolism in eukaryotic cells (Andersson et al. 2003; Gabaldon and Huyen 2003), but represent only one of the several versions of these organelles that are present in various eukaryotic organisms (Mentel et al. 2014). They all derive from the same unique event of endosymbiosis that occurred in ancestral oceans between proto-mitochondria and other prokaryotes (Müller et al. 2012; Williams et al. 2013). Leaving aside the dehydrogenases that convey electrons from nutrients to ubiquinone (Q) producing its reduced form, ubiquinol, the central and terminal portion of the mitochondrial respiratory chain produces most bioenergy in eukaryotic cells and shows strong similarity with that of aerobic proteobacteria (Sicheritz-Pontén et al. 1998; Emelyanov 2001). It consists of ubiquinol:cytochrome *c* reductase, commonly called *bc₁* complex, the soluble electron carrier cytochrome *c*, and its terminal bioenergy-producing oxidase, usually known as cytochrome *c* oxidase (COX), but nowadays defined as dioxygen reductase of *aa₃* type (Souza et al. 2012). Other mitochondrial enzymes that reoxidize either ubiquinol or cytochrome *c* without producing bioenergy are not considered in this review.

The fundamental difference that distinguishes the *bc₁* and *aa₃* enzyme complexes of mitochondria and proteobacteria is the presence of additional, supernumerary subunits that may contribute aspects of fine regulation in eukaryotic cells. In both structure and function, the core catalytic proteins are essentially the same in mitochondrial and proteobacterial complexes (Zhang et al. 1998; Harrenga and Michel 1999; Berry et al. 2004; Radzi Noor and Soulimane, 2012), possessing multiple transmembrane helices and various redox cofactors. Most of these catalytic proteins are retained in mitochondrial DNA (mtDNA), the principal vestige of the bacterial past of the organelles (Sagan 1967). However, the Rieske iron sulfur protein (ISP) and cytochrome *c₁* subunits of the *bc₁* complex have migrated into nuclear DNA early after the symbiotic transition of proto-mitochondria, followed by most subunits of the dehydrogenases, the assembly subunits of *aa₃*-type oxidase, and the catalytic subunits of adenosine triphosphate (ATP) synthase (Gray 2012; Burger et al. 2013). Consequently, the core subunits of *aa₃*-type oxidase, COX1, COX2, and COX3,

Table 1

Features of Bioenergetic Systems Specific to Bacteria that Sometimes Are Found in Mitochondria

System and Feature	Proteobacteria			Mitochondria (mtDNA)	Origin
	Alpha	Beta	Gamma		
COX operon					
a: fusion COX1 with COX3	Yes	Yes	Yes	3 ciliates	Firmicutes
a-I: operon linked with Act	Yes	2 only	Yes	No	Chloroflexi
a-II: fusion COX1 with COX4	Yes	Yes	9 only	1 fungus, ciliates? <i>Naegleria?</i>	Proteobacteria
a, a-I, a-II, a-b: fusion COX2-cyt c	Yes	Yes	Yes	1 sponge ^a	Firmicutes
a, a-II, a-b, b: <i>ctaG</i> (COX11) in operon	Yes	Yes	Yes	Jakobids	Firmicutes
a-b, b: <i>ctaB</i> (COX10) in operon	Yes	Yes	Yes	No	Firmicutes
a-b, b: <i>ctaG-ctaE</i> (COX11-COX3) synteny	Yes	No	Yes	Jakobids	γ -proteobacteria?
a-b, b: <i>ctaA</i> (COX15) in operon	2 only	Yes	Yes	1 Jakobide	Firmicutes
N-metabolism					
<i>NiaD</i> -like nitrate reductase alone	Yes	Yes	Yes	Fungi, Heterokonts, Amoebozoa	Actinomycetes
<i>BisC</i> -type nitrate reductase	Yes	Yes	Yes	1 anellide ^b	Actinomycetes?
<i>bc₁</i> complex					
Fusion of <i>cyt b</i> with <i>cyt c₁/c</i>	Yes	No	13 only	No	Planctomycetes
Long ISP Rieske (ISP2)	9 only	Yes	Yes	No	Alpha?
Whole <i>petABC</i> operon	Yes	Yes	Yes	<i>Daphnia</i> ^c	Cyanophyta

^aCOX2 fused with cytochrome *c* is found in the genome of the sponge *Amphimedon queenslandica* (accession: XP_003390963, 391 residues long) and is most closely related to the COX2 protein of COX operon a-b transition of γ -proteobacteria from the genus *Thioalkalivibrio*.

^bTypical bacterial *BisC*-type nitrate reductase is embedded in the genome of the anellide *Capitella teleta* (accession: ELU13386, 644 residues long); it is most closely related to the *BisC* reductase of γ -proteobacterial endosymbionts of the genus *Endozoicomonas*.

^cThe entire operon of a bacterial *bc₁* complex is present within the genome of the crustacean *Daphnia* (accession for ISP: EFX83890; Colbourne et al. 2011). See text for more information.

constitute the bioenergetic redox system that has remained most conserved between mitochondria and proteobacteria. Indeed, it appears to show the largest set of common features of potential evolutionary significance, as summarized in table 1. The gene order of these subunits appears to be almost random in the mtDNA of the great majority of eukaryotes, but follows defined sequences in some protists (Burger et al. 2013; Degli Esposti et al. 2014) and most proteobacteria. All such sequences most likely derive from the ancestral operon already present in Firmicutes such as *Bacillus subtilis* (Radzi Noor and Soulimane 2012; Degli Esposti et al. 2014).

The classification of COX gene sequences into defined operon types (fig. 1A, cf. Degli Esposti et al. 2014) has revealed new evolutionary information which had been previously overlooked using standard phylogenetic approaches (Andersson et al. 2003; Brochier-Armanet et al. 2009; Sousa et al. 2012; Burger et al. 2013). In particular, COX operon type b, which is characterized by the insertion of *ctaB*/COX10 and the conserved synteny *ctaG-CtaE* (COX11-COX3), is present only in α -proteobacteria; it lies in sister position of the transition operon a-b which is shared by β - and γ -proteobacteria. Recent analysis has shown the insertion of small hydrophobic subunits possibly related to COX4 between COX1 and COX3 in the gene sequences of β -proteobacterial COX operons, thereby providing a new distinguishing feature with respect to the operons of γ -proteobacteria (fig. 1A). The synteny COX11-COX3 previously observed in mtDNA of Jakobide

protists (Burger et al. 2013) thus becomes a feature that is common to mitochondria as well as α - and γ -proteobacteria, but no other lineage of the phylum proteobacteria (table 1).

Jakobide mtDNA shows the largest set of bacterial bioenergetic proteins among eukaryotes (Gray 2012), including another component of bacterial COX operons, *ctaA* (COX15; Burger et al. 2013). Intriguingly, the closest bacterial homolog of this protein has been found in the α -proteobacterium *Tistrella mobilis*, lying within its COX operon type b (Degli Esposti et al. 2014). So far, the presence of *ctaA* in this type of COX operon has been observed only in another α -proteobacterium, *Geminococcus roseus* (accession: NZ_ATYL01000000; Foessel et al. 2007). Hence, it can be considered a novel feature that is shared with the mitochondria of at least one protist, *Andalucia* (Burger et al. 2013, table 1). Its rare occurrence in both α -proteobacteria and protists contrasts with its frequency in COX operons of β - and γ -proteobacteria (fig. 1 and table 1), suggesting that *Tistrella* and *Geminococcus* might have a common ancestor with the bacteria that then became the mitochondrial organelles of protists. However, phylogenetic analysis of the *ctaA*/COX15 protein fails to sustain such a possibility, because the sequence of *Andalucia* COX15 does not form a monophyletic clade with other eukaryotes, while those of α -proteobacteria such as *Devosia* cluster with eukaryotic sequences rather than with those of other α -proteobacteria (not shown). Presumably,

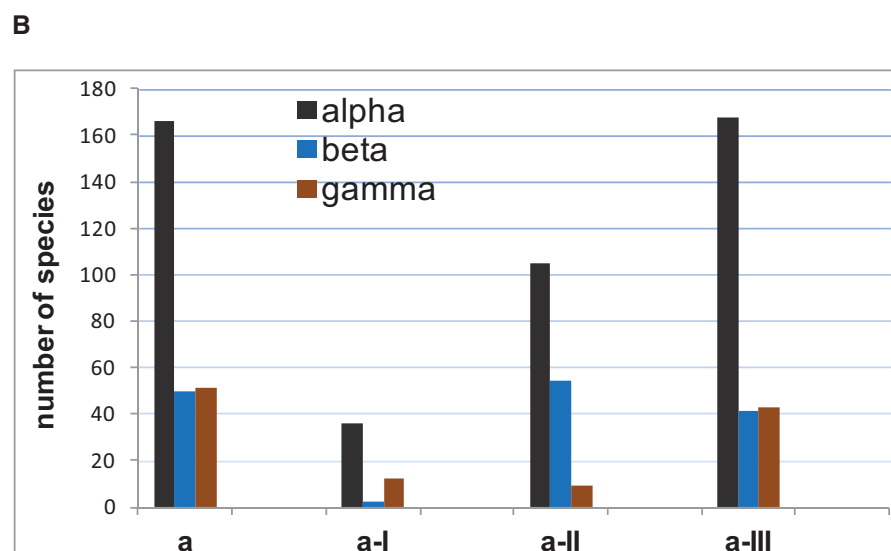
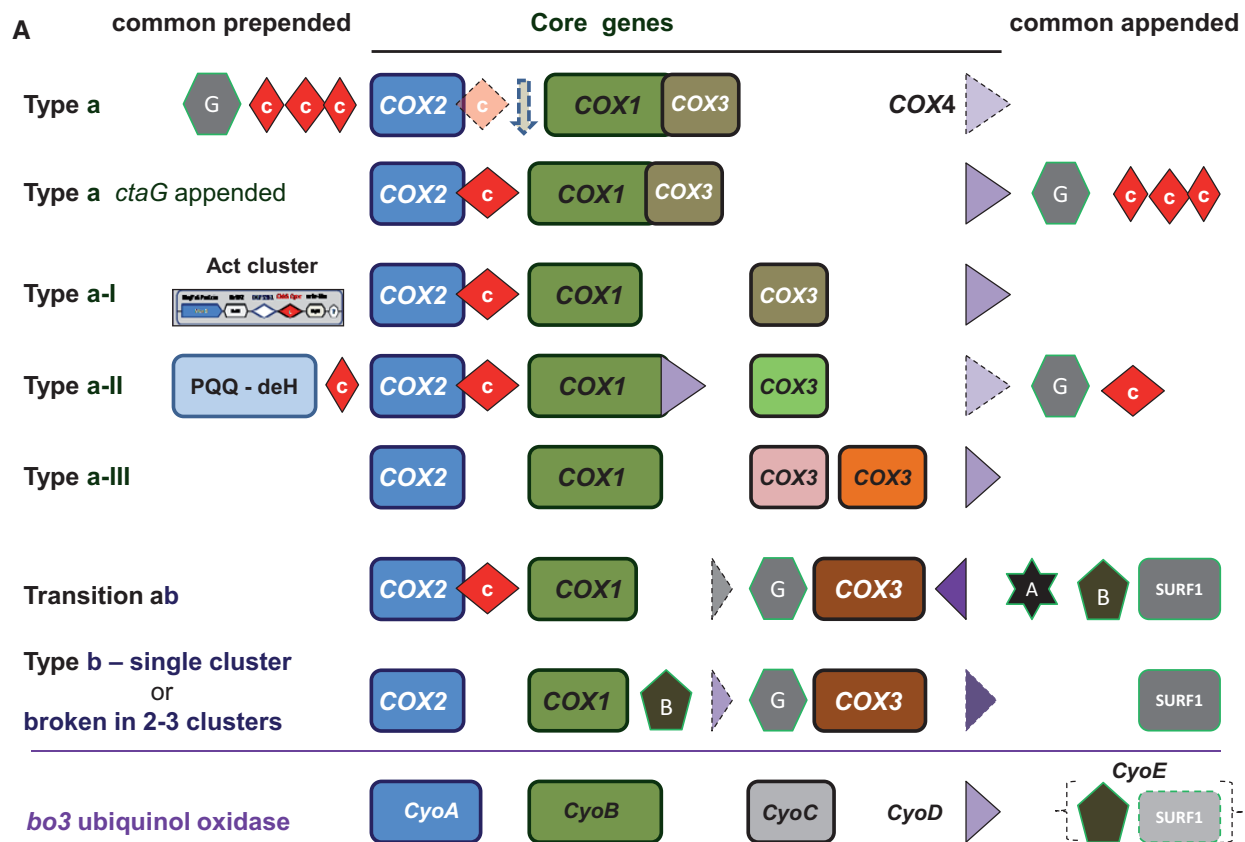


FIG. 1.—Classification and distribution of COX operons in proteobacteria. (A) COX operons defined by Degli Esposti et al. (2014) are graphically represented with the core genes COX1–4 (corresponding to *ctaBCDE* in *Bacillus*) and common other genes including those for assembling the catalytic groups of the oxidase: *ctaA* (star with A), *ctaB* (pentagon with B), *ctaG* (hexagon with G), and SURF-1 (grey square). The sequences falling within COX operon type a can be distinguished from the position of *ctaG* (supplementary fig. S1, Supplementary Material online). The canonical gene sequence *CyoA–E* of *bo3*-type ubiquinol oxidases is shown for comparison. See Matsutani et al. (2014) for a classification of these sequences, which are clearly different from those of COX operons (see also supplementary figs. S1 and S2, Supplementary Material online). (B) Distribution of COX operon type a in the classes of α -, β -, and γ -proteobacteria. Data were extracted from NCBI websites, accessed on September 15, 2014. The total number of species with available genomic data is comparable for α - and γ -proteobacteria (589 and 652, respectively), but approximately one-half (301) for β -proteobacteria. Act, alternative complex three (Refojo et al. 2010); PQQ, deH, methanol dehydrogenase.

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ctaA/COX15 proteins do not carry robust phylogenetic signals that are congruent with the evolution of mitochondria (cf. Hederstedt 2012), a situation that has been found also for other subunits of *aa₃*-type oxidase such as *COX2* and *ctaB/COX10*.

Refining the Evolutionary Path of COX Operons from Bacteria to Mitochondria

In contrast with the abovementioned subunits, *COX1* and *COX3* possess strong phylogenetic signals that are consistent with the general pattern of mitochondrial evolution, producing monophyletic clades for the mitochondrial homologs within the large group of COX operon type b (Degli Esposti et al. 2014). The molecular variations of *COX3* are particularly large, because bacterial homologs of the mitochondrial protein having seven transmembrane helices can be found only in operons b and a–b transition (Degli Esposti et al. 2014). All other bacterial forms of this protein are shorter (generally with five transmembrane helices) and could be considered ancestral homologs of mitochondrial *COX3*; indeed, they have been categorized in different subfamilies on the basis of their conserved domain features (CDD, Marchler-Bauer et al. 2011). One of these subfamilies is typical of *bo₃*-type ubiquinol oxidase, the operon sequence of which is shown at the bottom of figure 1A, while each of the two consecutive *COX3* proteins that are characteristically present in COX operon type a-III belongs to a different recognized subfamily: CD02865 (colored in pink, fig. 1A) and CD02864 (colored in orange, fig. 1A). Of note, the latter is often annotated as “bb3-type cytochrome c oxidase subunit IV” following an old nomenclature of *bo₃*-type oxidases (de Gier et al. 1994)—one of the several confusing annotations for COX genes (see below).

Although not recognized as distinct CDD-based subfamilies, the *COX3* subunits of COX operons type a-I and a-II cluster in separate clades of phylogenetic trees and show a different conservation pattern of key protein residues (Degli Esposti et al. 2014). In COX operon type a, instead, *COX3* is fused at the C-terminus of *COX-1*. The resulting fused *COX1–3* proteins are distributed in proteobacteria (fig. 1B and supplementary fig. S1, Supplementary Material online), as well as in *Thermus*, *Bacillus* (Radzi Noor and Soulimane 2012), and a few mitochondria from ciliates (Degli Esposti et al. 2014, table 1). The constant addition of new proteobacterial genomes has greatly expanded the number of *COX1–3* proteins (often, and inexplicably annotated as “cytochrome B561”) from the set previously considered by Degli Esposti et al. (2014). The analysis of these gene clusters has identified two subtypes that are clearly distinguished by the position of the assembly subunit *ctaG* with respect to the core catalytic subunits, as shown in supplementary figure S1, Supplementary Material online, and figure 1A according to

the operon nomenclature of Price et al. (2006). Intriguingly, several proteobacteria have representatives of both subtypes in their genome, for example, *Methylocystis* and *Nitrosococcus* (supplementary fig. S1, Supplementary Material online). The increasing complexity and presence of multiple forms within the same organism have hampered a clear definition of the possible evolutionary path of COX operons type a. However, recently I was able to trace this path by extending DELTABLAST (Boratyn et al. 2012) searches to 5,000 bacterial and mitochondrial species using the seven helices form of *COX3* from *Tistrella* as query (fig. 2). The phylogenetic trees thus obtained suggested the following evolutionary sequence: COX operon a, COX operon a-I, *bo₃*-type oxidase, COX operon a-III (with separate clades for the two consecutive *COX3* proteins), and a subset of COX operon type a-II, which forms the immediate ancestral precursor of the sister groups of operon b (that surrounds the mitochondrial clade) and a–b transition (including β and γ organisms; fig. 2). This evolutionary sequence of COX operons refines that previously presented in figure 5 of Degli Esposti et al. (2014), which was obtained with searches limited to 1,000 taxa; it is further confirmed by the extended analysis of *COX1* and *COX4* proteins (not shown).

The same trees also revealed the presence of COX operon type a-II in a group of γ methanotrophs including the genera *Methylomicrobium*, *Methylosarcina*, and *Methylobacter* (supplementary fig. S2, Supplementary Material online; cf. pink square in fig. 2, right). These organisms are closely related to the methanotrophic endosymbionts of deep-sea invertebrates and wetland plants (DeChaine and Cavanaugh 2006; Kip et al. 2011; Dedysh 2011; Cavanaugh et al. 2013) and form a separate clade from other methanotrophs of the *Methylococcales* order (Kip et al. 2011; Cavanaugh et al. 2013), which indeed do not have COX operon type a-II (supplementary fig. S2, Supplementary Material online). They also show a subset of four bioenergetic systems similar to that of α methylotrophs and methanotrophs (fig. 3). The subset is characterized by the presence of at least one type of *bd*-type ubiquinol oxidase and coincides with that previously suggested to form the most likely path of bioenergetic evolution of bacteria into mitochondria (Degli Esposti et al. 2014). Although previously represented by *Beijerinckia indica* alone, it is now recognized in additional organisms belonging to either α - or γ -proteobacteria which have methylotrophic metabolism in common, as illustrated in figure 3. Most of such organisms possess COX operons of type a and in some cases have the precursor of eukaryotic assimilatory nitrate reductase, *NiaD* (fig. 3). Nevertheless, there are multiple combinations of key elements of N-metabolism among the organisms that possess the *Beijerinckia* subset of bioenergetic systems, which would match the mitochondrial subset of fungi and protists after losing the *bd*-type oxidase (fig. 3, cf. Degli Esposti et al. 2014).

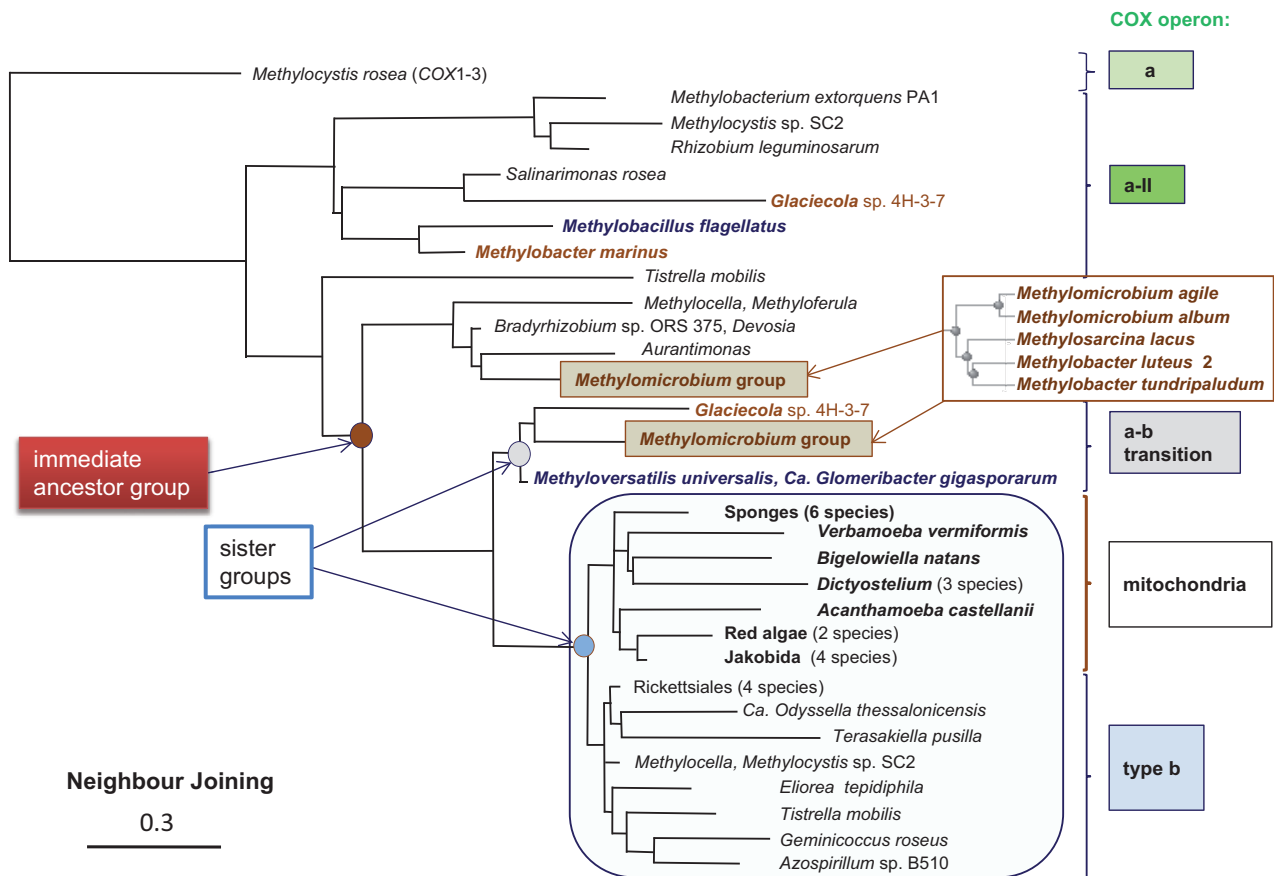


Fig. 2.—Phylogenetic tree of COX3 and evolutionary path of COX operons. The sequence of *Tistrella* COX3 (accession: YP_006372231, 269 residues long) was used as the query of a DELTABLAST search expanded to 5,000 proteins, including β - and γ -proteobacterial taxa for a total selection of 70 species. The *Methylococcus* group of five γ -proteobacterial taxa that is expanded on the right is the same in the branch of COX operon a–b transition and the subbranch of COX-operon a-II (arrow), which appears to be the immediate ancestral group to that containing mitochondria (enclosed in big square—see also supplementary fig. S2, Supplementary Material online). Only *Glaciecola* contains the same type of protein among other γ -proteobacteria, but in a COX operon that does not show methanol or related dehydrogenases (supplementary fig. S2, Supplementary Material online). The α -proteobacterial taxa in the same group include *Devosia* sp. DBB001, *Bradyrhizobium* sp. ORS 375, *Hyphomicrobium*, and *Aurantimonas*. *Methyloferula* and *Methylocella* have their COX3 proteins of COX operon type a-II that fall in the same group, together with several β -proteobacteria (supplementary fig. S2, Supplementary Material online). The outgroup sequence of *Methylocystis rosea* corresponds to the fused COX1–3 protein of 850 residues (accession: WP_018409174) belonging to a COX operon type a with prepended *ctaG* (supplementary fig. S1, Supplementary Material online, and fig. 1A). The α -proteobacterial subbranch close to the mitochondrial clade (species or group outlined in bold characters) contains representative of the order Rickettsiales (*Rickettsia prowazekii*, *Anaplasma*, *Neorickettsia*, and *Caedibacter*). Sponges include *Amphimedon queenslandica* and demosponges. Similar trees have been obtained with *Geminicoccus* COX3 or COX1 as a query.

Changes in Bioenergetic Systems of Proteobacteria in the Transition from Free-Living to Symbiont

Because mitochondria derive from ancestral bacteria that became endocellular symbionts, it is of value to analyze the genomic and metabolic changes in closely related bacteria that have undergone equivalent transitions from free-living to host-associated lifestyle (Sachs et al. 2011). There are several examples of such transitions in the literature, but often they are not relevant to the evolution of mitochondria. In

some cases, they refer to bacteria that do not possess the respiratory complexes of mitochondria but rather those typical of microaerophilic enterobacteriales, as in insect symbionts of the genus *Serratia* (Manzano-Marín et al. 2010) and acetic acid bacteria (Chouaia et al. 2014). In other cases, for instance *Teredinibacter turnerae* versus *Saccharophagus degradans*, the transition from free-living to endosymbiont is still ongoing and consequently not associated yet with evident bioenergetic changes (Yang et al. 2009). Conversely, the obligate endocellular parasites of the Rickettsiales order still await the recognition of free-living relatives, a major difficulty undermining the

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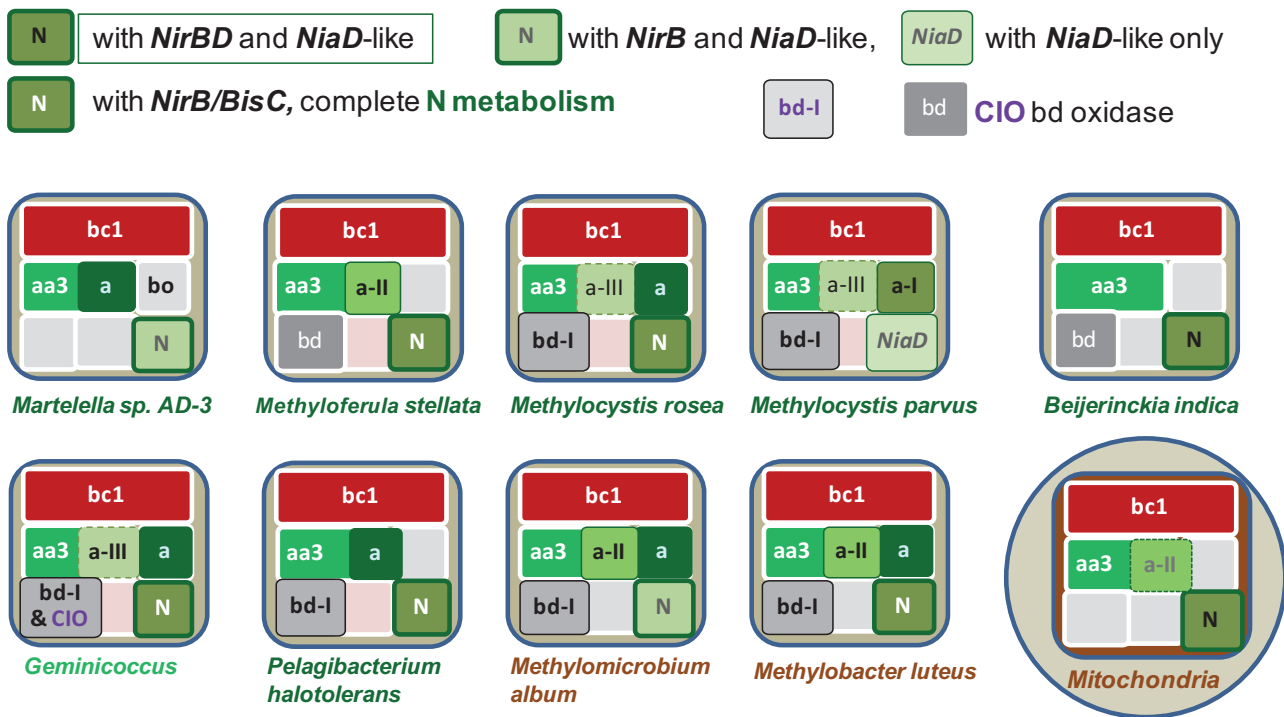


Fig. 3.—Bioenergetic systems of bacteria and mitochondria. Proteobacteria possessing only four bioenergetic systems, that is, only one more than those present in the mitochondria of protists and fungi, were identified from the latest NCBI resources (accessed September 15, 2014). Those possessing *ccb₃*-type oxidase and lying in pathway B are not presented here for they are unlikely to be in mitochondrial ancestry (Degli Esposti et al. 2014). COX operons of type a are overlaid on the area of *aa₃*-type oxidase. Different combinations of assimilatory, NAD(P)H-dependent nitrite (*NirB*) and nitrate reductase (labeled *NiaD* like when having redox modules that are precursors to those of the fungal *NiaD* enzyme; Degli Esposti et al. 2014) are color coded as indicated in the legend within the diagram. Specifically, organisms such as *Beijerinckia indica* that possess both the *NirBD* nitrite reductase and *NiaD*-like nitrate reductase are labeled with a different type of symbol for N-metabolism from those having the latter reductase combined with (not fused) *NirB* or without a *NirBD* homolog. Organisms having other nitrite or nitrate (e.g., *BisC*) reductases are differentially labeled too. Note that *Beijerinckia indica* does not possess COX operons of type a (Tamas et al. 2010) which are instead present in its close relatives such as *Methyloferula*, which is also shown because it has only four bioenergetic systems. Two major forms of *bd*-type oxidase that have been previously classified as *bd-I* and Cyanide-Insensitive-Oxidase (CIO, Degli Esposti et al. 2014) are annotated as shown in the legend within the diagram. Note that *Methyломicrobium* and *Methylobacter* are γ -proteobacteria and therefore have ISP proteins with multiple insertions that are not present in several α -proteobacteria and mitochondria (Degli Esposti et al. 2014).

proposal that proto-mitochondria originated from these α -proteobacteria (Andersson et al. 1998; Williams et al. 2007; Sasser et al. 2011; Ferla et al. 2013).

I found three transitions in β -proteobacteria that may be compared with the reconstructed evolution of mitochondria (Degli Esposti et al. 2014) and cover a phylogenetically divergent set of hosts, from unicellular eukaryotes to humans (fig. 4). These organisms belong to the large group of Burkholderiales and show the deletion of one or more terminal oxidases together with N-metabolism in the transition toward endosymbiotic lifestyles. The resulting subsets of bioenergetic systems remaining in symbionts/pathogens have, besides the common *bc₁* complex (fig. 4) the following: (1) a *ccb₃*-type oxidase together with the *aa₃*-type oxidase, as in the *Polynucleatus* symbiont of ciliates (Vannini et al. 2007); (2) only the *aa₃*-type oxidase, as in the fungal endosymbiont *Glomeribacter* (Ghignone et al. 2012); and (3) a *bd*-type

ubiquinol oxidase together with the *aa₃*-type oxidase, as in *Bordetella* pathogens. Hence, adaptation to a symbiotic lifestyle may follow pathways of serial loss of bioenergetic systems that resemble those previously deduced for proto-mitochondria: One ending with a *ccb₃*-type and another with a *bd*-type terminal oxidase (Degli Esposti et al. 2014).

However, proto-mitochondria retained a fully functional system of N-metabolism, which is instead lost in the examples of extant bacteria shown in figure 4. Conversely, N-metabolism is maintained in other bacteria that are phylogenetically close to methanotrophic endosymbionts (fig. 3). For example, *Methylocystis parvus* is very close to the *Methylocystis* strains that have been found as moss endosymbionts, which in turn are relatively distant to ecologically diverse organisms of the same genus such as *Methylocystis sp. SC2* (Dam et al. 2012; Belova et al. 2013). Notably, *M. parvus* does not possess the *bo₃*-type ubiquinol oxidase that is found in *Methylocystis sp.*

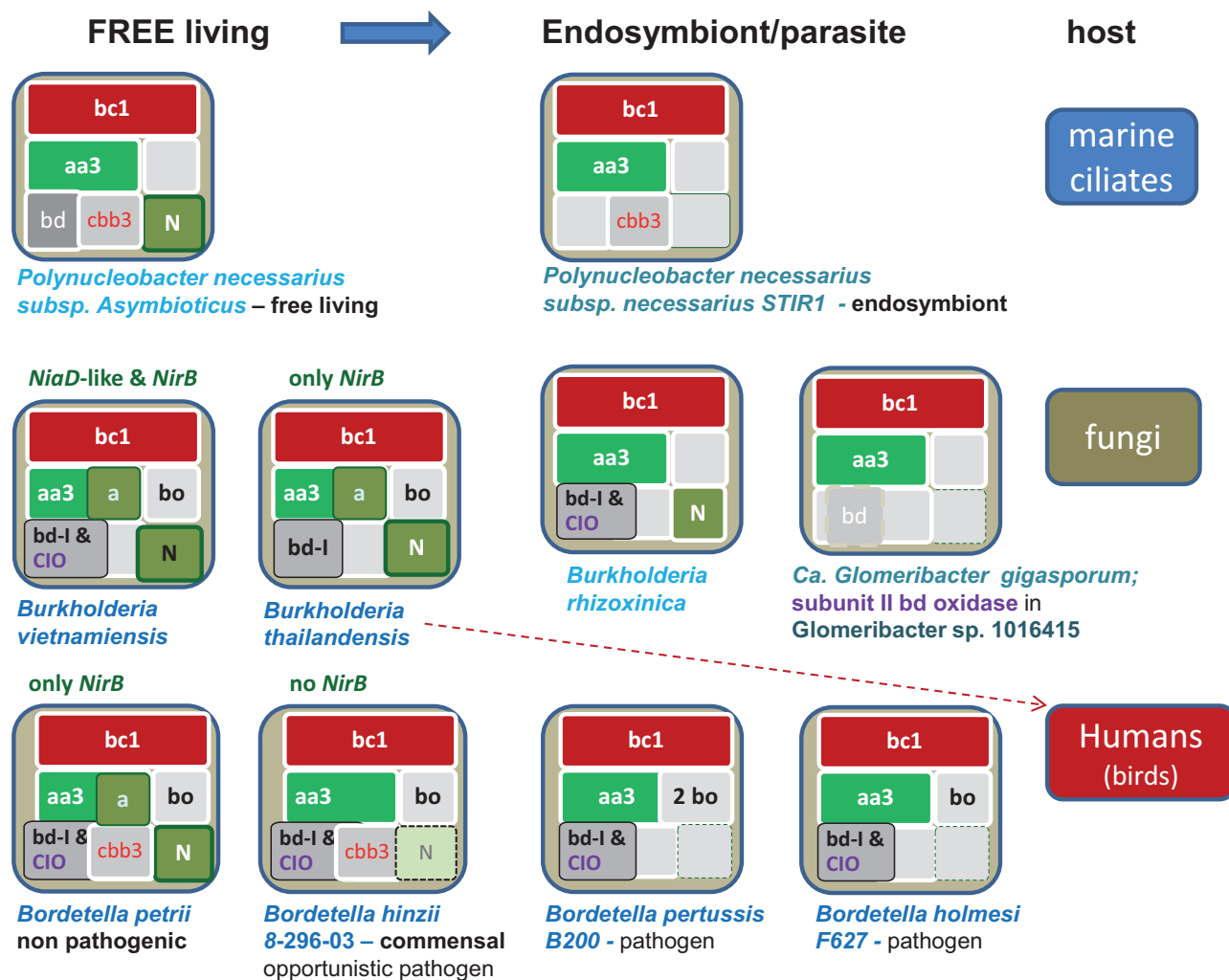


FIG. 4.—Reduction in bioenergetic systems in the transition from free-living to symbionts. Three different lineages of β -proteobacteria from the Burkholderiales order were analyzed on the basis of current genomic data and the reported lifestyle information indicating transitions to symbionts or pathogens (Vannini et al. 2007; Sachs et al. 2011; Ghignone et al. 2012). Bioenergetic systems are labeled as before (Degli Esposti et al. 2014) and variations in their elements of N-metabolism are color coded as indicated within figure 3. The red dash arrow indicates a behavior as human opportunistic pathogen of a plant-associated *Burkholderia*. See Lackner et al. (2011) and Ghignone et al. (2012) for the related fungal pathogens. Note that subunit II of *bd*-type oxidase is retained in the indicated *Glomeribacter* species as a likely relic of this bioenergetic system.

SC2 and other free-living members of the same group (fig. 3). Clearly, we need to wait until the completion of the genome of *Methylocystis bryophila* (Belova et al. 2013) or other moss endosymbionts to verify whether the specific loss of this bioenergetic system is a characteristic that methanotrophic α -proteobacteria may share with the presumed adaptation of proto-mitochondria to endosymbiosis (Degli Esposti et al. 2014). The same applies to the methanotrophic symbionts of deep-sea invertebrates (Fujiwara, Kawato, et al. 2010; Cavanaugh et al. 2013), some of which are closely related to the free-living γ -proteobacterium *Methylobacter*, also shown in figure 3. Intriguingly, these methanotrophic endosymbionts characteristically show stacks of intracytoplasmic

membranes (ICMs) that resemble mitochondrial cristae, as documented by the representative microscopic images collected in fig. 5 (Reed et al. 1980; Fujiwara, Takai, et al. 2000; Dunfield et al. 2010; Fujiwara, Kawato, et al. 2010; Dedysh 2011; Cavanaugh et al. 2013). Amazingly, this similarity has not been noted before.

In sum, *bd*-type ubiquinol oxidase is the bioenergetic system that appears to be most frequently retained in aerobic endosymbionts or pathogens that can be compared with mitochondria. Traces of its progressive loss are present in diverse proteobacterial lineages, such as the α -proteobacterium *Rickettsia* (Degli Esposti et al. 2014) and the β -proteobacteria *Bordetella* and *Glomeribacter* (fig. 4). These lineages include

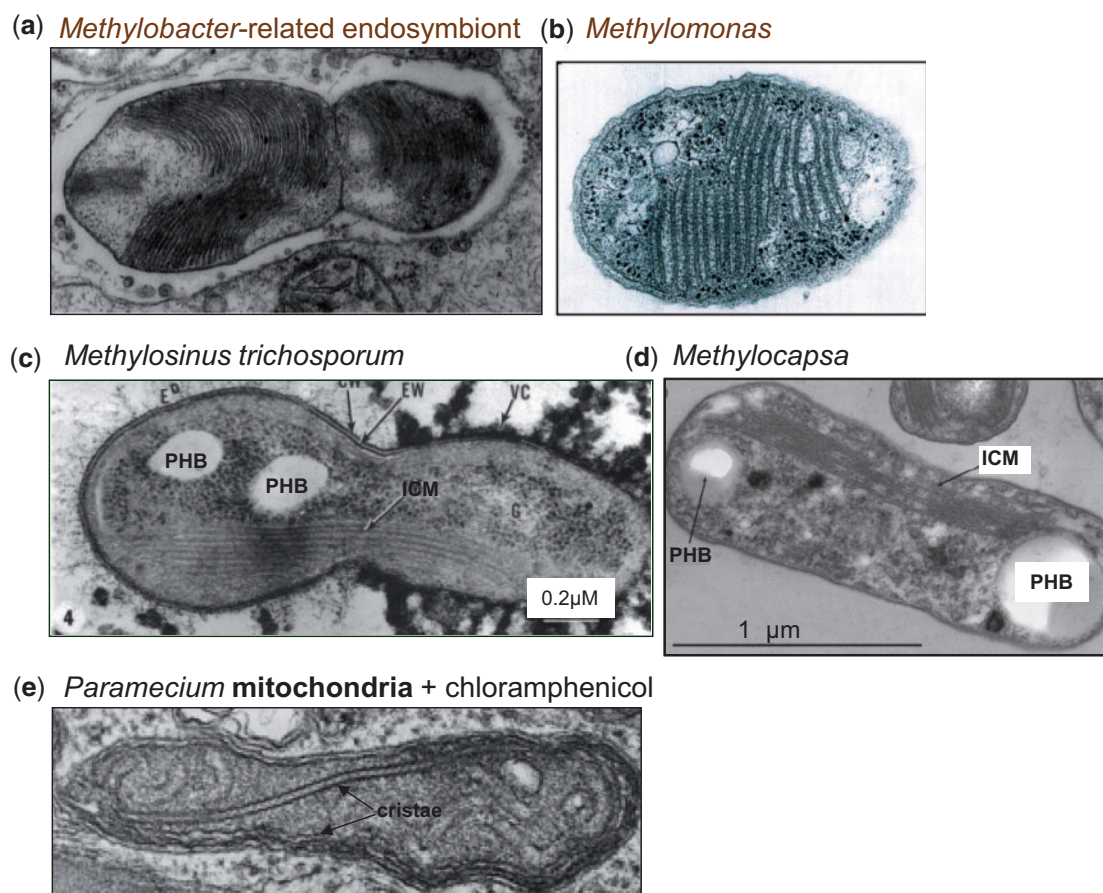


Fig. 5.—Membrane structures of methanotrophs resembling mitochondrial *cristae*. Electron microscopy images of methanotrophic bacteria refer to symbionts of clams (A; Fujiwara, Takai, et al. 2000) or plants of bog peats closely related to *Methylomonas* (B; Kip et al. 2011), or free-living *Methylosinus* (C, middle; Reed et al. 1980) and *Methylocapsa* (D; Dunfield et al. 2010). Note the resemblance of their ICMs with mitochondrial *cristae*, for comparison shown in a micrograph of the protist *Paramecium* (E; modified from Adoutte et al. 1972). Reserve deposits of poly hydroxy butyrate are labeled as PHB. Note: Images have been modified from the cited references.

species showing functionally intact *bd*-oxidases that lie aside species that either retain only one catalytic subunit or exhibit clear degradation of the gene cluster, as well as species that have lost the same gene cluster altogether. Moreover, I found the intact gene for one or both catalytic subunits of *bd*-type oxidase in the genome of some invertebrates, in particular the fruit fly *Ceratitis capitata* (accession: XP_004532875 for subunit I and XP_004532875 for subunit II), as well as the annelid *Capitella teleta* (accession: ELU02357) and the nematode *Caenorhabditis remanei* (accession: CRE_07978). Because *bd*-type oxidases are exclusively found in bacteria (Borisov et al. 2011), the presence of their genes in eukaryotic genomes must derive from the sequencing of DNA fragments belonging to associated, most likely symbiotic bacteria. In contrast, catalytic subunits of the *ccb3*-type oxidase have not been found in currently available genomes of metazoans. All these indications sustain the deduction that loss of *bd*-type

oxidase could be the last step in the bioenergetic evolution of proto-mitochondria (Degli Esposti et al. 2014).

The *bc₁* Complex of Bacterial Endosymbionts and Evolution of Its Catalytic Subunits

Compared with the bioenergetic systems of terminal oxidases, little is known about the changes in the *bc₁* complex that may be associated with symbiosis and its transition from free-living lifestyles. So far, I found only two cases that may provide some clues on this topic. The first case regards the presence of the entire *petABC* operon of the bacterial complex (coding for ISP, cytochrome *b*, and cytochrome *c₁* in sequence) within the genome of the small crustacean *Daphnia* (accession for ISP: EFX83890, DAPPUDRAFT_47869—Colbourne et al. 2011; table 1). The bacterial proteins of the *bc₁* complex are clearly

cytochrome/b6 analysis	characters													yeast numbering										haem c-1		haem c-2		cyt c1		
	Organism and protein (aa)	insert 184	W30	84	G100	F129	S140	T145	V146	H202	209-211	D229	E272	leader c1	cyt c-1	M ligand c-1	cyt c-2 c1	M ligand c-2	vs b/c1	vs b/c1										
	cytochrome b6	yes, V b6	Y- b6	W- b6	V- b6	G- b6	K- b6	I- b6	R- b6	DEL- b6	D	E	NO	CxxCH	MP	MP	MP	b not b6	b6 & not b	c fused										
yeast mtDNA cyt b	NO	W	N	G	F	S	T	V	H	NO	yes	E	NO	NO	NO	NO	NO	NO	12	0	no									
Saccharibacter	NO	W	G	yes	yes	yes	yes	yes	yes	NO	yes	yes	NO	NO	NO	NO	NO	NO	11	0	no									
Tistrella	NO	yes	yes	yes	yes	yes	yes	yes	yes	NO	yes	yes	NO	NO	NO	NO	NO	NO	12	0	no									
Phaeospirillum	NO	yes	yes	yes	yes	yes	yes	yes	yes	NO	yes	yes	NO	NO	NO	NO	yes	yes	16	0	yes									
Afiopia	NO	yes	yes	yes	yes	yes	yes	yes	yes	NO	yes	yes	yes	NO	NO	NO	yes	yes	17	0	yes									
Bradyrhizobium	NO	yes	yes	C	yes	yes	yes	yes	yes	NO	yes	yes	yes	NO	NO	NO	yes	yes	16	0	yes									
Nostoc cyt b6	yes	Y	W	V	V	G	K	I	R	yes	yes	yes	NO	NO	NO	NO	NO	NO	2	10	no									
Kueneia b6 256	yes	Y	W	V	V	G	yes	yes	R	end	yes	yes	NO	NO	NO	NO	NO	NO	5	7	no									
Kueneia Anammox 630	yes	Y	F	V	L	G	Q	yes	R	NO	yes	yes	yes	yes	yes	yes	yes	yes	9	8	yes									
KSU-1 Anammox 598	yes	Y	Y	V	L	G	Q	yes	R	NO	yes	yes	yes	yes	yes	yes	yes	yes	9	8	yes									
Schlesneria paludicola 625	yes	Y	F	V	L	G	K	yes	R	yes	yes	yes	yes	yes	MA	yes	yes	yes	8	9	yes									
Planctomyces maris	yes	F	Y	V	L	G	Q	I	R	NO	Y	yes	yes	yes	MS	yes	yes	yes	7	10	yes									
Isoaphaera pallida	yes	Y	F	V	L	G	K	yes	R	yes	yes	yes	yes	yes	yes	yes	yes	yes	7	10	yes									
Singulisphaera	yes	Y	F	V	L	G	K	yes	R	yes	A	yes	yes	yes	NO	NO	NO	5	12	yes										
Blastopirellula	yes	Y	W	V	V	S	S	yes	V	NO	H	D	NO	yes	yes	yes	NO	NO	4	13	yes									
Opiritus terrae	yes	Y	F	T	yes	A	V	yes	L	NO	yes	D	NO	yes	yes	NO	NO	NO	5	12	yes									
Leptospirillum ferrox. 460	yes	Y	W	V	V	yes	A	I	yes	R	yes	Q	A	NO	yes	yes	NO	NO	2	15	yes									
Nitrospira defluvi 441	yes	Y	W	T	yes	A	Q	I	R	yes	P	yes	NO	yes	yes	NO	NO	NO	4	13	yes									
Acidiphilium	NO	W	V	G	F	A	L	I	R	yes	yes	P	NO	NO	NO	NO	NO	NO	5	7	no									

Fig. 6.—Comparison of the molecular characters of cytochrome *b* from diverse bacterial lineages. The molecular characters which are conserved among all the cytochrome *b* proteins of the *bc*₁ complex but not in the paralog *b*₆ proteins (in green) are annotated following the numbering of the yeast sequence (Degli Esposti et al. 1993—see supplementary fig. S3a and b, Supplementary Material online for a representative alignment). The two c hemes that are present at the C terminus of some Planctomycetes’ proteins (Kartal et al. 2013) are designated heme c-1 and heme c-2. Fused cytochrome *c*₁ of α - and γ -proteobacteria align only with the heme-binding regions of heme c-2 (supplementary fig. S3b, Supplementary Material online). The amino acid changes that are structurally different from the conserved characters are highlighted in yellow. The total number of characters matching those conserved in either cytochrome *b* (*b* not *b*₆) or cytochrome *b*₆ (*b*₆ not *b*) are listed at the far right of the table—note that the maximal number is 17 for cytochrome *b* fused with *c*₁ and 12 for normal cytochrome *b*. *Leptospirillum ferrox.* refers to the 460 residues protein (accession: BAM07238) from *Leptospirillum ferrooxidans* C2-3, an organisms related to *Nitrospira* (Lücker et al. 2010). Note that *Acidiphilium* cytochrome *b* is degenerate for it does not have all the conserved histidines that function as heme ligands (annotated in supplementary fig. S3a, Supplementary Material online).

different from those of *Daphnia* mitochondria, but very close to those of a uncultivated α -proteobacterium from a ground-water metagenomic survey (accession: EKE09606 for ISP and EKE09607 for cytochrome *b* fused with cytochrome *c*₁—a protein 653 residues long; Wrighton et al. 2012). Although this metagenomic survey predominantly dealt with anaerobic bacteria, it also reported some potentially aerobic α -, β -, and γ -proteobacteria apparently related to known taxa (supplementary information in Wrighton et al. 2012). Sequence analysis of the ISP protein of the α -proteobacterium above indicates a closeness to Rhodospirillales that is partially confirmed by phylogenetic analysis of the fused cytochrome *b* protein. The latter shows an immediate ancestral position with respect to the cytochrome *b* proteins fused with cytochrome *c*₁ that are found in some Rhodospirillales and, characteristically, in most members of the Bradyrhizobiaceae family of α -proteobacteria (Thöny-Meyer 1997; supplementary fig. S3a and b, Supplementary Material online). Interestingly, the previously mentioned *T. turnerae*—a γ -proteobacterial endosymbiont of wood-boring clams—possesses multiple genes for cytochrome *b* fused with cytochrome *c*₁, only two of which are associated with an ISP gene thereby producing two separate operons for the *bc*₁ complex (Yang et al. 2009). Although similar proteins are present in closely related free-living bacteria, only *Teredinibacter* shows multiple genes coding for slightly different versions of ISP and fused cytochrome *b* proteins, a rare situation in proteobacteria (ten Brink et al. 2013). Among γ -proteobacteria, in fact, only the

genus *Acidithiobacillus* shows the duplication of *bc*₁ operon (ten Brink et al. 2013), without multiple versions of isolated ISP, which are instead found as a result of specific duplication of the ISP gene in Cyanophyta, as well as in some α - and β -proteobacteria (Schneider and Schmidt 2005). Of note, methanotrophs of the *Methylocystis* genus and some Bradyrhizobiaceae show vestiges of an ancient duplication and diversification of their Rieske ISP, because they retain a long ISP2 form that is now present within an isolated gene cluster resembling some of those present in *Teredinibacter* (Schneider and Schmidt 2005; Degli Esposti et al. 2014).

Whether or not duplication and diversification of the *bc*₁ operon can be considered among bioenergetic features associated with the transition from free-living to endosymbiosis, I take the opportunity raised by the above observations to provide an answer to the yet unresolved question: What’s the origin and evolutionary relevance of cytochrome *b* fusion with cytochrome *c*₁? Previous analyses focused on ancient bacterial lineages, finding diverse forms of cytochrome *b*₆—the “green” paralog of mitochondrial cytochrome *b* corresponding to its N-terminal domain (Degli Esposti et al. 1993; Lebrun et al. 2006)—fused with c-type cytochromes in Planctomycetes and Nitrospirales (Dibrova et al. 2013; ten Brink et al. 2013). In particular, anammox Planctomycetes such as *Kueneia* possess large sets of bioenergetic proteins fused with c-type cytochromes (Dibrova et al. 2013; Kartal et al. 2013). My detailed sequence analysis (fig. 6 and supplementary fig. S3, Supplementary Material online) shows a

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progressive diversification of proteobacterial cytochrome *b* proteins fused with cytochrome *c*₁ from some of these Planctomycetes proteins that are fused with two *c*-type cytochromes, for example, *Shlesneria* cytochrome *b/c* subunit (accession: WP_010584211 or ZP11092181—Guo et al. 2012), which has not been considered in recent surveys (Dibrova et al. 2013; ten Brink et al. 2013). This finding supports previous structural deductions suggesting that mitochondrial cytochrome *c*₁ may derive from a collapsed di-heme cytochrome *c* (Baymann et al. 2004).

Consequently, the answer to the above question is that cytochrome *b* fusion with cytochrome *c*₁ in proteobacteria derives from fused proteins present in Planctomycetes such as *Shlesneria* that have been probably inherited via vertical transmission, given the gradual changes in key amino acids (fig. 6)—but see ten Brink et al. (2013) for a different opinion. In any case, these fused proteins may represent the oldest version of the ancestors of mitochondrial cytochrome *b*, as in the case of fused COX1–3 for mitochondrial COX1 that have been mentioned before (cf. Degli Esposti et al. 2014). Within proteobacteria and outside the Bradyrhizobiaceae family, fused cytochrome *b/c* is found only in 13 γ -proteobacteria (table 1), two genera of Rhodospirillaceae, *Phaeospirillum* and *Magnetospirillum*, and in *Rhodovulum*, an organism normally classified among the Rhodobacterales but probably belonging to the Rhizobiales order (Ferla et al. 2013). The ISP proteins from these Rhodospirillaceae organisms and *Rhodovulum* show at least one conserved insertion that is not present in mitochondrial ISP and are therefore unlikely to be among the immediate ancestors of mitochondrial ISP (Degli Esposti et al. 2014). Conversely, the ISP sequences of Bradyrhizobiaceae appear to be as close to mitochondrial ISP as those of methylotrophs such as *Beijerinckia* and *Methyloferula* (cf. Degli Esposti et al. 2014). Such a circumstance underlines the importance of the molecular features of ISP in discriminating among possible candidates for the closest living relative of mitochondrial ancestors.

Conclusion—A Methanotrophic Past for Mitochondria?

The new information presented in this review (figs. 2, 3, and 5 and Supplementary figs. S1–S3, Supplementary Material online) supports and refines recent indications that extant methylotrophs may include the relatives of proto-mitochondria (Degli Esposti et al. 2014). This proposition contrasts with the majority of previous studies that had indicated different bacteria as the possible ancestors of mitochondria. Frequently, such studies have supported the possibility that proto-mitochondria originated from *Rickettsia* and its relatives (Andersson et al. 1998, 2003; Fitzpatrick et al. 2006; Williams et al. 2007; Sasser et al. 2011; Ferla et al. 2013). However, the possible mitochondrial origin from within the Rickettsiales, which include only obligate endosymbionts, is

viewed with increasing scepticism (Esser et al. 2004; Atteia et al. 2009; Abhishek et al. 2011; Gray 2012; Müller et al. 2012), because it suffers from the major problem of long-branch attraction distorting phylogenetic analysis. Moreover, in bioenergetic terms, all Rickettsiales have lost the elements of N-metabolism that are still present in mitochondria from fungi and heterokonts (Takaya 2009), while often retaining the noneukaryotic *bd*-type ubiquinol oxidase (Degli Esposti et al. 2014). This situation is similar to that encountered in various transitions from free-living to symbiotic lifestyles among extant bacteria (fig. 4), thereby suggesting phenomena of convergent evolution related to endocellular symbiosis. Finally, the proponents of the Rickettsiales origin for proto-mitochondria have as yet to provide a plausible answer to the obvious question: Which organisms were they a parasite of?

Methylotrophs are a general definition for chemolithotrophic bacteria that utilize C1 compounds as their carbon and energy source and include both methane and ammonia-oxidizing organisms (Chistoserdova et al. 2009). Methane oxidation arose when ambient oxygen levels increased dramatically on the planet, approximately at the time in which β - and γ -proteobacteria separated from the ancestors of current α -proteobacteria (Battistuzzi et al. 2004). Although methylotrophs or methanotrophs had not been considered before as possible ancestors of mitochondria, they now appear to be good candidates for proto-mitochondria, for the following reasons:

- they are evolutionary ancient, as just discussed, consistent with the accepted notion that proto-mitochondria evolved early after the separation of the γ - and β -lineages (Williams et al. 2007);
- they are related to autotrophic organisms forming permanent symbioses with invertebrate animals living in deep-sea habitats (Dubilier et al. 1999; Fujiwara, Takai, et al. 2000; Cavanaugh et al. 2013), as well as with mosses of northern wetlands (Kip et al. 2011; Dedysh 2011);
- they have a wide metabolic versatility which includes the cycles of glyoxylate, citrate, and serine that in eukaryotic lineages are segregated within organellar compartments that may also derive from mitochondria (Mohanty and McBride 2013);
- they have a bioenergetically efficient respiratory chain, even under low oxygen concentrations, for the presence of multiple terminal oxidases (Dam et al. 2012; Degli Esposti et al. 2014);
- finally, they characteristically contain “ICMs” derived from invaginations of their inner membrane (Hagen et al. 1966; Watson and Mandel 1971; Lynch et al. 1980; Reed et al. 1980; Fujiwara, Takai, et al. 2000; Dunfield et al. 2010; Dedysh 2011; Kip et al. 2011; Cavanaugh et al. 2013). Such tightly packed membranes resemble mitochondrial cristae (fig. 5) and are equally required to expand the surface available to plug in

respiratory complexes that provide bioenergy, together with membrane-bound methane mono-oxygenase (Chistoserdova et al. 2009).

It is surprising that such evident structural similarities between methanotrophs and mitochondria have passed unnoticed for almost 50 years (Hagen et al. 1966). However, the overlooked cristae-like structures of methanotrophs may constitute yet another example of how science has become compartmentalized in specialized fields, in this case a niche of microbiology and classical bioenergetics. No doubt, Lynn Margulis has encountered examples of the same kind in her pioneering work that in time established the endosymbiotic theory of eukaryotic cell evolution (Sagan 1967).

Taking together the timeline for bacterial evolution (Battistuzzi et al. 2004) with the restricted distribution of methanotrophic symbionts to α - and γ -proteobacteria (Fujiwara, Takai, et al. 2000; Kip et al. 2011; Dedysh 2011) and the list of rare bioenergetic features shared by proteobacteria and mitochondria, which are more common between α - and γ - than between α - and β -proteobacteria (table 1), it seems possible that proto-mitochondria have evolved from the common ancestors of methanotrophic α - and γ -proteobacteria. The phylogenetic trees of COX proteins (fig. 2) support this possibility. The invention of the metabolic pathways of methane and ammonia oxidation immediately followed the large increase in ambient oxygen that occurred around 2 billion years ago, allowing autotrophic ways of life which are now retained by the abovementioned methanotrophs (Battistuzzi et al. 2004; Chistoserdova et al. 2009; Vlaeminck et al. 2011). These bacteria also possess the largest variety of COX operons and molecular forms of their catalytic subunits (supplementary figs. S1 and S2, Supplementary Material online), as a result of multiple events of operon and gene duplication. Indeed, the reconstruction of the molecular evolution of COX3 proteins and their binding strength for oxygen-modulating phospholipids seems to recapitulate a progressive adaptation to increasing levels of O₂, gauged in terms of decreasing oxygen affinity to maintain maximal efficiency of the oxidase reactions (Riistama et al. 1996; Degli Esposti et al. 2014).

These considerations refer to the symbiont independently on the host organism that was involved in the eukaryogenic event leading to the evolution of our cells. Recent evidence points to an archaeal nature for such a host (Williams et al. 2013) without defining ecological and metabolic features that may help indicating the type of symbiosis that it may favor. Certainly, this host was adapted to anaerobic conditions, because the evolution of proto-mitochondria and the earliest eukaryotic cells occurred when oxygen levels were still very low in the oceans (Johnston et al. 2009; Müller et al. 2012). It is therefore plausible that mitochondrial progenitors derive from organisms that had experimented with a wide variety of oxygen-reacting systems and thus retained great plasticity

in their adaptation to environments poor in oxygen, a trait that is partially retained in eukaryotes adapted to anaerobic environments (Mentel et al. 2014). Hence, those progenitors were probably related to the ancestors of extant proteobacterial methanotrophs, many of which have the capacity of establishing endosymbiosis with “primitive” animals such as gutless worms, as well as primitive plants such as mosses. A methanotrophic past for mitochondria is thus looming; future research will clarify its validity and evolutionary implications.

Supplementary Material

Supplementary figures S1–S3 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org>).

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