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From Alphaproteobacteria to Proto-Mitochondria

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Introduction

The class of alphaproteobacteria is the second largest among proteobacteria after that of gammaproteobacteria, according to the number of genomes and proteins present in current NCBI databases and also in terms of taxonomic richness (Schulz et al. 2017), but it is the most diverse in both phylogenetic breadth and functional properties (Garrity et al. 2005, Gupta and Mok 2007, Williams et al. 2007, Ferla et al. 2013, Degli Esposti and Martinez-Romero 2017). Alphaproteobacteria include some of the most widespread and economically important prokaryotes, from agriculture to biotechnology and human health. Indeed, entire groups of this class are noxious pathogens of humans and animals with some, in particular the Rickettsiales, living solely as obligate endocellular parasites of eukaryotes, from protists to humans. Alphaproteobacteria share the propensity to intimately associate with plants and animals with other bacteria, but this propensity is so diffuse among their various phylogenetic groups that it has sustained the concept that proto-mitochondria originated within this bacterial class (Yang et al. 1985, Esser et al. 2004, Wu et al. 2004, Fitzpatrick et al. 2006, Williams et al. 2007, Atteia et al. 2009, Gray et al. 1999, Gray 2012, Ferla et al. 2013).

Initially, the alpha subdivision was dominated by purple non sulfur bacteria, such as *Rhodobacter* and *Rhodospirillum*, which have played a major role in the history of microbiology (Truper and Pfennig 1981, Woese et al. 1984). Contrary

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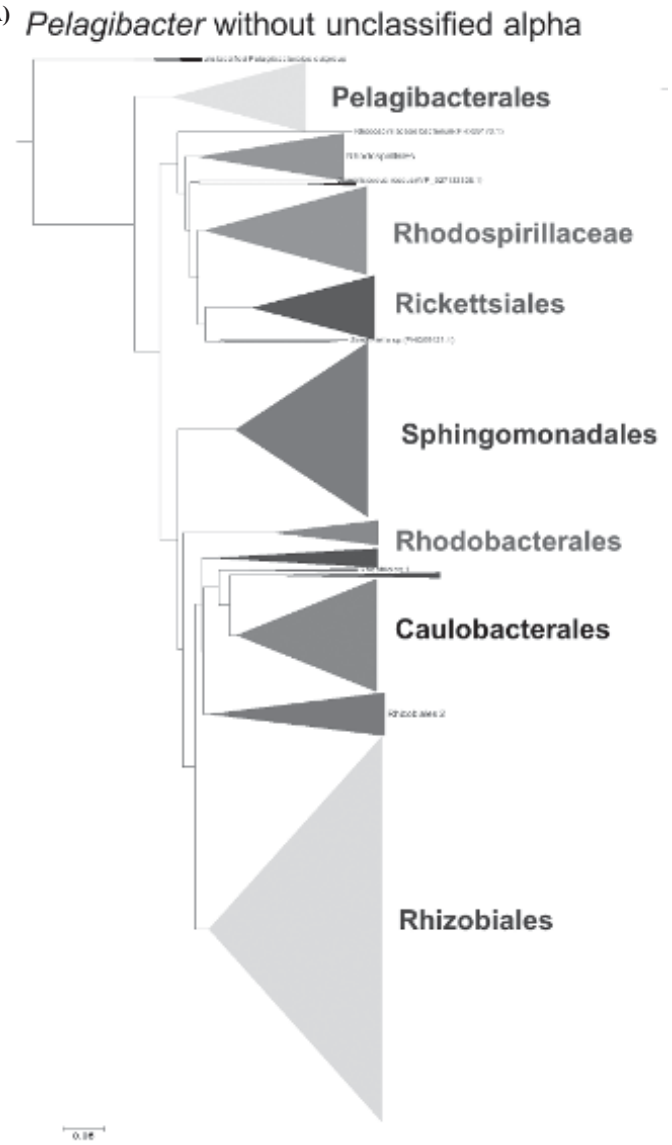
to the initial observations (Woese et al. 1984, Woese 1987), the great majority of the two thousand taxa now classified within the class (Chapter four) are not photosynthetic (Louca et al. 2016, Degli Esposti and Martinez Romero 2017). This underlies the emerging concept that photosynthesis has been acquired and lost multiple times via Lateral Gene Transfer (LGT), in proteobacteria as in other bacterial *phyla* (Martin et al. 2018).

Alphaproteobacteria are subdivided in an increasing number of orders and unclassified taxa. The latest PATRIC web repository https://www.patricbrc.org/view/Taxonomy/28211#view_tab=taxontree (accessed on 9 Dec 2017) lists a total of 16 orders of alphaproteobacteria. The orders with the largest number of taxa remain, in decreasing number of available genomes: Rhizobiales, Rhodobacterales, Sphingomonadales, Rhodospirillales and Rickettsiales, followed by Caulobacterales and Pelagibacterales. These are the same orders considered 10 years ago by Williams et al. (2007), following almost exactly their phylogenetic sequence from the latest divergent to the most basal. The basic phylogenetic tree of alphaproteobacteria was established in previous works (Lee et al. 2005, Gupta 2005), which had not considered *Pelagibacter* and its relatives. The phylogenetic position of these common marine bacteria has remained controversial since the first tree of reference reported by Williams et al. (2007), in part due to the diversity of molecular approaches used (Ferla et al. 2013, Luo 2015). However, a major factor influencing the relative phylogenetic position of *Pelagibacter* and its relatives, now forming the order of Pelagibacterales, is the taxonomic sampling of the alphaproteobacterial organisms considered, as exemplified by phylogenetic trees of the ubiquitous cytochrome *b* protein (Fig. 1).

The taxonomic span or breadth of alphaproteobacteria has increased considerably in the last few years, owing to programs designed to reduce the human bias in current genomic databases and the dramatic increase in metagenomic information on previously unclassified organisms (see Table 1 in Chapter four, and also later). It now appears established that *Magnetococcus* and its relatives (grouped in the order of Magnetococcales; Bazylinski et al. 2013a) form the basal branch of the class (Schübbe et al. 2009, Ferla et al. 2013, Degli Esposti et al. 2014, Wang and Wu 2015). When these and various unclassified alphaproteobacteria are considered, the phylogenetic trees contain Pelagibacterales in an intermediate branch, as shown in Fig. 1B—in agreement with some articles (Viklund et al. 2012, 2013, Luo 2015)—rather than in a deep branch as reported in other papers (Williams et al. 2007, Georgiades et al. 2011 Smith et al. 2012, Ferla et al. 2013) and shown in Fig. 1A, where Magnetococcales were not picked by the initial blast search.

The issue of the taxonomic position of Pelagibacterales is of particular importance for the origin of proto-mitochondria, since the works that consider these organisms as basal to the alpha class also imply that Pelagibacterales subtended the phylogenetic origin of proto-mitochondria (Thrash et al. 2011, Georgiades et al. 2011, Ferla et al. 2013). This topic will be elaborated further in the final part of the chapter because of its relevance to the the origin of proto-mitochondria.


(A) Pelagibacter without unclassified alpha
Pelagibacterales
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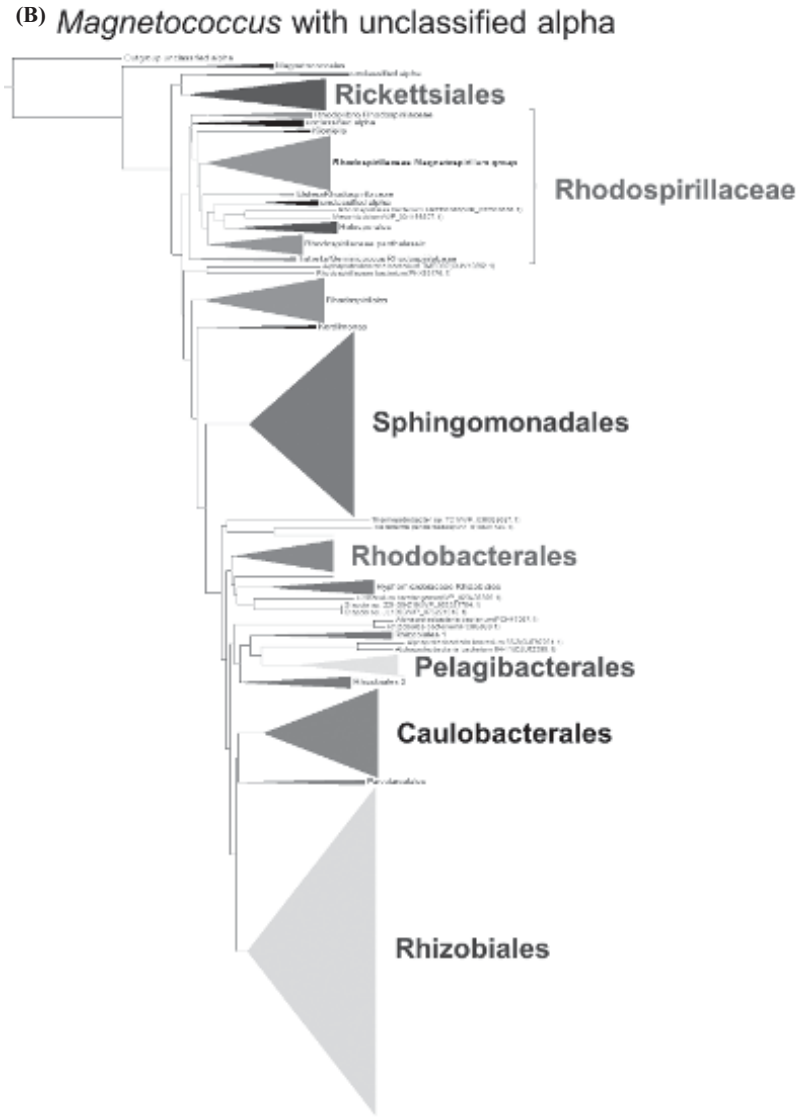

Fig. 1. Phylogenetic tree of alphaproteobacteria using cytochrome *b* as the universal marker. (A) The NJ tree was obtained by a wide DeltaBLAST (Boratyn et al. 2012) using as a query the cytochrome *b* of *Pelagibacter ubique* over all major taxa of alphaproteobacteria excuding Magnetococcales and unclassified alphaproteobacteria. (B) The NJ tree was obtained by a wider DeltaBLAST than in A, using as a query the cytochrome *b* of *Magnetococcus marinus* including Magnetococcales and unclassified alphaproteobacteria.

An Iron Wire in the Evolution of Alphaproteobacteria

The controversy surrounding the origin of proto-mitochondria and the taxonomic position of Pelagibacterales mentioned above has overshadowed the significance of Magnetococcales in the evolution of alphaproteobacteria, especially in regard to their functional properties. Some of these properties have already been discussed in Chapter five in comparison with deep-branching gammaproteobacteria. The type organism of Magnetococcales was previously characterized as microaerophilic magnetic coccus, strain MC-1 (Frankel et al. 1997). Renamed as *Magnetococcus marinus*, it entered the phylogeny of alphaproteobacteria the same year (Esser et al. 2007) in which the controversial issue regarding Pelagibacterales vs. proto-mitochondria started (Williams et al. 2007). Although earlier reported to be at the basis of the alphaproteobacterial lineage (Schübbe et al. 2009), *Magnetococcus* has been considered in major phylogenetic works only since 2013 (Bazylinski et al. 2013a, Ferla et al. 2013, Degli Esposti et al. 2014, Ji et al. 2017). Some initial uncertainties regarding the affiliation to the alpha class (Esser et al. 2007, Schübbe et al. 2009, Thiergart et al. 2012) have been set aside by the consistent finding of its basal position among alphaproteobacteria in phylogenetic trees obtained with different markers (Bazylinski et al. 2013a, Ferla et al. 2013, Degli Esposti et al. 2014, Morillo et al. 2014, Wang and Wu 2015, Ji et al. 2017).

Genomic analysis of *Magnetococcus* has shown a high degree of chimaerism, since the affiliation of all coded proteins produce a mosaic picture in regard to their closest homologs, with only about one third of proteins showing close affiliation to alphaproteobacteria (Esser et al. 2007, Schübbe et al. 2009, Ji et al. 2017). This result has been recently confirmed by using a simplified version of all reciprocal blast approach focused on proteins for energy metabolism (Degli Esposti 2017). When not arising from a secondary evolutionary event as in *Ca.* Tremblaya (see Chapter six), genome chimaerism may be considered a telltale of antiquity for a bacterium, as in the case of Acidithiobacillia (Chapter five). On one side, chimaerism reflects taxonomically deep evolutionary relationships with ancestral lineages, while on the other side it derives from affiliations with more modern taxa of the same class. Intriguingly, the same applies to magnetotaxis, which is the distinctive trait of *Magnetococcus* (Schübbe et al. 2009, Ji et al. 2017). Of note is that *Magnetospirillum magneticum* has been reported to display the second largest degree of genomic chimaerism among alphaproteobacteria after *Magnetococcus* (Esser et al. 2007).

Magnetococcus and other Magnetococcales share magnetotaxis with three genera of alphaproteobacteria belonging to the family Rhodospirillaceae - notably *Magnetospirillum*, where this peculiar trait was first described, as well as *Magnetovibrio* and *Magnetospira* (Bazylinski et al. 2013a, b, Lefèvre and Bazylinski 2013, Lin et al. 2017a). Among the whole *phylum* of proteobacteria, magnetotaxis is additionally present in some deltaproteobacteria such as *Geobacter magneticus* (Lefèvre et al. 2012, Lin et al. 2017a) and a few gammaproteobacteria (Lefèvre and Bazylinski 2013, Leão et al. 2016). However, it is relatively common

in organisms of the *phylum* Nitrospirae (Jogler et al. 2011, Lin et al. 2017a,b, Wang and Chen 2017, Lin et al. 2017c—cf. Chapter three), from which it is supposed to have been vertically transmitted to proteobacteria (Lefèvre et al. 2013, Zeytuni et al. 2015, Lin et al. 2017a).

Magnetococcus and Magnetospirilli, as well as magnetotactic Nitrospirae, characteristically contain an array of intracellular vesicles enriched in Fe compounds such as magnetite, the magnetosomes (Lefèvre et al. 2013, Bazylinski et al. 2013b, Li et al. 2014, Dziuba et al. 2016, Uebe and Schüler 2016, Kolinko et al. 2016, Lin et al. 2017a). Fundamentally, magnetosomes serve to orient the bacteria towards water columns with oxygen gradient, so as to locate areas containing micromolar concentration of oxygen which are optimal for their growth (Lefèvre et al. 2013, Bazylinski et al. 1988, 2013b, Li et al. 2014, Uebe and Schüler 2016, Lin et al. 2017a). Hence, these microoxia-seeking bacteria are facultatively anaerobes in the most poignant significance of the term (cf. Chapter two). Besides sulfur oxidation, a trait already discussed in Chapter five, *Magnetococcus* shares with Magnetospirilli another metabolic trait that is typical of anaerobes, strictly or facultatively, namely the ionmotive Rnf complex (Biegel et al. 2011). The Rnf complex was originally discovered in *Rhodobacter* and hence named Rhodobacter nitrogen fixation (Biegel et al. 2011), since it physiologically reduces the ferredoxin that feeds electrons into the nitrogenase reaction. Reduction of ferredoxin is the reverse reaction of the thermodynamically more favourable reduction of NADH and is driven by the ionmotive function of the enzyme complex, which can pump either Na^+ or H^+ across the membrane (Biegel et al. 2011). It is likely that the Rnf complex predominantly functions in the same way in magnetotactic alphaproteobacteria, for both *Magnetococcus* and Magnetospirilli have the nitrogenase complex and therefore are capable of fixing nitrogen as free-living diazotrophs (Schübbe et al. 2009, Lefèvre et al. 2013, Bazylinski et al. 2013b, Dziuba et al. 2016). See also Chapter three, four and five for Rnf distribution among other facultatively anaerobic bacteria.

From Molecular Phylogeny to Functional Evolution of Alphaproteobacteria

A survey of the distribution of the Rnf complex in currently available genomes (January 2018) has revealed its presence in 27 alphaproteobacterial taxa, 21 more than eight years ago (Biegel et al. 2011). The majority of Rnf-containing taxa belong to the family Rhodospirillaceae, especially Magnetospirilli, but also four metagenomic *Azospirillum* strains that have been found in human gut and are strictly anaerobes (Fig. 2). These organisms are *Azospirillum* sp. CAG:239 and sp. CAG:260 (Nielsen et al. 2014, Degli Esposti et al. 2016), which are closely related to *Azospirillum* sp. 51_20 and *Azospirillum* sp. 47_25, respectively (Brown et al. 2016). Remarkably, they neither have membrane quinones nor cytochromes and thus rely only on the Rnf complex for pumping ions across the membrane to drive ATP production via protonmotive ATP synthase, as presented in Fig. 2A of

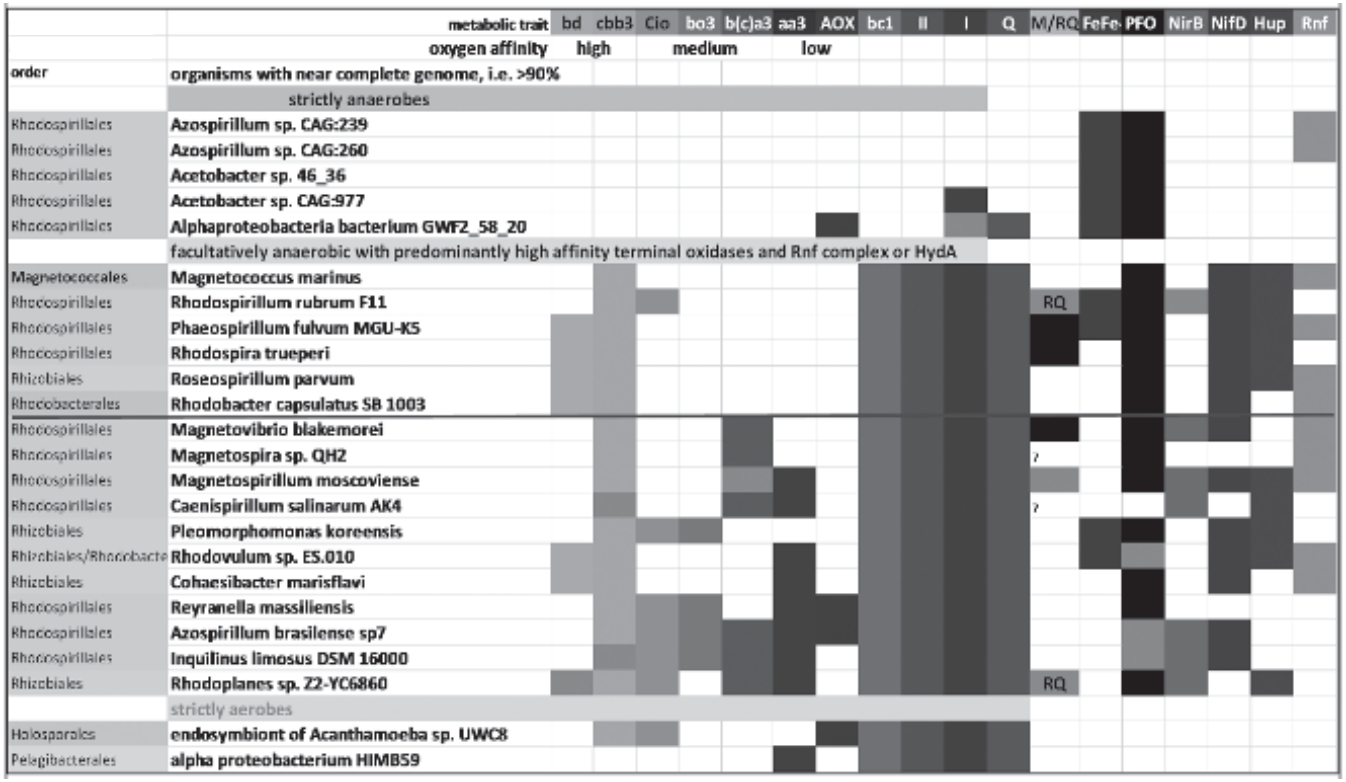

Fig. 2. Metabolic traits of selected alphaproteobacteria. The trait abbreviated as b(c)a3 includes HCO Oxidases of both the B family (ba3) and A2 type oxidases of the A family (aa3 oxidases) that often have a cytochrome *c* fused with subunit 2 as in *Bacillus* caa3 oxidases. Other traits are abbreviated as in chapter five.

Chapter two. Such a simplified protonmotive circuit is typical of strictly anaerobes of the Firmicutes *phylum*, in which ferredoxin is reduced by catabolic enzymes or hydrogenases (Biegel et al. 2011). In such metagenomic *Azospirillum* strains, ferredoxin is reduced primarily by Pyruvate-Ferredoxin Oxidoreductase (PFO; Fig. 2, cf. Chapter four) and can also be re-oxidized by one or more forms of [FeFe]-hydrogenases that are coded in their genomes and appear to be related to those present in anaerobic eukaryotes such as *Entamoeba* (Degli Esposti et al. 2016). This metabolic path is shared with strictly anaerobic prokaryotes such as Clostridiales and also aerotolerant sulfate-reducing deltaproteobacteria (see Chapter four), as well as strict anaerobes of the betaproteobacteria class such as *Sutterella* (see Chapter six). Therefore, it constitutes a basal trait inherited by proteobacteria from ancestral lineages of anaerobic prokaryotes, surviving as a metabolic adaptation to an anaerobic niche like the animal and human gut, which in several aspects appears to be a nutrient-rich environment favouring the survival of strictly anaerobes (Nielsen et al. 2014, Degli Esposti and Martinez-Romero 2017).

The scattered taxonomic distribution of the anaerobic traits that are concentrated in the above mentioned *Azospirillum* strains (Fig. 2) suggests important considerations regarding the functional evolution of alphaproteobacteria: (1) the progenitors of extant Rhodospirillaceae might have been strictly anaerobes and thus likely to be the most ancestral of the class—possibly even 'older' than the Great Oxygenation Event (GOE, Chapter two); (2) ancestral alphaproteobacteria adapted to the microoxic conditions that followed the GOE by acquiring or inheriting high affinity terminal oxidases such as the cbb3 oxidase present in *Magnetococcus* (Fig. 2); (3) subsequent adaptation to increased levels of oxygen entailed the additional acquisition of low affinity terminal oxidases and then a progressive loss of anaerobic traits. In this rationale, extant alphaproteobacteria that combine anaerobic traits such as PFO, [FeFe]-hydrogenases and the Rnf complex with high affinity terminal oxidases could be considered closest to the ancestors of the class. Genomic analysis indicates that such a combination is currently present in a handful of contemporary bacteria besides *Magnetococcus* (Fig. 2): photosynthetic Rhodospirillaceae closely related to *Rhodospirillum* like *Phaeospirillum* (Imhoff 2005, Degli Esposti and Martinez-Romero 2017); *Roseospirillum parvum*, a photosynthetic member of the Rhizobiales order that is classified within the family Rhodobiaceae (Glaeser and Overmann 1999, Permentier et al. 2000); and *Rhodobacter capsulatus*, the type species of Rhodobacterales (Gupta 2005, Williams et al. 2007). At difference with *Magnetococcus*, these organisms additionally contain an ubiquinol oxidase of bd-I type (Fig. 2), which has an oxygen affinity comparable to that of cbb3 oxidases (see Chapter two).

While the absence of low affinity terminal oxidases of the A family is typical for *Rhodospirillum* and related organisms (Imhoff 2005, Degli Esposti et al. 2014), it is exceptional in the case of *Rhodobacter capsulatus*, since its closely related *R. sphaeroides*, as most other members of the parent Rhodobacteraceae family, have one or more oxidases of the A family (Pereira et al. 2001, Degli Esposti et al. 2014, Degli Esposti and Martinez-Romero 2017). Indeed, the first crystal

structure of a family A oxidase has been obtained in *Paracoccus denitrificans*, a non-photosynthetic member of the same family (Iwata et al. 1995, Pereira et al. 2001). Therefore, it is quite possible that *R. capsulatus* has lost its original low affinity oxidase as a result of secondary evolutionary events, as in the case of other alphaproteobacteria (Fig. 2, cf. Degli Esposti and Martinez-Romero 2017). The same concept applies to *Roseospirillum parvum*, since all its relatives of the Rhodobiaceae family have at least one low affinity terminal oxidase of the A family (Degli Esposti and Martinez-Romero 2017). Following these considerations, then *Rhodospirillum/Phaeospirillum* (Imhoff et al. 1998) remain the alphaproteobacterial taxa that constitutively possesses the rare combination of anaerobic traits with high affinity terminal oxidases only, as in *Magnetococcus*. Hence, *Phaeospirillum* and *Rhodospirillum* may be viewed, functionally, as the most ancestral members of the alphaproteobacteria class, besides Magnetococcales.

Similar to sulfur-oxidizing taxa among the gammaproteobacteria (Chapter five), the genomic distribution of functional traits strongly indicates that the family Rhodospirillaceae is among the most basal in alphaproteobacteria, for it contains taxa possessing all the anaerobic and aerobic traits that are present in the whole class (Fig. 2). This conclusion is supported only in part by phylogenetic trees of ubiquitous proteins such as cytochrome *b* (Fig. 1), but could be in accord with the evidence that members of the family Rhodospirillaceae do not follow the tree topology of the rest of the Rhodospirillales order (see Ferla et al. 2013, and reference therein). However, it contrasts many reported trees of alphaproteobacteria in which members of the Rhodospirillaceae diverge later than either Rickettsiales or Pelagibacterales (Gupta and Mok 2007, Williams et al. 2007, Ferla et al. 2013, Viklund et al. 2013, Wang and Wu 2015). Those trees are often based upon rRNA phylogenies and consequently suffer from the dichotomy between ribosomal genes and the phenotypic traits of bacterial organisms, a problem that has been discussed previously in Chapters four and five of the book. The streamlined nature of the genome of Pelagibacterales, as well as the eroded and reduced genome of intracellular parasites of the Rickettsiales and Holosporales order, do not leave much space for metabolic traits that are no more used after diversification from ancestral alphaproteobacteria (Emelyanov 2003, Wu et al. 2004, Georgiades et al. 2011, Wang and Wu 2015). Indeed, the majority of the taxa classified under these orders currently have an aerobic metabolism that is based upon low affinity terminal oxidases, as mitochondria (Kurland and Andersson 2000, Emelyanov 2003, Giovannoni et al. 2005b, Boussau et al. 2004, Georgiades et al. 2011, Morris and Schmidt 2013). This functional evidence is in stark contrast with the basal position assigned to Rickettsiales or Pelagibacterales in traditional phylogenetic trees of alphaproteobacteria (Fig. 1, cf. Williams et al. 2007), a problem which will be discussed further in regard to the origin of proto-mitochondria at the end of this chapter.

The problems just discussed raise a fundamental question: how can we recognise secondary loss of aerobic traits or their possible acquisition by LGT rather than vertical inheritance from ancestral lineages? Evidently, phylogenetic trees

cannot provide an adequate answer (Chapter one). Integrated approaches are thus required, as pioneered by Degli Esposti et al. (2014). Central to these approaches is the systematic classification of the diverse variants of COX and other terminal oxidases that are present in alphaproteobacteria and define the plasticity of their aerobic metabolism (Imhoff 2005, Morris and Schmidt 2013, Degli Esposti 2014, Wang and Wu 2015). The following section will provide an updated view of the currently known variety of heme *a*-containing terminal oxidases.

The Variety of Heme a Oxidases in Alphaproteobacteria

The variety of aerobic metabolism present in alphaproteobacteria has been recognised at the very beginning of their classification on the basis of 16S rRNA in the 80s. In his classical review on bacterial evolution, Woese (1987) remarked: 'Aerobic metabolism also appears to have arisen a number of times in the alpha subdivision alone', citing Woese et al. (1984). Compared with the delta subdivision, alphaproteobacteria obviously emerged as a fundamentally aerobic lineage of proteobacteria, even if its initial members were predominantly facultatively anaerobes and photosynthetic (Schultz and Weaver 1982, Woese et al.1984, Woese 1987, Boussau et al. 2004, Imhoff 2005, Garrity et al. 2005). After the sequencing of the first *Rickettia* genome (Andersson et al. 1998), strictly aerobes became a fundamental part of the alphaproteobacterial lineage (Müller and Martin 1999, Kurland and Andersson 2000, Boussau et al. 2004, Gupta 2005). However, strictly aerobes constitute a minority of today's alphaproteobacteria with sequenced genomes, as previously found for other bacteria (Morris and Schmidt 2013). The great majority of alphaproteobacteria, therefore, are facultatively anaerobes or microaerobic (Morris and Schmidt 2013, Martin 2017). These definitions of aerobic metabolism, presented in Chapter two of the book, do not clarify how such a wide set of organisms live in so many different environments, adapting to oxygen levels that vary from basically zero—as in parts of animal guts, underground niches and sulfidic ocean zones—to fully aerated, as in the photic zone of the oceans (Louca et al. 2016). Such adaptations primarily depend upon the genomic endowment and functional expression of terminal oxidases that differ in structure, regulation and fundamental biochemical properties, in particular the affinity for oxygen (Morris and Schmidt 2013, Degli Esposti et al. 2014, Degli Esposti and Martinez-Romero 2017). The different types of bacterial terminal oxidases have been introduced in Chapter two. Here the subtypes and variants of heme *a* oxidases are discussed in detail, since their presence and combination with other terminal oxidases determine the environmental adaptation of alphaproteobacteria, as previously mentioned.

Terminal oxidases containing heme *a* belong to family A and B according to the classification of Pereira et al. (2001), now widely accepted (Sousa et al. 2012, Gao et al. 2012, Sharma and Wikström 2014, Degli Esposti 2014). The classification is based upon conserved signatures of the proton channels that are present in the structure of the largest catalytic subunit COX1 (Iwata et al. 1995), which is shared by all Heme Copper Oxygen reductases, HCO (Pereira et al. 2001). Family B oxidases

lack one of these channels (Pereira et al. 2001, Radzi Noor and Soulimane 2012) and consequently have a reduced capacity of proton pumping (Han et al. 2011, Sharma and Wikström 2014). However, this structural distinction is becoming blurred, following the accumulation of COX1 sequences in the last few years, especially from metagenomic studies (see following section of this chapter). It thus appears that there is a continuum in the variations of COX1 sequences associated with proton channels and their function, from the cbb3 oxidases of the C family to the most ancestral forms of aa3 oxidases of the A family (Ducluzeau et al. 2014). What is becoming particularly interesting in the context of the evolution of aerobic metabolism is that COX1 proteins now document the gradual transition from family B to family A oxidases in current genomes of alphaproteobacteria. Species of this class contain all the variants of both family A and B that have been recognized so far, including a novel operon for type A2 oxidases that has been found in a few alphaproteobacteria and other bacterial taxa (Fig. 3A, *vedi infra*). This operon is currently named CyoCAB from the sequence of the Conserved Domain (CD—Marchler-Bauer et al. 2015) definition of its subunits: CyoC for COX3, CyoA for COX2 and CyoB for COX1, with a Cu-assembly protein of the SCO (Synthesis of Cytochrome *c* Oxidase) family intermixed between CyoC and CyoA. The CD of CyoA, CyoB and CyoC are usually associated with ubiquinol oxidases belonging to type A1 (Matsutani et al. 2014), as shown in Fig. 3B, but they are assigned also to catalytic subunits of cytochrome *c* oxidases of the same A family.

The real peculiarity of the CyoCAB operon resides in the sequence of its genes, since the great majority of gene clusters for heme *a*-containing oxidases start with COX2 (Degli Esposti 2014) and rarely contain SCO proteins, for example in aerotolerant *Desulfovibrio* (Fig. 3B and Chapter four). The operon was initially discovered in the genome of two marine magnetotactic Rhodospirillaceae, *Magnetovibrio* (Bazyliski et al. 2013b, Trubitsyn et al. 2016) and *Magnetospir*a (Ji et al. 2014), which do not have the low affinity cytochrome *c* oxidases of A1 type that is present in freshwater Magnetospirilli (Figs. 2 and 3B - M.D.E., M. Mentel, W.F. Martin and F. Sousa, manuscript in preparation). *Magnetovibrio* has a more pronounced anaerobic physiology than Magnetospirilli (Bazyliski et al. 2013b); accordingly, its genome contains the complete set of enzymes for the biosynthesis of menaquinone as in *Phaeospirillum* genome (Fig. 2, cf. Duquesne et al. 2012, Degli Esposti 2017). Notably, menaquinone is typical of anaerobic respiratory chains (see Chapters two and five). The evidence just mentioned suggests that CyoCAB operon oxidases may have higher affinity for oxygen than type A1 oxidases of Magnetospirilli, or have a specialized function related to Fe metabolism. At present, these possibilities remain speculative, since no biochemical data on the enzyme coded by the CyoCAB operon is available yet.

However, some analogies could be drawn with cytochrome *c* oxidase I of *Aquifex*, which appears to have the same gene sequence as the CyoCAB operon of *Magnetovibrio* (Fig. 3A). This oxidase, which has been classified as a typical A2 type by Pereira et al. (2001), has a gene cluster concatenated with that of cytochrome *c* oxidase II or cox2, which is classified instead among family B

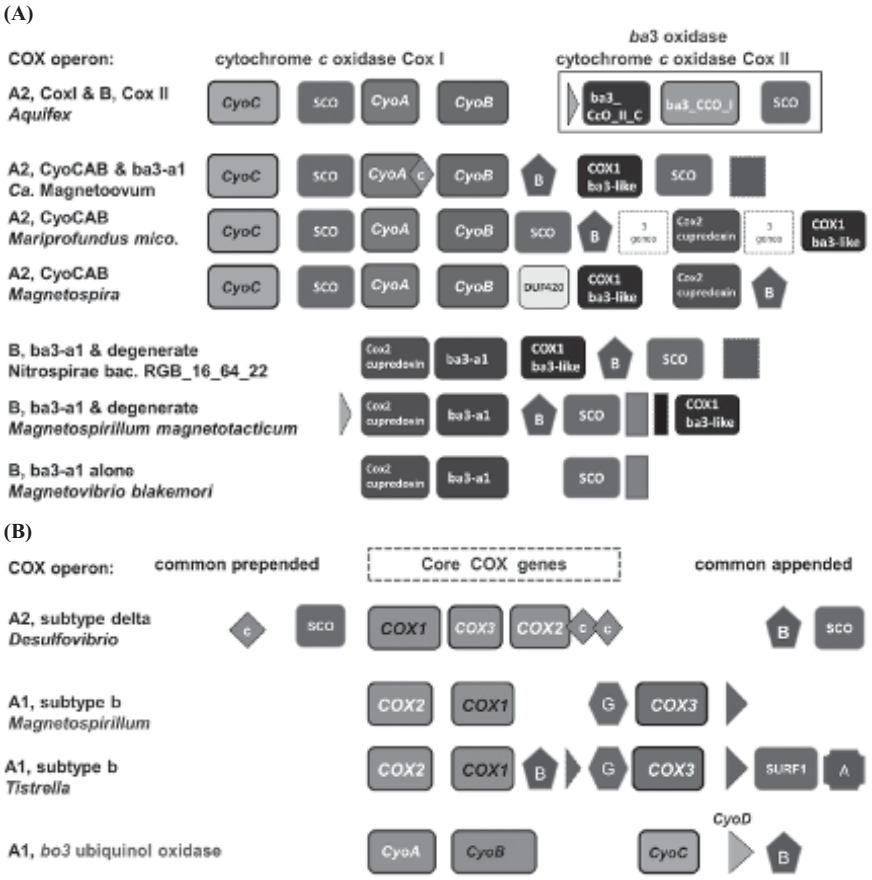

Fig. 3. Gene clusters for terminal oxidases of family A and B. (A) Variants in heme *a*-containing terminal oxidases of various phyla. (B) Subtypes of family A terminal oxidases, from *Desulfovibrio* to alphaproteobacteria. Modified from Degli Esposti (2014). Symbols with A and B identify ctaA and ctaB, respectively.

oxidases (Pereira et al. 2001, Prunetti et al. 2011, Gao et al. 2012). The latter has been characterized biochemically after purification and displays the dual function of oxidizing either ubiquinol or cytochrome *c* (Gao et al. 2012), a hybrid property that may be present in other family B oxidases. Intriguingly, the CyoCAB operon of *Magnetovibrio* and *Magnetospira* is associated with a deranged form of what appears a B family oxidase that is structurally different from cox2 of *Aquifex*, since its subunit 1 does not have the conserved Y280 and also the subsequent doublet of histidine ligands for CuB (M.D.E., unpublished results). A similarly deranged oxidase is associated with the gene cluster of another subtype of B family oxidases that is present in Magnetospirilli (Fig. 3A, cf. Degli Esposti et al. 2014), which retains the doublet of histidine ligands but not the conserved Y280. The absence of this conserved residue has been recognized earlier in the COX1

subunit of some B family oxidases (Sousa et al. 2012), including that present in *Magnetospirillum* (Ducluzeau et al. 2014), and considered to indicate the loss of cytochrome *c* oxidase function. The *Magnetospirillum* B family oxidase, without conserved Y280, corresponds to the so-called cytochrome *a*1-like that is expressed in *Magnetospirillum magnetotacticum* under microoxic conditions (Tanimura and Fukumori 2000). Because this *Magnetospirillum* oxidase had been previously labelled cytochrome *a*1-like (Tanimura and Fukumori 2000), it will, henceforth, be called subtype **ba3-a1** (Fig. 3). Its homologs are generally present in taxa having the CyoCAB operon, thereby indicating possible evolutionary connections between the two different enzymes. To uncover these connections, a detailed analysis of their distribution, genetic neighborhood and protein sequence has ben undertaken, obtaining the following information.

CyoCAB operon oxidases are present in two other alphaproteobacteria besides *Magnetovibrio* and *Magnetospira: Terasakiella* sp. *PR1*, taxonomically classified among the Rhizobiales (Han et al. 2106) but containing Mam proteins typical of magnetotactic organisms (Kolinko et al. 2012, 2016), and the unclassified alphaproteobacterium RIFOXYD12_FULL_60_8 (Anantharaman et al. 2016 , Fig. 4). In these four species, the operon is associated with one or more subunits of the above mentioned deranged B family oxidase. This association is not present in the genome of other proteobacterial organisms that have the CyoCAB operon: strains of the ancestral betaproteobacterium *Ca.* Accumulibacter (see Chapter six), a couple of gammaproteobacteria such as *Sulfuritalea hydrogenivorans*, several metagenomic deltaproteobacteria such as deltaproteobacteria bacterium GWA2_55_82 (Anantharaman et al. 2016) and, remarkably, the same zetaproteobacteria that also have heme *a* synthase (Fig. 4, cf. Table 2 in Chapter four). In the latter group, however, *Mariprofundus micogutta* has both subunits of a deranged B family oxidase intermixed with other genes downstream the CyoCAB operon (Fig. 3A), suggesting genomic erosion of the latter oxidase. In the genome of *Candidatus* Magnetoovum, a magnetotactic Nitrospirae (Kolinko et al. 2016), the gene for subunit 1 of the same deranged oxidase (labelled ba3-like in Fig. 3) follows that of the ctaB gene at the end of the CyoCAB operon (Fig. 3A, top). This gene sequence is unusual, since other Nitrospirae have either a ba3-a1 oxidase concatenation with deranged oxidases, or CyoCAB operons alone as in deltaproteobacteria. However, all Nitrospirae do not have the *ctaA* gene for heme *a* synthase. Consequently, CyoCAB operon and also ba3-a1 oxidases present in this *phylum* cannot have heme *a* prosthetic groups. Future biochemical analysis will verify whether these oxidases actually have only *b* hemes, similar to the cbb3 oxidases, or rather resemble the bo3 oxidases, since the majority of Nitrospirae do have the *ctaB* gene for heme *o* biosynthesis, often in association with the gene clusters of the oxidases (Fig. 3A).

Overall, the association of the above mentioned oxidases does not follow a recognizable pattern, even if it may reflect some ancient event in the evolution of these and other terminal oxidases. Conversely, the occurrence of concatenated clusters of HCO appears to be relatively frequent in diverse bacterial groups,

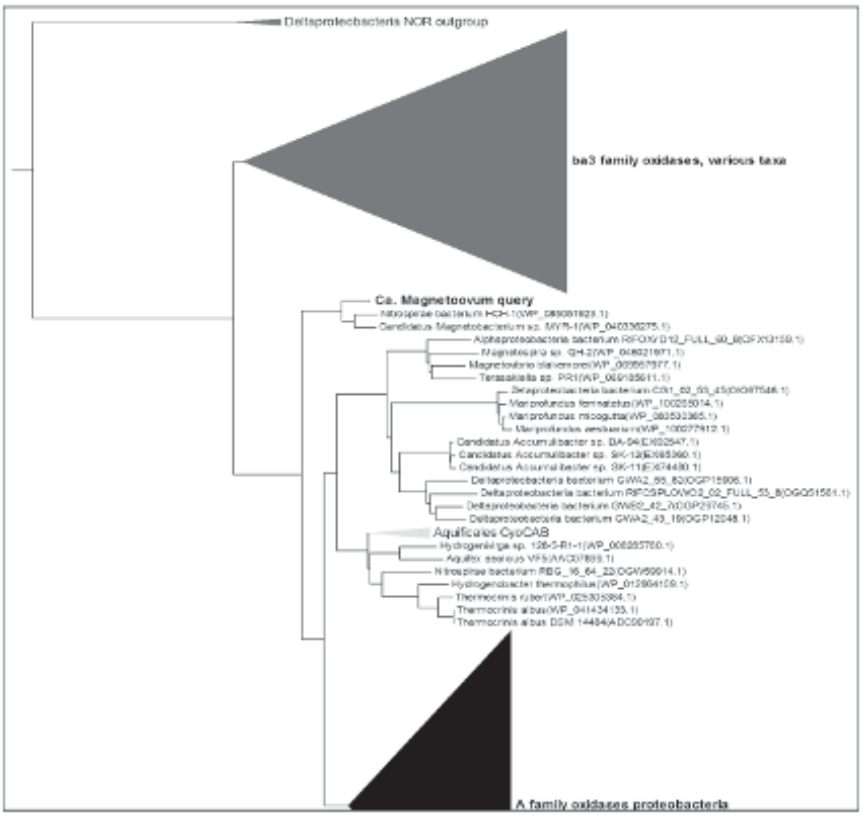

Fig. 4. Phylogenetic tree of CyoB oxidases. The NJ tree derived from a phylogenetically broad blast of the COX1 subunit of the CyoCAB operon of family A cytochrome *c* oxidases. The tree was rooted by the outgroup of NOR oxidases at the top. Note that the Ca. Magnetoovum (cf. Fig. 3) protein is the most ancestral in CyoCAB clade.

as previously documented for the concatenated family A oxidases present in alphaproteobacteria such as *Methylocella* (Degli Esposti et al. 2014). However, the concentration of these unusual oxidases in Fe-metabolizing organisms—magnetotactic Nitrospirae, magnetotactic alphaproteobacteria and Fe^{II}-oxidizing zetaproteobacteria—suggests an ancient connection with Fe chemolithotrophy. Of note is that the uptake of Fe and its subsequent incorporation in magnetosomes require oxidoreduction reactions involving terminal oxidases (Lefèvre et al. 2013, Li et al. 2014, Kolinko et al. 2016, Lin et al. 2017a), which may also be contributed by CyoCAB operon or B family oxidases. These enzymes may have an intermediate affinity for oxygen compared with the high affinity of cbb3 oxidases that are present in the majority of the above taxa (cf. Li et al. 2014), thereby enabling the removal of oxygen over a wide range of ambient concentrations so as to limit spontaneous Fe^{II} auto-oxidation with respect to its uptake and metabolism in bacterial cells

(Fullerton et al. 2017, Chiu et al. 2017—see also the section on zetaproteobacteria in Chapter four of the book).

Ultimately, the CyoCAB operon may represent an ancient form of cytochrome *c* oxidase that originally evolved in response to increasing levels of oxygen in primordial seas and then survived subsequent evolutionary changes in species living in niches preserving the original conditions. This possibility has implications for the evolution of alphaproteobacteria and, in a broader perspective, of the aerobic metabolism in prokaryotes.

Examples of Common and Well Known Alphaproteobacteria

After having considered the phylogeny and functional evolution of the alphaproteobacteria lineage, a brief review of some of the most common and representative members of the class is presented. Within alphaproteobacteria, there are some of the most widely spread bacteria in Nature, namely *Pelagibacter* and *Wolbachia*. The former thrives in oceans, the latter inside insects and nematodes. Methylotrophic *Methylobacterium* that consumes the plant wall biosynthesis byproduct methanol and most of nitrogen-fixing rhizobia are also successful and widespread members of alphaproteobacteria.

Pelagibacter

Pelagibacter was first reported as the SAR11 cluster organisms having a novel 16S rRNA gene sequence within the alphaproteobacteria, originally obtained from the Sargasso Sea by a culture-independent approach (Giovannoni et al. 1990). SAR11 clade bacteria are the most abundant group of planktonic cells in marine systems, accounting for a third of cells present in surface oceanic waters; in some regions they are up to 50% of the total surface microbial community (Morris et al. 2002). Hence, they were considered as the first candidate for the most successful organism on Earth (Morris et al. 2002, Giovannoni et al. 2005b, Giovannoni and Vergin 2012). *Pelagibacter* constitutes a separate phylogenetic group within the alphaproteobacteria (Williams et al. 2007, Ferla et al. 2013) but, as mentioned earlier in this chapter, its phylogenetic position has been variable (Ferla et al. 2013). Although *Pelagibacter* isolates are genetically diverse, analysis of their genome showed that they share a large core and extensive synteny of various gene clusters, perhaps due to strong genomic streamlining (Grote et al. 2012, Giovannoni et al. 2005b) since they do not have pseudogenes and are small, with an average size of 1.3 Mb. *Pelagibacter* genomes are AT-rich as other bacterial genomes that are reduced, for example those from insect endosymbionts presented in Chapter five.

Pelagibacter cells are small with seemingly efficient surface/volume ratios (Steindler et al. 2011). Their small cell volume may be related to efficient adaptation to low nutrient marine environments, with high affinity transporters and a very efficient metabolism to use organic matter in oligotrophic conditions. Notably, *Pelagibacter* has a proteorhodopsin that pumps protons upon absorption of light

and increases the supply of ATP during periods of carbon limitation (Giovannoni et al. 2005a), similar to some marine CFB (see Chapter three). SAR11 strains have genes for glycolysis and C1 metabolism, require reduced sulfur compounds for growth (Tripp et al. 2008) and show a conditional auxotrophy for glycine. Originally, *Pelagibacter* was uncultured, but now it may be cultured (Carini et al. 2013), thus allowing transcriptomic and proteomic analysis that have helped to clarify its metabolism (Smith et al. 2013). *Pelagibacter* has phages, including novel viruses just recently described, which apparently do not have effects on diminishing the *Pelagibacter* populations in the ocean (Mizuno et al. 2016).

Wolbachia

Among the various obligate parasites of the Rickettsiales order, *Wolbachia* organisms are the master manipulators of sex in insects (Werren et al. 2008) and responsible for insect speciation in some cases (Telschow et al. 2005). Estimates on the degree of insect infection by *Wolbachia* range from 16 to 76% (Dobson 2003). Since insects are the dominant animals on Earth, then their associated *Wolbachia* are numerous and widely distributed. The origin of wolbachias in nematodes is unclear. *Wolbachia* symbionts of nematodes provide vitamins and ATP to their hosts and are essential for host development (Darby et al. 2012). Remarkably, part of the eye damage inflicted in humans by the filarial nematode of river blindness is due to *Wolbachia* parasites (Tamarozzi et al. 2011, Gillette-Ferguson et al. 2004). Hence, antibiotics against *Wolbachia* are being used in the fight of river blindness (Hoerauf et al. 2003). The dissemination of *Wolbachia* in mosquitos has been studied (de Oliveira et al. 2017) with increasing interest because the bacteria exert effects on the immune response of the insects (Dieme et al. 2017, Pan et al. 2018) and affect their transmission of virus. For example, mosquitoes infected with *Wolbachia* were less susceptible to infections by dengue (Moreira et al. 2009, Mousson et al. 2012, Geoghegan et al. 2017, Joanne et al. 2017, Terradas et al. 2017), but unfortunately they became more susceptible to West Nile virus infections (Dodson et al. 2014). Thus, the use of *Wolbachia* to prevent viral transmission to humans has been questioned (Popovici et al. 2010).

It is estimated that *Wolbachia* organisms evolved around 500 million years ago, before the divergence of the slow growing *Bradyrhizobium* and the fast growing rhizobia. Only a low number of genes are conserved between rhizobia and wolbachias (E.M-R., unpublished data). None of the conserved genes were located in the *Rhizobium* extrachromosomal replicons such as plasmids, except for one common gene that was located in a rhizobial chromid. This suggests that *Rhizobium* plasmids were genomic innovations that occurred after the split of *Wolbachia* and rhizobia along the evolution of today's alphaprotobacteria. In contrast to the large genomes of rhizobia, normally over six Mb, *Wolbachia* genomes are reduced to small sizes ranging from 0.86 to 1.5 Mb (Ramírez-Puebla et al. 2015). Although maternal inheritance of *Wolbachia* is present in various insects, horizontal transfer of *Wolbachia* transfer between different insects, and maybe also to plants, has

been documented. For instance, parasitic wasps may transfer *Wolbachia* between different insect species (Cook and Butcher 1999).

Wolbachia organisms have been taxonomically organized into supergroups, many of which include only one or few strains, with one species formally recognized, *W. pipentis*. Most *Wolbachia* strains remain uncultured and their effects exerted on the hosts are just beginning to be understood. For example, the mechanisms that underlie male killing and the masculinization inhibition in insects that are induced by *Wolbachia* infection have been explored (Fukui et al. 2015). Taxonomically, these intracellular bacteria are classified within the family of Anaplasmataceae, despite recent additions and re-organization to the Rickettsiales order (Szokoli et al. 2016).

Methylobacterium

Known *Methylobacterium* bacteria were originally designated as PPFMs, for pink pigmented facultative methylotrophs (Holland and Polacco 1994). When plant mutants in the urease gene were tested for their enzymatic activity, urease was still detected, indicating the presence of a bacterial protein of resident methylobacteria (Holland and Polacco 1992). Methylobacteria are common endophytes that use methanol as carbon and energy source; methanol is released as a byproduct of plant wall synthesis. They grow selectively in media with added methanol without any other carbon source needed—see Chapter two for more information on methylotrophy. The characteristic pink color of *Methylobacterium* is due to carotenoids, but the genes involved in their biosynthesis are not known (Van Dien et al. 2003). Proteomic studies have revealed rare proteins that are present in methylobacteria and may be involved in the production of carotenoids (Kumar et al. 2014).

Methylobacterium is the type genus of its own family of Methylobacteraceae, which currently contain four other genera, the most widespread of which is *Microvirga* forming root-nodules (Ardley et al. 2012). There are also taxa revealed from metagenomic studies that are associated with the family (Beck et al. 2015).

Methylobacteria produce the plant hormones cytokinins (Lidstrom and Chistoserdova 2002) and also auxins (Ivanova et al. 2001). They thus promote growth in inoculation assay in plants, as well as in mosses (Tani et al. 2012). No specificity in the bacteria-plant interaction has been revealed; however, not all the strains of *Methyobacterium* promote the growth of all plant host species (Tani et al. 2012). In one case of scotch pines, methylobacteria have been found as intracellular symbionts (Koskimaki et al. 2015); they also become intracellular when they harbor *nod* genes (similar to those from rhizobia) and form nitrogen fixing bacteroids in few legume nodules, for example *Methylobacterium nodulans* in different *Crotalaria* species and *Microvirga* in lupinus (Ardley et al. 2012). Intriguingly, methylobacteria that are capable of forming nodules are not pink pigmented (Sy et al. 2001). They probably arose by acquiring *nod* genes from rhizobia via LGT.

Rhizobia

Rhizobium was initially called *Bacterium radiobacter* by Beijerinck. Nowadays, rhizobia is a common term used to collectively define a set of genera that are capable of forming nitrogen-fixing nodules in legumes (Poole et al. 2018). Taxonomically, rhizobia belong to different families of the order Rhizobiales, including Rhizobiaceae (*Rhizobium*), Phyllobacteraceae (*Mesorhizobium*) and Bradyrhizobiaceae. Overall, there are fifteen genera that contain such species (as mentioned in Chapter six), but nodulation is not universally distributed among these species. Nodulation is not uniformly distributed even among strains from a single nodulating species, which may thus contain non-symbiotic isolates (Segovia et al. 1991). The mechanisms underlying nodulation have been studied for a long time and are known to depend on *nod* genes; these genes code for Nod factors, variants of which could explain, to some extent, the specificity observed in the symbiosis of rhizobia with legumes. Nevertheless, there are rhizobial species that have a broader host range (Pueppke and Broughton 1999, Ormeño-Orrillo et al. 2012) and legumes that may be nodulated by many different species (Martinez-Romero 2003). There is phylogenetic incongruence among rhizobial housekeeping genes and symbiosis genes that may be explained by LGT, especially of *nod* and *nif* genes among bacterial species, conferring novel specificities and metabolic capabilities (López-Guerrero et al. 2012, Rogel et al. 2011).

The concept of symbiovar refers to the bacterial specificity for host plants that may be encoded in symbiosis plasmids, chromids or in symbiosis islands, which are contained in the chromosomes, for example in bradyrhizobia (Rogel et al. 2011). Novel symbiovars have been further described for different rhizobial species (Martinez-Hidalgo et al. 2015, 2016, Cobo-Diaz et al. 2014). LGT of symbiosis islands has been evidenced in *Mesorhizobium* (Sullivan and Ronson 1998, Nandasena et al. 2006) and in *Bradyrhizobium* (Parker 2012). *Bradyrhizobium* taxa belong to the phylogenetically deep branching family of Bradyrhizobiaceae, which is metabolically much more versatile than the Rhizobiaceae (one of its genus is a specialized nitrifier while another has the Fe^{II}-oxidizing trait, see Chapter two). Generally, bradyrhizobia have a few, if any, plasmids (Cytryn et al. 2008). In contrast, lateral transfer of plasmids seems to play a key role in the evolution of *Rhizobium*, *Sinorhizobium* and *Agrobacterium*. These taxa, indeed, are the latest diverging not only within the Rhizobiales order, but among all alphaproteobacteria (Williams et al. 2007, Ferla et al. 2013). In some rhizobia, up to around 50% of the genome is contained in extrachromosomal elements (Lopez-Guerrero et al. 2012). Moreover, plasmids have been exchanged among symbiotic *Rhizobium* and pathogenic *Agrobacterium* converting the latter pathogen into a nitrogen-fixing legume symbiont that carries the plant specificity of the donor (Martinez et al. 1987).

Besides forming nodules, rhizobia are soil and root colonizers and they are also found on the surface or inside seeds (Perez-Ramirez et al. 1998, López-López et al. 2010, Mora et al. 2014). In seeds, non-symbiotic rhizobia (López-López et al. 2010) have been found in addition to different symbiotic rhizobial species (Mora

et al. 2014). Bacteria in seeds may be tolerant to desiccation, a desired trait when producing industrial rhizobial inoculants for agricultural crops. Rhizobia may colonize non-legume roots in large numbers and even promote their growth in a non-nodule based symbiosis, thus acting as other PGPR bacteria. Some strains of *Rhizobium leguminosarum* have been found associated also with rice roots (Yanni et al. 1997). Conversely, some strains of *Rhizobium phaseoli* are natural endophytes of maize (Gutierrez-Zamora and Martinez Romero 2001, Rosenblueth and Martinez-Romero 2004). In both cases, the rhizobium-cereal symbiosis was seemingly promoted by the succession or association of legume and non-legume crops that occurs in traditional agricultural systems.

Outstandingly, genes for rhizobial nodulation are only expressed in the presence of the host plant, or of its exuded molecules such as root flavonoids. In turn, Nod factors synthesized from enzymes encoded in rhizobial *nod* genes are inducers of plant gene expression (Journet et al. 1994, Smit et al. 2005). Among the genes thus expressed are the early nodulins that participate in a signaling cascade in plants, which culminates with the formation of a novel root or stem structure called nodule (Oldroyd 2013). In a few legumes, nodulation of photosynthetic bradyrhizobia may proceed without the involvement of Nod factors (Giraud et al. 2007). In nodules, rhizobial bacteroids (the term bacteroids indicates differentiated nitrogen fixing symbionts) are fed dicarboxylic acids by the plant, mainly malate or succinate (Ronson et al. 1981, Poole et al. 2018). Nitrogen fixation would not occur outside of the nodules, with few peculiar exceptions. Nitrogen fixation increased when plants were exposed to high CO_2 concentrations (Fischinger et al. 2010) or high light intensity, suggesting that the phosynthate supply (Baysdorfer and Bassham 1985) may limit nitrogen fixation. The respiratory chain of rhizobia changes upon differentiation into bacteroids in the nodules, which contain very low but constant concentrations of oxygen. Concomitant with the over-expression of the *nif* genes for N_2 fixation, bacteroids up-regulate their cbb3 oxidase, originally found in rhizobia as mentioned in Chapter two, while down-regulating other terminal oxidases with lower affinity for O_2 (Degli Esposti and Martinez-Romero 2016). As an example, Fig. 5 shows the respiratory chain of a well-known nodulating rhizobium of peas, *Rhizobium leguminosarum*.

In some cases, especially in legumes grown in temperate climates such as peas or alfalfa, bacteroids achieve a terminal differentiation process and become no longer viable (Mergaert et al. 2006, Montiel et al. 2016). In those cases, plant peptides similar to antimicrobial peptides participate both in the bacteroid differentiation process and in their subsequent loss of viability (Alunni and Gourion 2016). Efficiency of nitrogen fixation is higher in symbiosis associated with terminal differentiation, which seems to be a late acquisition in plant evolution as tropical legumes do not show such a process (Mergaert et al. 2006, Montiel et al. 2016).

Rhizobia deliver fixed nitrogen to plants in the form of ammonium, which is then processed by plant nitrogen assimilation enzymes (Ohyama and Kumazawa

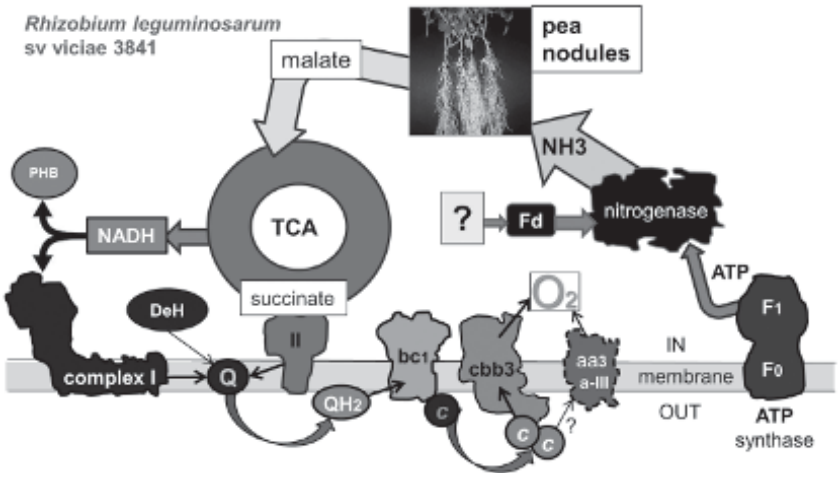

Fig. 5. Respiratory chain of a typical nodulating *Rhizobium*. The picture was modified from Degli Esposti and Martinez-Romero (2016).

1978, Poole et al. 2018). Amino acids have also been reported as rhizobial products that are delivered to plants, but these results turned out to be controversial (Waters et al. 1998, Li et al. 2002, Prell and Poole 2006, Dunn 2015). In some legumes, allantoin and allantoic acid are produced from fixed nitrogen precursors in non-infected legume cells and used for the transport of nitrogen compounds to aerial parts of the host plant (Herridge and Peoples 1990). However, this process occurs only in tropical legumes due to the low solubility of these ureides at low temperatures. Different types of rhizobial secretion systems have a role in the symbiotic interaction (Nelson and Sadowsky 2015). The diversity of nutrients from roots and soils seems to have been a driver to promote rhizobial differentiation and speciation in such a way to avoid bacterial nutrient competition (López-Guerrero et al. 2013). It is not uncommon that different strains from a single species have diverse phenotypic traits with regard to carbon and nitrogen metabolism. A consequence of this is that phenotypes are not good characteristic traits for a species description in taxonomy studies (Ormeño-Orrillo et al. 2013). Furthermore, most of the genes encoding nutritional capabilities in the rhizosphere are encoded in plasmids or genome islands; therefore, they may be easily lost or gained, supporting the concept that gene content constitutes the main variation among rhizobial genomes.

Rhizobia became one of the most studied symbiosis models in microbiology with their own developments in genetic strategies (Olson et al. 1985, Martínez-Romero et al. 1990, Hernandez-Lucas et al. 2002). Such strategies and further functional genomic and metabolic analysis (Degli Esposti and Martinez-Romero 2016) will continue to open wide and far reaching possibilities towards rhizobial biotechnological and commercial applications (Jiménez-Gómez et al. 2018).

Rhodospirillum

Rhodospirillum rubrum is the last example of a common, cultivated member of the alphaproteobacteria class that will be presented here. It is a photosynthetic bacterium of intense red colour living in diverse freshwater environments, including mud and sludge. *R. rubrum* was discovered over a century ago (Molisch 1907) for its characteristic spiral shape, hence *spirillum* from the Latin word for spiral (Imhoff 2005). The red colour derives from pigments associated with the photosynthetic apparatus, which is particularly developed when the bacterium grows under anoxic conditions (Imhoff 2005). *R. rubrum* has been used since the 60s as a model organism for studying anoxygenic photosynthesis, as well as other bioenergetic systems of electron transport. This is due to its extreme metabolic versatility and genomic plasticity in response to oxygen (Imhoff 2005, Munk et al. 2011). *Rhodospirillum* is also the type genus of the Rhodospirillaceae family and its parent Rhodospirillales order (Imhoff 2005), which have been previously mentioned along this chapter. Its best studied species is *R. rubrum*, which was identified early as a prototypic member of the alpha subdivision of proteobacteria (Woese et al. 1984, Woese 1987, Lee et al. 2005, Imhoff 2005). Recently, this organism has been exploited also for the biotechnological production of bio-plastics based upon its storage compounds (Karmann et al. 2016, Heinrich et al. 2016).

R. rubrum is also notable for its production of the rare amino analog of ubiquinone, rhodoquinone (Moore and Folkers 1965)—see also the end of chapter four. This quinone, usually abbreviated as RQ, is present together with ubiquinone in the membranes of *R. rubrum* and its closely related *Pararhodospirillum photometricum*, formerly belonging to the same genus (Imhoff 2005, Lakshmi et al. 2014), and in a few other alphaproteobacteria (Hiraishi and Hoshino 1984, Hiraishi et al. 1995, Okamura et al. 2009a,b, Srinivas et al. 2014). Rhodoquinone has a redox potential much lower than that of ubiquinone and therefore is very suitable for anaerobic electron transport, which can use multiple donors and acceptors in *R.rubrum* owing to the richness of redox enzymes coded in its genome (Imhoff 2005, Munk et al. 2011). However, this bacterium does not possess low affinity terminal oxidases (Fig. 2), thereby explaining its preference for a facultatively anaerobic lifestyle. Its relative *P. photometricum* actually cannot grow above micromolar concentrations of O_2 (Imhoff 2005). *R. rubrum* will be further mentioned in the final part of this chapter for it has been proposed as a possible relative of proto-mitochondria (Esser et al. 2004, Atteia et al. 2009, Thiergart et al. 2012, Abishek et al. 2013).

New Information from Unclassified Alphaproteobacteria of Metagenomes

There has always been a strong human bias in the taxonomic representation of bacterial genomes in NCBI databases due to medical and economic reasons (Mukherjee et al. 2017). This bias has been progressively reduced by ongoing efforts

to fill the sequence space of under-represented groups of bacteria that have been discovered in environmental and microbiome studies (Rinke et al. 2013, Schulz et al. 2017, Mukherije et al. 2017, Spang et al. 2017). Current genome databases still maintain a disproportion of taxonomic richness, measured in number of taxa defined on the basis of thresholds for rRNA similarity (Schulz et al. 2017), vs. phylogenetic diversity of proteins and their function (Anton et al. 2014, Louca et al. 2016, Probst et al. 2017, Bergauer et al. 2017, Bowers et al. 2017). However, it appears that the increase in phylogenetic diversity contributed by the rapid expansion of metagenomic assembled genomes (MAGs) is approaching saturation for the major proteobacterial classes of gamma-, beta- and alphaproteobacteria (Schulz et al. 2017, Mukherije et al. 2017, Parks et al. 2017).

Although an actual representation of bacterial taxa in environmental communities may be obscured in metagenomic studies of soil and other solid samples, due to the differential lysis of distinct species, such problems can be minimized using methodological strategies (de Castro et al. 2011, Parks et al. 2017). In the case of alphaproteobacteria, significant gains in phylogenetic diversity have been obtained in metagenomic studies of seawater (Tully et al. 2017, Parks et al. 2017) and groundwater environments (Ananthamaran et al. 2016, Probst et al. 2017, Schulz et al. 2017, Parks et al. 2017). Unexpected aspects of what appear to be primordial features of alphaproteobacteria have recently emerged from the analysis of the increasing wealth of genomic information derived from metagenomic studies (Brindefalk et al. 2011, Degli Esposti et al. 2016, Anantharam et al. 2016, Probst et al. 2017, Tully et al. 2017, Bergauer et al. 2017, Eme et al. 2017). For example, MAG's of unclassified alphaproteobacteria occupy the deepest branches in the great majority of phylogenetic trees of proteins required for the biosynthesis of membrane quinones (Ravcheev and Thiele 2016, Degli Esposti 2017).

The recent work by Parks et al. (2017) has added several novel MAG's, called UBA for Uncultivated Bacteria and Archaea, to both classified and unclassified groups of alphaproteobacteria. Of note, UBA taxa are not equally distributed in the various genera of the family of Rhodospirillaceae which has been discussed previously in this chapter. While only one UBA is associated with the *Rhodospirillum* genus, which previousy had four recognized species (Imhoff 2005), 37 UBA have been associated with the genus of predatory *Micavibrio*, doubling the number of its strains identified before (Parks et al. 2017); this clearly indicates that the phylogenetic diversity of *Micavibrio* is much larger than previously estimated. Although *Micavibrio* is usually considered an unclassified alphaproteobacterium (Davidov et al. 2006), phylogenetic trees of bioenergetic proteins such as cytochrome *b* indicate its close association with some MAG's from groundwater metagenomes (Probst et al. 2017) and members of the Rhodospirillaceae family such as *Nitrospirillum* (see later Chapter eight). A potential association of *Micavibrio*-like environmental rRNA's to Nitrospirae (Dolinšek et al. 2013) may thus be discarded. The discovery of several new *Micavibrio* taxa living in diverse environments (Parks et al. 2017) is of particular interest to the origin of mitochondria, since this predatory alphaproteobacterium has been suggested to be a relative of proto-mitochondria

(Davidov et al. 2006). The issue of the origin of proto-mitochondria is discussed below—see also Chapter eight.

Alphaproteobacteria and Proto-mitochondria

This section will summarize the approaches and findings obtained by studying the bacterial ancestry of mitochondria from the bacterial angle, i.e., the guest in the symbiogenic process leading to the eukaryotic cell (Margulis 1970). Subsequent Chapter eight will deal with the complementary approach of studying mitochondria and their origin from the eukaryotic angle, i.e., from the host side of the symbiosis, focusing on eukaryogenesis itself (Ettema 2016). The fundamental object of the current section is to provide evidence sustaining the concept that proto-mitochondria originated from alphaproteobacteria (Gray et al. 1999, Gray 2012), a concept that has been assumed along this and other chapters of the book. Previous evidence included the similarity in rRNA (Yang et al. 1985, Ferla et al. 2013) and the homology between alphaproteobacterial proteins and those coded in the mitochondrial DNA (mtDNA) of eukaryotes (Andersson et al. 1998, Gray et al. 1999, Emelyanov 2003, Willams et al. 2007, Brindefalk et al. 2011, Gray 2012, Wang and Wu 2015). Additional evidence has been obtained recently by genomic analysis of metabolic traits such as ubiquinone biosynthesis (Pelosi et al. 2016, Degli Esposti 2017), structure of the COX operon (Degli Esposti 2014) and regulatory subunits of ATP synthase that are functionally equivalent in the mitochondrial and alphaproteobacterial enzyme complex (García-Trejo et al. 2016, Mendoza-Hoffmann et al. 2018). This evidence is summarized in Table 1.

The items listed in Table 1 also include the presence of RQ, a membrane quinone initially discovered in *R. rubrum* (see the previous section dedicated to this

Table 1. Proteins and traits that are shared by mitochondria and alphaproteobacteria.

Protein and metabolic trait	Present in other proteobacteria	Reference
UbiL, ubiquinone biosynthesis	NO	Pelosi et al. 2016
CoQ9, ubiquinone biosynthesis	NO	Degli Esposti 2017
AOX, alternative ubiquinol oxidase, energy conservation	a few gammaproteobacteria	Atteia et al. 2004
Cox11/CtaG assembly of cytochrome c oxidase	NO	Degli Esposti et al. 2014, Pittis and Gabaldon 2016
Subunit zeta ATP synthase, energy conservation	NO	Garcia-Trejo et al. 2016, Mendoza-Hoffmann et al. 2018
Supernumerary subunit B17.2 13 kDa of complex 1	NO	Yip et al. 2011
Rhodoquinone, energy conservation	a few beta protecbacteria	Hiraishi and Hoshino 1984, Müller et al. 2012

bacterium) and then found in several metazoans adapted to anaerobiosis (Müller et al. 2012). Of note is that the presence of RQ in eukaryotes and its exclusive distribution in alphaproteobacteria, plus a few betaproteobacteria (which anyway do not share with mitochondria the proteins shown in Table 1), would in principle exclude the origin of proto-mitochondria from any other bacterial group. The fundamental question then becomes: from which lineage of alphaproteobacteria did proto-mitochondria come from?

This question remains open today since there is no consensus on the answer. Various studies (see Thiergart et al. 2012 for an earlier survey) have pinpointed to different orders of alphaproteobacteria that might include close relatives to proto-mitochondria, from Rhizobiales (Yang et al. 1985) to Pelagibacterales (Williams et al. 2007). An exhaustive study based on the phylogeny of the 16S rRNA gene has indicated that proto-mitochondria would be a sister group of contemporary Rickettsiales, both having Pelagibacterales as common ancestors (Ferla et al. 2013). This phylogeny would be consistent with the popular view initiated by Lynn Margulis (1970) that mitochondria derive from bacteria with strong aerobic metabolism (Andersson et al. 1998, Gabaldon and Huynen 2003, Boussau et al. 2004, Brindefalk et al. 2011, Wang and Wu 2015). However, several problems considerably weaken the possibility of a close relatedness between Pelagibacterales, Rickettsiales and proto-mitochondria.

Regarding the closeness between Rickettsiales and mitochondria, the first major problem is biological and is often overlooked, for example by Ferla et al. (2013). The genome of Rickettsiales is fast evolving and rapidly eroding because these bacteria are obligate intracellular parasites, a condition which prevents the evolutionary adaptation to changing environmental conditions that the genome of free-living bacteria must undergo for the species to survive and reproduce. Once established within the primordial eukaryotic cells, proto-mitochondria also became isolated from the outside environment and thus evolved a lifestyle equivalent to that of intracellular parasites (Ku et al. 2015). Indeed, genes encoded by mtDNA have a higher mutation rate than nuclear-encoded genes that end up in mitochondria (Gray et al. 1999, Wang and Wu 2015). Inevitably, this high mutation rate and the compositional bias in gene sequence due to a very high AT content—a genomic feature which is shared by mitochondria, Rickettsiales and Pelagibacterales (Andersson et al. 1998, Gray 2012, Grote et al. 2012, Rodríguez-Ezpeleta and Embley 2012), as well as gammaproteobacterial symbionts of insects (Chapter five)—produce artifacts in phylogenetic trees of either RNA or protein-coding genes (Gray 2012, Rodríguez-Ezpeleta and Embley 2012, Viklund et al. 2012, 2013). The artifacts consist of evident tree distortions technically called long branch attraction, which we have previously encountered in Chapter five in relation to sulfur-oxidizing symbionts. Sequences of either genes or proteins of fast evolving organisms are attracted to each other in any type of phylogenetic tree, producing erroneous sister clades (Philippe and Forterre 1999, Gray 2012, Wang and Wu 2015). A clear example of long-branch attraction between Rickettsiales and mitochondria is seen in the 16S rRNA trees reported by Ferla et al. (2013).

A similar situation applies to Pelagibacterales of the SAR11 clade and Rickettsiales, both of which have genome reduction and high AT content as discussed by Rodríguez-Ezpeleta and Embley (2012). Consequently, even if Pelagibacterales are free-living, and thus would not have the same accelerated mutation rate as endocellular parasites, their unusual genomic features, including very high AT content, likely derive from phenomena of convergent evolution, rather than reflecting evolutionary relatedness (Rodríguez-Ezpeleta and Embley 2012, Degli Esposti et al. 2014). The phylogenetic placement of the group of Pelagibacterales within the alphaproteobacteria has been debated extensively, as mentioned in the Introduction of this chapter (cf. Fig. 1). However, it appears that the concept of their affiliation to Rickettsiales (Williams et al. 2007, Grote et al. 2012, Ferla et al. 2013) has progressively given way to the more realistic possibility that they constitute a highly derived, but not very ancient lineage among alphaproteobacteria (Rodríguez-Ezpeleta and Embley 2012, Degli Esposti et al. 2014, Wang and Wu 2015). Although their taxonomic position remains essentially unresolved, it is becoming more central than originally thought by increasing the phylogenetic diversity of the alphaproteobacterial taxa with which they are compared, as shown by the tree in Fig. 1B. In any case, detailed phylogenetic analysis has progressively indicated that the quest for the closest relatives to mitochondria should point to a direction different from Pelagibacterales and related oceanic taxa, to paraphrase the conclusion by Rodríguez-Ezpeleta and Embley (2012).

This may well apply to a small group of oceanic metagenomic bacteria that Brindefalk et al. (2011) reported to display the closest bioenergetic proteins coded by mtDNA. These bacterial sequences were later identified to cluster with the taxon of alphaproteobacterium HIMB59 (Grote et al. 2012, Viklund et al. 2013), which is currently classified among Pelagibacterales, according to NCBI websites (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=744985 accessed 9 March 2018).

The study of Brindefalk et al. (2011) represents a paradigm for investigating the closest living relatives of proto-mitochondria by analyzing the sequence of major bioenergetic proteins encoded in mtDNA. This approach was pioneered by the same group of Andersson (Andersson et al. 1998) and later expanded by also including mitochondrial proteins that are now encoded in the nuclear DNA of eukaryotes (Esser et al. 2004, Thiergart et al. 2012, Wang and Wu 2015, Degli Esposti 2016). However, extensive experimentation has clarified that not all mitochondrial proteins have the same capacity of resolving deep phylogenetic relationships, while some are much better than others. These include cytochrome *b*, COX1 and ND5 among mtDNA-encoded proteins (Zardoya and Meyer 1996, Esser et al. 2004, Degli Esposti et al. 2014, Havird and Santos 2014), as well as the Nad7/NuoD/49kDa subunit of complex I (Brindefalk et al. 2011, Degli Esposti 2016) and the cytochrome *c*1subunit of the bc1 complex (Degli Esposti 2016). Such proteins have strong phylogenetic signal, a technical term which indicates a sequence length above 200 amino acids providing a combination of highly conserved residues required for function with hyper-variable stretches corresponding to external or transmembrane

regions, which can undergo large sequence changes without altering the structure-function of the protein. Concatenation of these proteins with other mitochondrial sequences generally does not add significant phylogenetic resolution (Esser et al. 2004), while taking away information from those regions that do not have detailed 3D structures of reference to guide their proper alignment (see Chapter one). Indeed, trees based upon concatenated sequences of proteins often fail to resolve the order of deep branches (Thiergart et al. 2014), a problem of particular severity for the ancestry of mitochondria, which undoubtedly evolved from an early branching group of alphaproteobacteria.

Using concatenations of hybrid sets of mtDNA-coded and nuclear coded mitochondrial proteins with diverse phylogenetic signal, Wang and Wu (2015) have placed proto-mitochondria within the Rickettsiales order. They claimed to have reduced the almost intractable problem of long branch attraction by increasing the phylogenetic depth of their analysis with newly sequenced genomes of Rhodospirillales and Rickettsiales (Wang and Wu 2015). However, the overall set of taxa examined was limited and biased, thereby undermining the conclusion of Wang and Wu (2015). Indeed, if a similar analysis were to be repeated today with all the available genomes of unclassified proteobacteria, the results and consequent conclusion would be different (cf., Fig. 1).

The truth of the matter is that the branching order of mitochondrial proteins vs. their alphaproteobacterial homologs depends upon the phylogenetic signal of the same proteins, the breadth of the taxonomic sampling of both the bacterial and eukaryotic species analyzed and the depth of the rooting taxa. Even small changes in any of these major variables can produce largely different trees, with diverse topologies of the closest nodes to the mitochondrial clade (Rochette et al. 2014, Burki 2014, Derelle et al. 2015). Ultimately, proto-mitochondria arose within the alphaproteobacterial lineage, most likely a few hundred million years after the separation from other proteobacteria and just before the branching of gamma and beta lineages (Degli Esposti 2016). Very few extant taxa can be related to the ancestral alphaproteobacteria that lived just before or at the same time of proto-mitochondria. It thus looks unlikely that their contemporary relatives can be clearly found by even the most sophisticated phylogenetic analysis based upon trees (Müller et al. 2012). The examination of the distribution of metabolic traits among extant species (Fig. 2) provides an alternative source of information, which clearly points to contemporary members of the Rhodospirillaceae family as likely relatives of those ancestral alphaproteobacteria from which proto-mitochondria then arose. Next chapter will verify this possibility further from the angle of the host in the eukaryogenesis process.

To conclude this chapter, the only certainty we currently have regarding the origin of mitochondria from the standpoint of the bacterial symbionts is that proto-mitochondria originated from an early lineage of ancestral alphaproteobacteria. Consequently, the phylogenesis of alphaproteobacteria is intimately linked to the origin of mitochondria. However, phylogenetic trees can provide limited information due to their intrinsic limits and the problems discussed in the final part

of the chapter. Alternatively, functional analysis extended to the ever increasing wealth of genomic data does provide valuable information, which so far pinpoints to facultatively anaerobes of the Rhodospirillaceae family as the most likely relatives of proto-mitochondria. This possibility is consistent with the results of early studies (Esser et al. 2004, Thiergart et al. 2012) and in part (regarding the Rhodospirillaceae *Tistrella* and the origin of ubiquinone, cf. Degli Esposti 2017) with those previously reported (Degli Esposti 2014). However, it definitively contrasts with several other studies that suggested a mitochondrial ancestry around or within the Rickettsiales order of intracellular parasites, a proposition that would never be able to answer the logical question: which organism were *Rickettsia*-like proto-mitochondria parasites of?

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