EVOLUTION OF MAJOR METABOLIC INNOVATIONS IN THE PRECAMBRIAN

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Abstract. A combination of the information on the metabolic capabilities of prokaryotes with a composite phylogenetic tree depicting an overview of prokaryote evolution based on the sequences of bacterial ferredoxin, 2Fe–2S ferredoxin, 5S ribosomal RNA, and *c*-type cytochromes shows three zones of major metabolic innovation in the Precambrian. The middle of these, which reflects the genesis of oxygen-releasing photosynthesis and aerobic respiration, links metabolic innovations of the anaerobic stem on the one hand and, on the other, proliferation of aerobic bacteria and the symbiotic associations leading to the eukaryotes. We consider especially those pathways where information on the structure of the enzymes is known. *Halobacterium* and *Thermoplasma* (archaebacteria) do not belong to a totally independent line on the basis of the composite tree but branch from the eukaryote cytoplasmic line.

1. Introduction

Nowhere among living organisms are the metabolic features so diverse as in the prokaryotes. However, information about phylogenetically useful traits has been scarce. It is not surprising, therefore, that proposed schemes that depict evolutionary development of metabolic pathways (Klein and Cronquist, 1967; Stanier, 1968; Broda, 1971; Hall, 1971; Hall *et al.*, 1975; Hartman, 1975; Dickerson *et al.*, 1976; Morris, 1977; Margulis, 1981) differ in the order in which these capabilities appear. In this article, we assume that the phylogenetic schema that we have derived from relevant protein and nucleic acid sequences is essentially correct (Dayhoff and Schwartz, 1980). By combining this schema with information on the metabolic capabilities of prokaryotes in which these molecules occur, we infer a phylogenetic sequence of major metabolic events appearing in Precambrian cellular life. We have assumed that, during prokaryote evolution, gene transfer between major groups followed by permanent acceptance is insignificant for genes of basic metabolic importance. We have examined pathways where there is information on the chemical structures of the enzymes, and have assumed that gene products whose structures have been conserved have remained relatively unchanged in their basic functions. The alternative, which is that a conserved molecule previously carried out a different function that it has lost, may prove correct in a few instances. However, it is

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undoubtedly rare, and our assumption of continuity of function is necessary to the development of a sequence of metabolic innovations.

2. Metabolic Features of Precambrian Strict Anaerobes

The composite tree depicting an overview of prokaryote evolution based on sequences of bacterial ferredoxin, 2Fe–2S ferredoxin, 5S ribosomal RNA, and *c*-type cytochromes is presented in Figure 1. The lower portion, which is based largely on bacterial ferredoxin sequences, places strictly anaerobic fermentative bacteria such as clostridia, *Megasphaera elsdenii*, and *Peptococcus aerogenes* next to the root. The position of the root was deduced by taking into account a gene doubling present in all ferredoxins (Tanaka *et al.*, 1966; Eck and Dayhoff, 1966). These results are consistent with what is generally assumed – that the primitive atmosphere was reducing (Broda, 1975a) and that fermentative obligate anaerobes were the first organisms to appear on Earth (Oparin, 1957), some 3.5 to 4 Gyr (10^9 yr) ago (Schopf, 1970; Ponnamperuma, 1972).

Ferredoxins, iron-sulfur proteins that have an extremely electronegative redox potential, around -400 mV (Tagawa and Arnon, 1962), mediate a number of oxidation-reduction reactions (Yasunobu and Tanaka, 1973). Common metabolic pathways in fermentative anaerobes in which ferredoxins take part are phosphoroclastic splitting of pyruvate leading to acetyl phosphate (Mortensen, 1964) and reductive carboxylation of an acyl-CoA derivative to an α-keto acid (Buchanan, 1972). It is possible that, during prokaryote evolution, glycolysis followed by phosphoroclastic split met the demands for ATP and reducing power needed for biosynthetic reactions. It is noteworthy that *Clostridium thermoaceticum* (Ljungdahl and Wood, 1978) and *C. formicoaceticum* (O'Brien and Ljungdahl, 1972) have the ability to carry out the total synthesis of acetate from carbon dioxide during fermentation. These organisms, in addition, contain cytochrome *b* and menaquinone (Gottwald *et al.*, 1975), but the role of these electron carriers, at least in *C. formicoaceticum*, appears to be in the reduction of fumarate rather than in the reduction of CO_2 to acetate. Although neither of these species appears in Figure 1, it seems reasonable to group them near other clostridial species and, thus, we infer that the ability to fix CO_2 heterotrophically, to synthesize heme, and to respire anaerobically using fumarate as terminal electron acceptor were early innovations in prokaryote evolution.

In Figure 1, two nitrogen-fixing clostridial species, *C. pasteurianum* and *C. butyricum*, emanate from a branch near the base of the tree. Among the obligate anaerobes, many species of *Clostridium*, a few of *Chlorobium*, and most of *Desulfovibrio* have the ability to fix N_2 (Postgate, 1974; Mortensen and Thorneley, 1979). The sequence of electron transfer to reducible substrates in the reduction of N_2 to $2NH_3$, which involves the nitrogenase system, is now well understood. This reduction requires not only four ATP molecules per electron pair but also six electrons from a low-potential reductant such as ferredoxin; both the dissociating

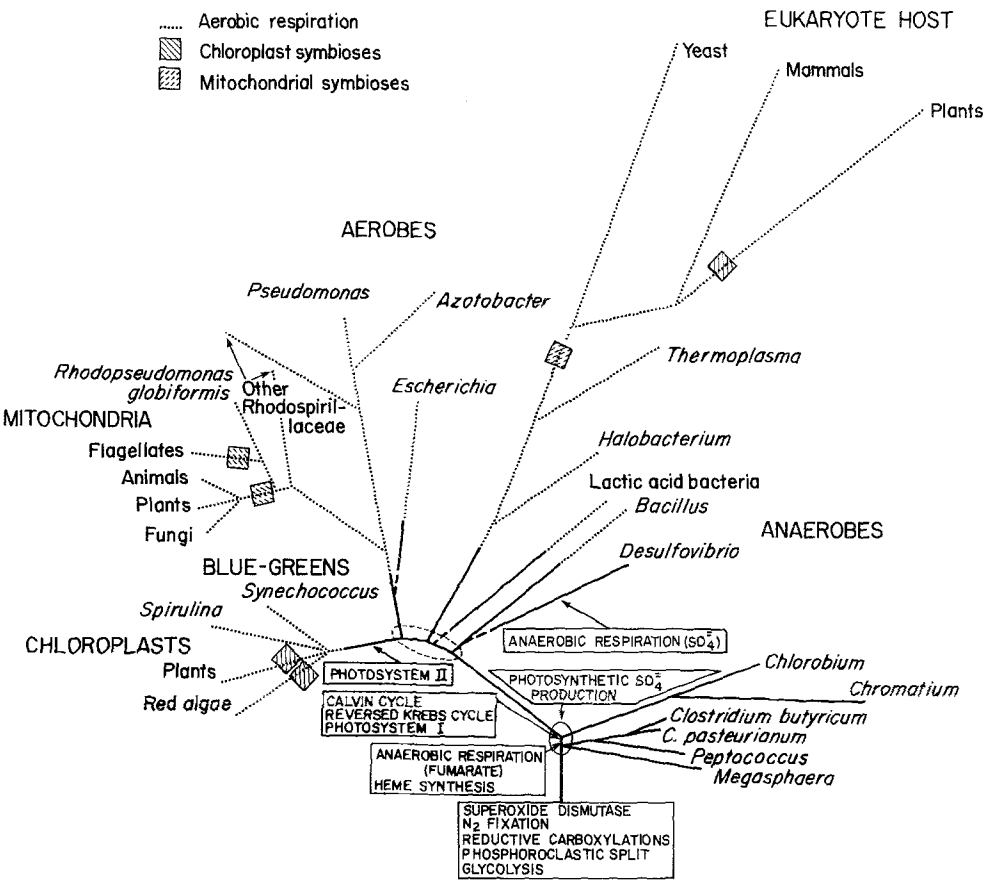

Fig. 1. Phylogenetic tree of prokaryotes and eukaryotes. The sequence of metabolic innovations over evolutionary time is shown. The tree is based on bacterial ferredoxin, 2Fe–2S ferredoxin, 5S rRNA, and *c*-type cytochrome sequences. Individual trees for these four sets of sequences were constructed using the least-squares matrix method. The trees derived for each set were scaled so that overlapping positions were comparable and the composite tree was constructed (Dayhoff, 1976; Schwartz and Dayhoff, 1978; DeMoulin, 1979; Schwartz and Dayhoff, 1979; Dayhoff and Schwartz, 1980; Dayhoff and Schwartz, 1981; Schwartz and Dayhoff, 1981). References to sequence data are given in Dayhoff, 1972, 1973, 1976, 1978. The *Thermoplasma* 5S rRNA sequence was determined very recently (Luehrsen *et al.*, 1981). We redetermined the tree shown here to include this sequence. The exact order of branching in the dotted region is not clearly resolved. Dashed branches reflect an uncertainty in the exact attachment of the branch. The eukaryote host branch, derived from 5S rRNA, clearly contains the archaebacteria *Thermoplasma acidophilum* and *Halobacterium salinarium*. Lactic acid bacteria include *Lactobacillus brevis* and *Streptococcus faecalis*. The bacterial species shown are *Megasphaera elsdenii, Peptococcus aerogenes, Clostridium pasteurianum, C. butyricum, Chromatium vinosum, Chlorobium limicola, Bacillus stearothermophilus, Halobacterium salinarium, Escherichia coli, Pseudomonas fluorescens, Azotobacter vinelandii*, 12 groups of Rhodospirillaceae described in Bergey's Manual of Determinative Bacteriology. 8th edition, *Rhodopseudomonas globiformis, Spirulina maxima, Synechococcus* ATCC 27144. Flagellate mitochondria are from *Euglena* and *Crithidia*. The eukaryotes include several representative species.

components of nitrogenase, namely Fe protein and MoFe protein, act as electron carriers (Mortensen and Thorneley, 1979). Although these nitrogenase proteins appear to belong to a class of proteins separate from the ferredoxins (Brill, 1979;

Tanaka *et al.*, 1977), they also contain iron-sulfur clusters. Thermodynamic data indicate that iron sulfides were plentiful on the primitive earth (Osterberg, 1974). Moreover, the observations that in many cases one nitrogenase component of a bacterial species complements the other component from a different bacterial species (Detroy *et al.*, 1968) *in vitro* and that even components from noncomplementary combinations such as *C. pasteurianum* and *Azotobacter vinelandii* interact with each other (Emerich and Burris, 1976) would suggest that the structures of the nitrogenase components from different organisms have been conserved. Furthermore, interspecies homology of nitrogenase genes has been established through hybridization of *Klebsiella pneumoniae* nitrogen-fixation (*nif*) gene fragments with DNAs from a variety of widely divergent nitrogen-fixing bacterial species (Ruvkun and Ausubel, 1980). From these results, we infer that these interacting types of iron-sulfur proteins appeared early in the evolutionary history of prokaryotes and thus that nitrogen fixation is a primitive characteristic on this tree.

Figure 1 shows Chlorobiaceae (green sulfur bacteria) and Chromatiaceae (purple sulfur bacteria) separating from the anaerobic stem early in prokaryote evolution. The presence of stromatolites in the 3.4–3.5 Gyr old Pilbara Block of Western Australia and the possibility that these stromatolites came from non-oxygen-producing photosynthetic flexibacteria would be consistent with an early evolution of anaerobic photosynthesizers (Walter *et al.*, 1980). Chlorobiaceae and Chromatiaceae are able to carry out anoxygenic photosynthesis using photosystem I; reduced sulfur compounds in general act as electron donors. In *Chlorobium limicola*, for example, light-excited bacteriochlorophyll (Bchl) directly produces a strong reductant that acts as a primary electron acceptor (Dutton and Prince, 1978; Knaff, 1978) from which electrons flow to NAD^+ via ferredoxin and flavoprotein NAD^+ reductase. The sulfur compounds, in turn, replenish electrons to Bchl via cytochromes b_{564} and c_{553} (Dutton and Prince, 1978). In *Chromatium vinosum*, on the other hand, light-excited Bchl feeds electrons to a primary electron acceptor from which they pass via ubiquinone, cytochrome b_{560} and cytochrome c_{555} back to Bchl (Knaff, 1978). During this cyclic electron transport, ATP is generated, which in turn could be used to power the thermodynamically unfavorable electron flow from a donor such as succinate to NAD^+ (Olson, 1978; Fuller, 1978). Cytochrome c_{553} apparently forms a part of the substrate-linked noncyclic system (Knaff, 1978). For the assimilation of CO_2, these photosynthetic bacteria may use either the reductive carboxylic acid cycle (Buchanan, 1972) or the reductive pentose phosphate (Calvin) cycle (Fuller, 1978). Clearly, these results imply that photosystem I, including cyclic, noncyclic, and reverse electron-delivery systems, and the pathways for CO_2 assimilation were early innovations.

Figure 1 (middle portion) shows *Desulfovibrio gigas* separating from the anaerobic stem after the divergences of other obligate anaerobes. It is remarkable that *Desulfovibrio*, like clostridial species, has retained many properties of the anaerobic stem. These include a phosphoroclastic system, a hydrogenase, and a nitrogenase system as well as the ability to carry out ferredoxin-linked carboxylations and dissimilatory (energy-producing) fumarate reduction (Morris, 1977; Thauer *et al.*,

1977). However, this group is unique in being able to carry out the dissimilatory reduction of sulfate. This complex process involves the intermediary formation of adenosine 5′-phosphosulfate and is mediated by a specific electron carrier protein called cytochrome c_3 (Peck, 1974). Clearly, in agreement with the suggestion by Peck (1974), Figure 1 indicates that sulfate-producing photosynthesizers arose prior to sulfate-reducing *Desulfovibrio*. Recently, Schidlowski (1979) has obtained isotopic evidence in the upper Archean of the Aldan Shield, Siberia (3 Gyr old) and in the Michipicoten and Woman River banded iron formations of Canada (2.8 Gyr) that suggests that sulfate reducers arose between 2.8 and 3 Gyr ago.

3. Genesis of Aerobic Respiration

Figure 1 (middle portion) shows three groups of facultative anaerobes, *Bacillus*, lactic acid bacteria, and *Escherichia coli*, diverging from the trunk at about the same time as the blue-greens (cyanobacteria). In recent years, the notion that photodissociation of water could have generated small but significant amounts of oxygen in early Precambrian times (Schwartz and Dayhoff, 1978; Berkner and Marshall, 1965; Towe, 1978) has gained some acceptance. The high degree of similarity in the *N*-terminal sequences of superoxide dismutases (SODs) between Fe- and Mn-enzymes of *E. coli*, mitochondrial Mn-enzyme, and *Bacillus stearothermophilus* Mn-enzyme (Harris and Steinman, 1977) makes it likely that the ancestor at the base of the *Bacillus* branch possessed a functional SOD (Brock and Harris, 1977; Hewitt and Morris, 1978). The widespread occurrence of Fe-SODs in obligate anaerobes suggests that these enzymes may have evolved even earlier in the anaerobic stem (Hewitt and Morris, 1978; Gregory *et al.*, 1978; Lumsden and Hall, 1975).

It is generally believed that enough free oxygen to support respiration began to appear only because of the development of oxygen-releasing photosynthesis in blue-greens (Cloud, 1968). This assumption implies that the common ancestors in the dotted region at the base of the blue-green branch were anaerobes whose descendants eventually independently acquired aerobic respiration when the atmosphere became oxygenic, including the lines to *Bacillus*, lactic acid bacteria, *E. coli*, and the line to *Thermoplasma* and the eukaryote host as well as that to the blue-greens. While the diversity of terminal oxidases suggests independent adaptations to oxygen, this diversity in itself does not prove it. Sequence data from terminal oxidases could establish whether there was a conserved structural basis for this function.

The adaptation to oxygen would involve a cytochrome-dependent electron transport chain in *Bacillus* (Miki and Okunuki, 1969) and a flavin-terminated respiratory system in the lactic acid bacteria (Dolin, 1961). In Figure 1 (middle portion), *Lactobacillus* and *Streptococcus* both lie on the lactic acid bacterial branch. This is consistent with the observation that there is a close immunological relatedness individually among aldolases (London and Kline, 1973), lactate dehydrogenases (Gasser and Gasser, 1971), and malic enzymes (London *et al.*, 1971) in some species

belonging to these two genera. Mycoplasmas, which have the smallest genome size of all free-living organisms (Morowitz and Wallace, 1973), show some similarity in their metabolic capabilities to lactic acid bacteria in that they ferment glucose, accumulate lactate, and possess a flavin-terminated respiratory system (Neimark, 1979). They probably diverged from the middle portion of the tree, a position consistent with the evidence from phenylalanine tRNA sequences. Recent work (Searcy *et al.*, 1981) on the metabolism of *Thermoplasma*, an aerobe, indicates that respiration primarily utilizes flavin oxidases. This capacity resembles that of the microbodies of eukaryotic cells.

Although the exact position of the *E. coli* branch is unclear, it is nevertheless obvious that this branch acquired oxygen-terminated electron transport pathways independent of those of either *Bacillus* or the blue-greens. In fact, there are two such pathways in *E. coli*: one operating at conditions of high aeration and terminating in an oxidase, cytochrome *o*, and the other operating at lower oxygen tensions and terminating in an oxidase, cytochrome *d* (Haddock and Jones, 1977). Clearly, consistent with the suggestions of Broda (1971) and Dickerson (1980) these results suggest that at least the diverse terminal components in aerobic respiration are polyphyletic in origin.

It is noteworthy that some members of the genus *Bacillus* (*B. stearothermophilus* in Figure 1) and a number of coliform bacteria (*E. coli* in Figure 1) are able to carry out dissimilatory nitrate reduction (Schulp and Stouthamer, 1970). In fact, nitrate respiration is a capability widely distributed among facultative anaerobes, and even in obligate anaerobes such as *Clostridium perfringens* (Ishimoto *et al.*, 1974), *Veillonella alcalescens* (Inderlied and Delwiche, 1973), and *Selenomonas ruminantium* (De Vries *et al.*, 1974) nitrate may serve as a terminal electron acceptor. In the absence of any known nonequilibrium process that produces significant amounts of nitrates in a reducing atmosphere, thermodynamic considerations require an oxygenic environment (Broda, 1975b) for nitrates to have arisen. This would suggest that the pathways found in anaerobes for respiration using nitrate as a terminal electron acceptor were later acquisitions. Any attempt to locate the origin of nitrate respiration must take into account whether or not nitrate reductase *A*'s in different prokaryotes are similar. Amino acid or nucleotide sequence data of this enzyme would be helpful here.

4. Metabolic Features of Precambrian Aerobes

It seems likely that blue-greens arose prior to 2 Gyr ago, when geological evidence indicates that free oxygen began to accumulate in the atmosphere (Cloud, 1968). From Figure 1 (upper portion), it is seen that the blue-greens and Rhodospirillaceae (purple nonsulfur bacteria) arose from the anaerobic stem as a branch distinct from that of anaerobic photosynthetic bacteria. However, it is known that a number of blue-greens (Garlick *et al.*, 1977) as well as the Rhodospirillaceae are capable of anoxygenic photosynthesis. Clearly, this property is an attribute of the anaerobic

stem that both these branches have retained. Branches leading to *Bacillus*, lactic acid bacteria, coliform bacteria, and pseudomonads have lost this property. Hydrogenase activity, N_2 fixation, and chemoheterotrophy are other anaerobic attributes that some blue-greens (Schneider *et al.*, 1960; Fujita and Myers, 1965; Stanier, 1973) as well as Rhodospirillaceae (Yoch, 1978; Sojka, 1978) have retained from the anaerobic stem. Eventually, the ancestral stock of blue-greens began to use water as an indispensable electron donor for the photoassimilation of CO_2. This oxygen-releasing photosynthesis in blue-greens involves two photosystems (Hill and Bendall, 1960) with chlorophyll a and phycobiliproteins acting as light-collecting pigments (Krogmann, 1973). The chemical identities and order of participation of redox carriers in these photosystems are fairly well understood. Whereas photosystem I was an early acquisition of the anaerobic stem, photosystem II, which is unique to this group, utilizes a *b*-type cytochrome, plastoquinone, a *c*-type cytochrome, and plastocyanin as redox carriers. In blue-greens, the reductant of CO_2 appears to be NADPH rather than NADH as in other photosynthetic bacteria. Although blue-greens do utilize oxygen as the terminal electron acceptor in respiration (Fogg *et al.*, 1973), many of them do not possess a complete Krebs cycle (Stanier, 1973).

Figure 1 (upper portion) encompasses two bacterial groups: Rhodospirillaceae and Pseudomonadaceae. The Rhodospirillaceae possess a photosystem similar to that of the anaerobic purple sulfur bacteria. Both these groups have energy-linked photosystem I involving, at the least, ubiquinone, a *b*-type cytochrome, and a *c*-type cytochrome; both have cyclic, noncyclic, and reverse electron-supply systems (Dutton and Prince, 1978). The major difference is that in purple sulfur bacteria cyclic and noncyclic electron-delivery systems converge at the photooxidizable reaction center, and in purple nonsulfur bacteria the point of convergence appears to be at cytochrome c_2 (Dutton and Prince, 1978).

Because purple nonsulfur bacteria and pseudomonads are aerobes, it can be assumed that the common stem of these organisms, which already possessed a photosynthetic electron transport system, acquired an oxygen-terminated electron transport chain as well. In fact, both these processes have been shown to be similar in *Rhodopseudomonas capsulata* (Marrs and Gest, 1973a, b) in that they share a common electron-carrying arm consisting of ubiquinone, *b*- and *c*-type cytochromes, iron-sulfur proteins, and cytochrome c_2. However, in aerobic respiration, reduced flavoprotein generated by the entry of NADH + H^+ starts the electron cascade, and a cytochrome oxidase complex, either cytochrome *o* or aa_3, terminates it.

Once aerobic respiration and the Krebs cycle had become fully established in the prokaryotic stem, the potential was provided for deriving many times more ATP than from fermentation. This innovation opened many new niches to the aerobic stock.

Furthermore, much larger and more complex cell types developed, giving rise to eukaryotes probably around 1.4 Gyr ago (Schopf and Oehler, 1976). There have been two major theories concerning the origin of eukaryotes. One is that eukaryotes arose as a result of serial endosymbiosis among prokaryotes (Margulis, 1970), and the other is that they arose by compartmentalization of the DNA within the cytoplasm of a

single line of prokaryotes (Raff and Mahler, 1972; Uzzel and Spolsky, 1974). The sequence data clearly support the endosymbiotic theory, which pictures mitochondria and chloroplasts as once having been free-living prokaryotes not unlike *Rhodopseudomonas globiformis* and blue-greens, respectively (Figure 1, upper portion). Polyphyletic origins of these organelles through such symbioses have been discussed in detail in recent communications from this laboratory (Dayhoff and Schwartz, 1981; Schwartz and Dayhoff, 1981). A eukaryotic branch emanating from the middle portion in Figure 1 represents the host line for the endosymbioses. The 5S rRNAs used in constructing this portion of the tree were isolated from cytoplasmic ribosomes, one of the three ribosomal systems found in eukaryotes. Two bacterial species, *Halobacterium salinarium* and *Thermoplasma acidophilum*, clearly diverge from the eukaryotic host line. This position is reinforced by the observation that parts of the protein-synthesizing machinery of *Halobacterium* resemble those of eukaryotes (Bayley and Morton, 1978) and that *Thermoplasma* uses histone-like proteins to stabilize its DNA, lacks a rigid cell wall, contains an actin-like skeleton, and is an obligate aerobe that appears to depend mainly on flavin oxidases for its oxygen metabolism (Searcy *et al.*, 1981). The other organisms that diverge from the middle region of Figure 1 include fermentative facultative anaerobes, some of which also have flavin-terminated respiratory systems. These have evidently been conserved in the host line, supporting the suggestions of Raff and Mahler (1972), that the eukaryote cytoplasm had made a primitive adaptation to the use of oxygen, and of de Duve (1969), that flavin oxidases present in microbodies of all eukaryotes are vestiges of the original cytoplasmic respiratory pathways.

On the basis of both their 16S ribosomal RNA catalogue and their unusual lipids, *Halobacterium*, *Thermoplasma*, and methanogenic bacteria have been classified by others as a unique group of organisms that are different from either prokaryotes or eukaryotes (Woese *et al.*, 1978; Magrum *et al.*, 1978; Fox *et al.*, 1980). From the 5S rRNA sequence evidence, there is no reason to classify *Halobacterium* and *Thermoplasma* in an entirely separate group. These forms clearly diverge from the line to the eukaryote host. Apparently there is insufficient information in the 16S ribosomal RNA catalogues to show the details of such a distant relationship. There is no sequence information available from methanogens. These organisms share a number of metabolic traits with prokaryotes, for example, *Methanosarcina barkeri* (Kuhn *et al.*, 1979) produces a cytochrome *b* and *Methanospirillum hungatii* (Fuchs and Stupperich, 1978) possesses a partial reductive carboxylic acid cycle.

Figure 1 (upper portion) place purple nonsulfur bacteria near eukaryote mitochondria. In particular, *Rhodopseudomonas globiformis* diverges from the flagellates *Euglena* and *Crithidia* close to their point of divergence from one another. Clearly, the ancestors of *R. globiformis* qualify as free-living prokaryotes that gave rise to eukaryote mitochondria, probably in more than one symbiotic event. Similarly, phylogenetic histories based on 2Fe–2S ferredoxin and cytochrome c_6 sequences identify blue-greens as the free-living organisms that gave rise to chloroplasts and indicate that more than one symbiotic event has occurred.

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