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A new α-proteobacterial clade of *Bdellovibrio*-like predators: implications for the mitochondrial endosymbiotic theory

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Summary

***Bdellovibrio*-and-like organisms (BALOs) are peculiar, ubiquitous, small-sized, highly motile Gram-negative bacteria that are obligatory predators of other bacteria. Typically, these predators invade the periplasm of their prey where they grow and replicate. To date, BALOs constitute two highly diverse families affiliated with the δ-*proteobacteria* class. In this study, *Micavibrio* spp., a BALO lineage of epibiotic predators, were isolated from soil. These bacteria attach to digest and grow at the expense of other prokaryotes, much like other BALOs. Multiple phylogenetic analyses based on six genes revealed that they formed a deep branch within the α-*proteobacteria*, not affiliated with any of the α-proteobacterial orders. The presence of BALOs deep among the α-*proteobacteria* suggests that their peculiar mode of parasitism maybe an ancestral character in this proteobacterial class. The origin of the mitochondrion from an α-*proteobacterium* endosymbiont is strongly supported by molecular phylogenies. Accumulating data suggest that the endosymbiont's host was also a prokaryote. As prokaryotes are unable to phagocytose, the means by which the endosymbiont gained access into its host remains mysterious. We here propose a scenario based on the BALO feeding-mode to hypothesize a mechanism at play at the origin of the mitochondrial endosymbiosis.**

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Introduction

Predation among prokaryotes has not been extensively explored and the only well-known obligate predatory-bacteria group is the *Bdellovibrio*-and-like organisms (BALOs). *Bdellovibrio*-and-like organisms are small, highly motile Gram-negative bacteria that obligatorily prey on other Gram-negative bacteria. In their typical life cycle free-swimming cells invade the periplasm of the prey, grow, replicate and then differentiate to progeny cells that lyse the host to start a new cycle (Jurkevitch, 2000). *Bdellovibrio*-and-like organisms are commonly found in diverse habitats including soil, fresh water, seawater, sewage and animal feces. They form the two distant and internally diverse families, Bacteriovoracaceae and Bdellovibrionaceae, classified as the order Bdellovibrionales, and cluster within the δ-*proteobacteria* class (Davidov and Jurkevitch, 2004). Their high diversity, ubiquity and presumed long evolutionary history suggest a possible ecological and evolutionary impact on microbial communities.

Given that phagocytosis is unknown in prokaryotes, the association of BALOs with their hosts was proposed to be analogous to early events of the endosymbiotic acquisition of eukaryotic cell organelles. If prey bacteria could have avoided digestion by the predators, stable symbioses between the two types of organisms might have developed (Guerrero *et al*., 1986; Margulis, 1993). Molecular data support an origin of the mitochondrion in an α-*proteobacterium* (Gray *et al*., 1999). This, and the discovery of amitochondriate phagocytotic eukaryotes (Cavalier-Smith, 1987) caused the abandon of the 'predatory endosymbiosis hypothesis'. However, recent and accumulating data suggest that all characterized amitochondrial eukaryotes are derived from mitochondriate ancestors (van der Giezen *et al*., 2005), implying that the proteomitochondrion was acquired by a prokaryotic host (Embley and Martin, 2006; Martin and Koonin 2006).

In this article, we report on *Bdellovibrio*-like predatory bacteria that form a deep branch within the α-*proteobacteria*. As it is widely accepted that mitochondria evolved from an α-*proteobacterium*, a re-examination of a possible

relationship between predatory bacteria and the endosymbiotic origin of the mitochondrion is proposed.

Results

Characterization of epiobiotic predators

Six strains of predatory bacteria: EPB, EPC2, EPC4, ESA, ESB and ESC were isolated from soil in north-eastern Israel using *Pseudomonas corrugata* or *Pseudomonas syringae* as prey. Similar to known BALOs, they were small, vibrio or rod-shaped, highly motile obligate predators (Fig. 1A) that did not grow in the absence of prey, neither in poor or rich media. The prey range of strain EPB was tested on a variety of potential Gram-negative and Gram-positive host bacteria. Among the organisms examined, only *Pseudomonas* strains were found to be preyed upon, as previously reported by Lambina and colleagues (1982). The predators attached to the preys' outer membrane (Fig. 1B), but unlike most BALOs they did not penetrate the periplasmic space, and divided by binary fission (Fig. 1C). Attacked prey cells were totally consumed, leaving empty cell remains (Fig. 1B). The predators possessed a single polar flagellum, but distinctively from BALOs, this flagellum was not sheathed and did not exhibit the typical damped waveform of BALOs (Thomashow and Rittenberg, 1985). These morphological characters resembled those of *Micavibrio*, a genus first described in 1982 (Lambina *et al.*, 1982) but little mentioned in the literature. Based on morphology, enzymatic activity and other metabolic features, it had been suggested that *Micavibrio* was related to *Bdellovibrio* (Lambina *et al.*, 1982; 1983; Afinogenova *et al.*, 1986), and it was tentatively placed within the Bdellovibrionaceae (Garrity *et al.*, 2004). Instead, a phylogenetic analysis based on the almost complete 16S rRNA gene sequences of our isolates and from *Micavibrio aeruginosavorus* strain ARL-13, showed that *Micavibrio* clustered within the α-*proteobacteria* class, forming a deep branch within this clade (Fig. 2). The *Micavibrio* group was constituted of three subgroups with a maximum of 1.4% sequence dissimilarity (Fig. 2). The closest related sequences diverged by more than 11% and originated from environmental clones from diverse habitats (Fig. 2 and Table S1 in the *Supplementary material*). *Micavibrio* and these associated sequences, while forming a strongly supported cluster did not constitute a stable assemblage with any of the known α-proteobacterial groups. The minimal divergence between *Micavibrio*'s 16S rRNA sequences and any other cultured bacterial sequences was about 14%.

Most BALOs exhibit a periplasmic growth stage, i.e. they grow and divide within the space between the inner and the outer membranes of their Gram-negative prey (Fig. 1F). However, and similarly to *Micavibrio*, *Bdellovibrio* strain JSS, a predator of *Caulobacter crescentus*, did not display a periplasmatic growth stage. Instead, it remained extracellular during its full cell cycle (Fig. 1D). A 16S rRNA phylogeny of this bacterium clearly set it within the clade Bdellovibrionaceae (Fig. 2). Its closest relative

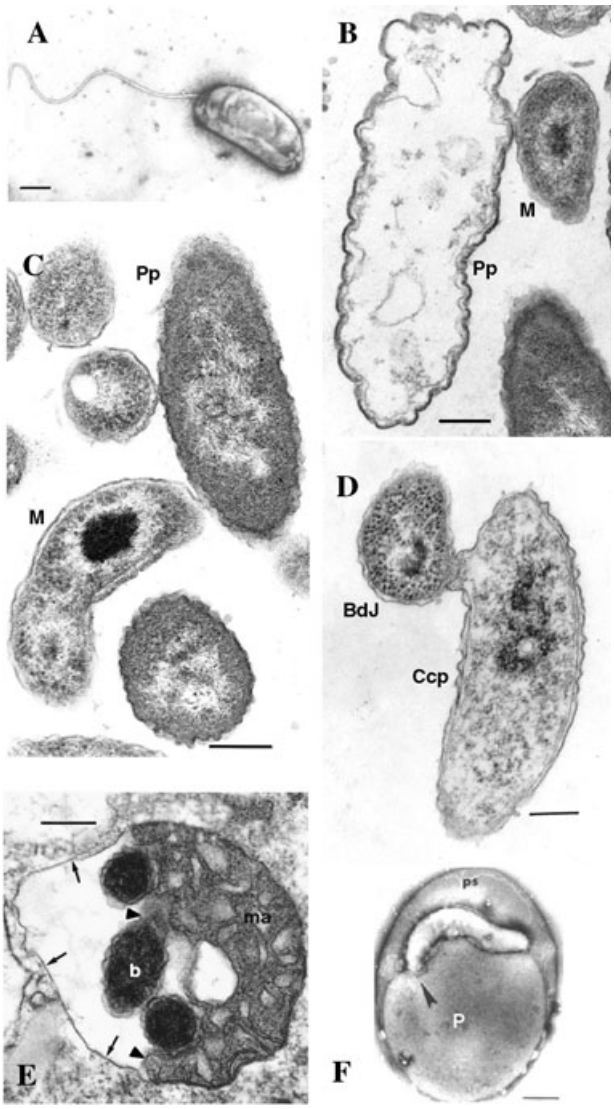

Fig. 1. Electron micrographs of epibiotic and periplasmic *Bdellovibrio*-like predators.
A. A free-swimming *Micavibrio* sp. EPB cell. Bar = 0.2 μm.
B. A *Micavibrio* sp. EPB (M) cell attached to an emptied *Pseudomonas corrugata* (Pp) prey cell. Bar = 0.5 μm.
C. A *Micavibrio* sp. EPB predator (M) dividing by binary fission while attached to a *P. corrugata* prey cell (Pp). Bar = 0.5 μm.
D. *Bdellovibrio* sp. JSS (BdJ), an epibiotic predator attached to a *Caulobacter crescentus* (Ccp) prey. Bar = 0.2 μm.
E. An Iric ES1 bacterium (b) infecting a mitochondrion. It is located between the outer (arrows) and inner (arrow heads) membranes of the mitochondrion (ma). Bar = 0.3 μm (Sacchi *et al.*, 2004, by permission).
F. A *Bdellovibrio bacteriovorus* within the periplasm of an *Escherichia coli* prey cell. ps, periplasm; p, prey's protoplast. Bar = 0.2 μm (Abram *et al.*, 1974, by permission).

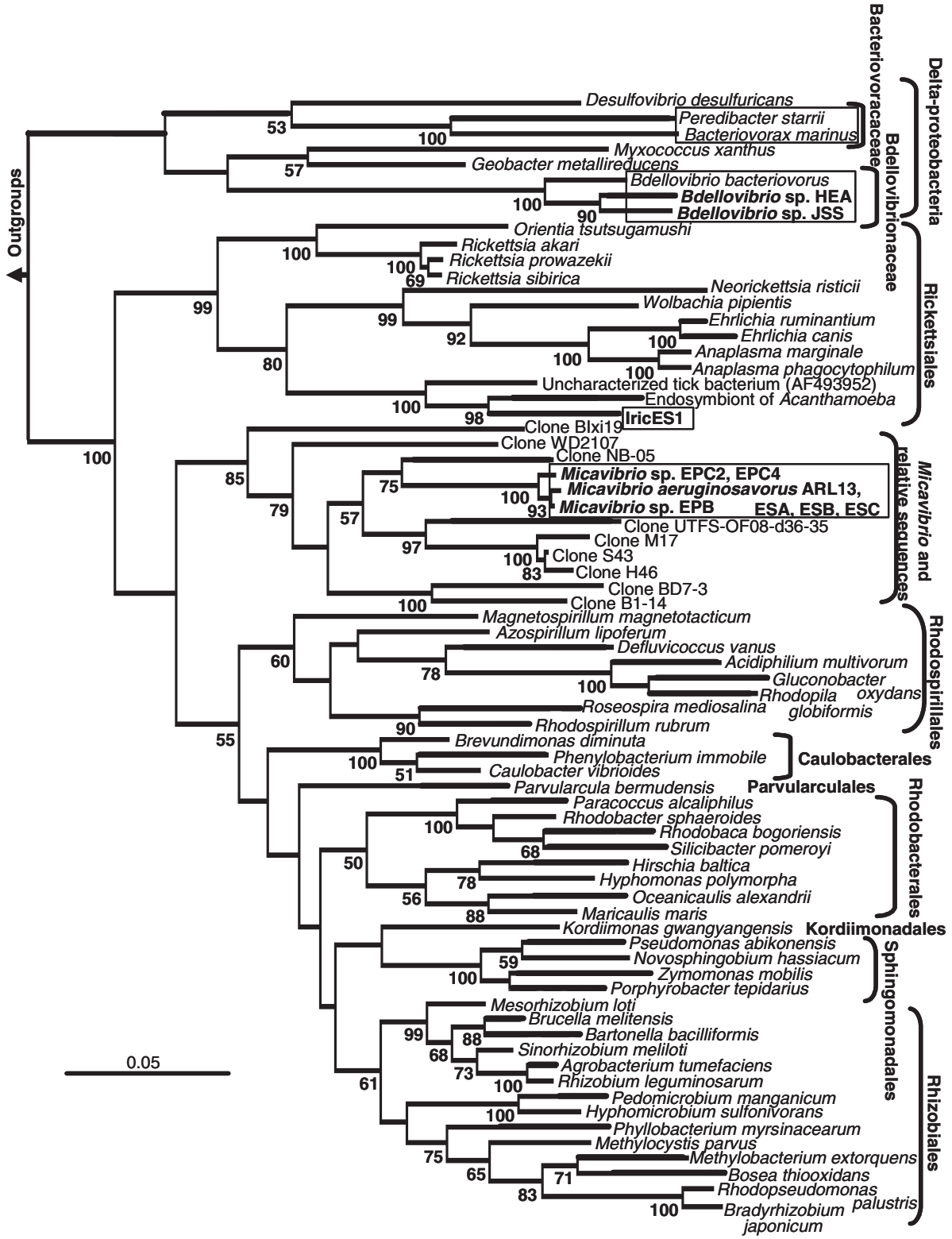

Fig. 2. Phylogenetic SSU rRNA tree showing the affiliation of *Micavibrio* and other *Bdellovibrio*-like predators within the α-*proteobacteria*. Representatives δ-*proteobacteria* are also included. The tree is based on maximum-likelihood (FastDNAml) analysis, using a 50% conservation filter. Numbers below nodes represent parsimony BP (1000 bootstrap replications, values below 50% are not indicated). *Bdellovibrio*-like predators are marked by rectangles. The following collection of bacteria was used as the outgroup for treeing: *Clostridium nexile, Thermoanaerobacter ethanolicus, Bacillus megaterium, Deinococcus grandis, Aquifex aeolicus, Sphaerobacter thermophilus, Spirochaeta aurantia, Synechococcus* sp. PCC7002, *Fusobacterium gonidiaformans, Chlamydophila pneumoniae, Helicobacter rappini* and *Nautilia lithotrophica*.

was strain HEA (Fig. 2), a periplasmic predator of *Escherichia coli* (not shown). This therefore suggests that the transition from an epibiotic to a periplasmic mode of predation (or vice versa) may not involve profound changes.

Multilocus phylogenetic analyses

The resolving power of single-gene phylogenies is limited and SSU rRNA sequences often do not provide satisfactory resolution for determining the phylogenetic positions of deep-branching lineages. To further define the phylogenetic position of *Micavibrio*, partial regions of the *rpoB, atpD, cox2, cox3* and *cob* genes from *Micavibrio* strain EPB were amplified and sequenced. The analyses included diverse representatives from the major α-proteobacterial groups and were based on sequences totalling 2557 amino acids.

Phylogenetic trees obtained with maximum likelihood (ML) and Bayesian analyses on the combined sequences exhibited the same topologies (Fig. 3): the *α-proteobacteria* class was divided into five stable clades: (1) The four orders: Rhizobiales, Rhodobacterales, Caulobacterales and Sphingomonadales formed together the largest stable clade [bootstrap percentages (BP) = 84%, posterior probability (PP) = 1]; (2) Rickettsiales + mitochondria (BP = 100%, PP = 1); (3) *Micavibrio*; (4) Rhodospirillales except for *Gluconobacter* (Rhodospirillaceae) (BP = 58%, PP = 0.77); and (5) *Gluconobacter oxidans* (Acetobacteraceae). The interrelationships between these clades were poorly resolved. The results suggested that the most basal α-proteobacterial orders are Rickettsiales and Rhodospirillales. *Micavibrio* appeared at an intermediate phylogenetic position as a sister to clades 1 + 2 and 4 (Fig. 3). Thus, in agreement with the 16S rRNA phylogeny, it placed *Micavibrio* as a deep branch within *α-proteobacteria* class without close affiliation to any known lineage.

Indels have been proved to be powerful markers in phylogeny (Gupta, 1998; 2005). A 25-amino-acid insertion was found at the same position in the rpoB proteins of *Micavibrio*, and Rhodospirillales (including *Gluconobacter*) that was absent from all the other known orthologous protein in the *α-proteobacteria* (see Fig. S1 in the *Supplementary material*). This suggests that *Micavibrio* and Rhodospirillales may form sister clades.

In these series of phylogenetic analyses, mitochondrial sequences strongly clustered with those originating from the Rickettsiales (BP = 100, PP = 1). However, the possibility that their grouping is the result of long-branch attraction cannot be totally rejected (Gray *et al*., 1999; Burger and Lang, 2003). A comparison of alternative hypotheses revealed that clustering of mitochondria with Rhodospirillales, as recently suggested (Esser *et al*., 2004) was significantly less likely (pSH < 0.001) than the best topology (Fig. 3). Among the 105 trees representing all the possible

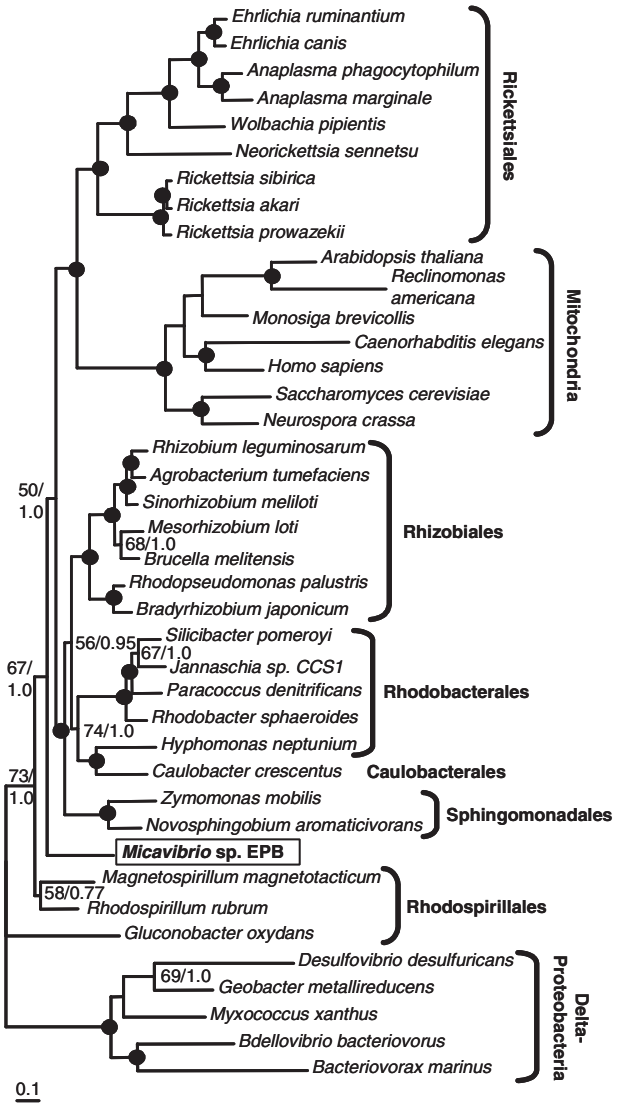

Fig. 3. Maximum likelihood (ML) concatenated amino acid tree showing the affiliation of *Micavibrio* and mitochondria within the *α-proteobacteria*. For each node the ML bootstrap percentage (BP) and the Bayesian posterior probabilities (PP) are given, respectively, at the right and left of the slash. Nodes getting a BP > 80% and a PP > 0.99 are marked with a full circle. BP below 50% and PP below 0.75 are not indicated.

relationships between the five above mentioned clades, 35 trees appeared to be significantly less likely than the best tree (pSH < 0.05). However, the reconstruction of the majority rule consensus of these 35 trees did not indicate that any clade combination was significantly worse.

A super-network constructed of individual ML protein trees (Fig. 4 and Fig. S2 in the *Supplementary material*) well reflected the conflicts observed between the various ML topologies (Fig. S3) which mainly involved relationships between the five above mentioned α-proteobacterial clades. Such conflicts between individual ML trees that

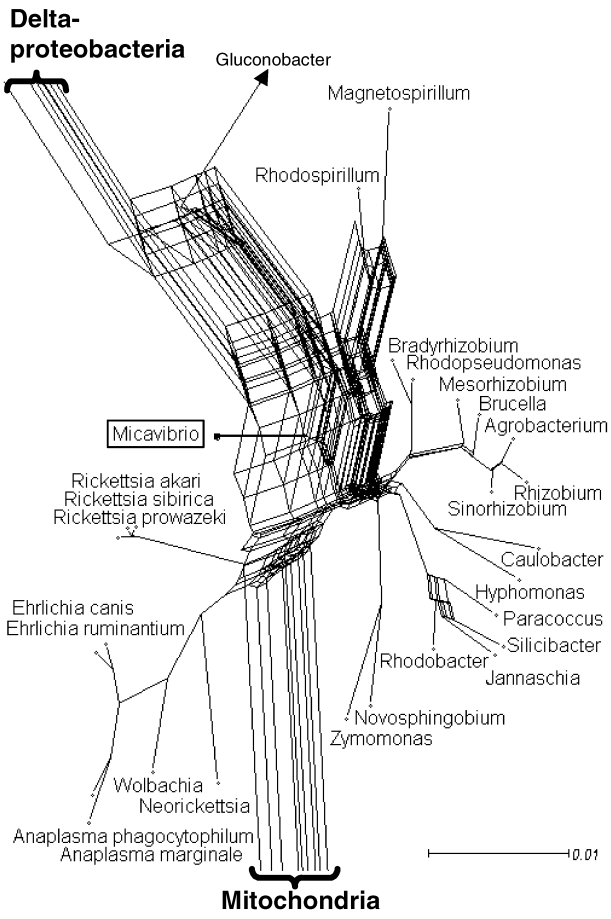

Fig. 4. A phylogenetic super-network showing the affiliation of Micavibrio and mitochondria within the α-*proteobacteria*. The network obtained by applying the Z-closure method to the five protein ML trees. Only the detailed relationships among the α-*proteobacteria* are presented. The whole network is shown in Fig. S3 in the *Supplementary material*. The branches leading to *Gluconobacter*, δ-*proteobacteria* and mitochondria have been cut. Full names are shown in Fig. 3.

are not supported by high BP values may result from a restricted number of informative characters available in the databases. The super-network was however, in good agreement with the concatenated protein and the 16S rRNA trees (Figs 2 and 3): *Micavibrio* was centrally positioned within it (Fig. 4), confirming its early split within the α-*proteobacteria*.

Discussion

Deep-branching α-proteobacterial predators and the mitochondrial ancestor

In this study, *Micavibrio* spp., small, highly motile, obligate predatory bacteria that were isolated from soil in Israel and from sewage in Russia (Lambina *et al*., 1983) and resemble the known BALOs were shown to constitute a deep branch within the α-*proteobacteria* class. This branch was not affiliated to any known α-proteobacterial group, and the deepest α-proteobacterial orders, i.e. Rhodospirillales and Rickettsiales (Gupta, 2005; Fitzpatrick *et al*., 2006) seemed to represent *Micavibrio*'s closest cultured groups (Figs 2–4).

Recently, another α-*proteobacterium* (IricES1), residing as a symbiont in the ovarian tissues of *Ixodes* ticks, was seen invading and consuming mitochondria in a mode similar to that exhibited by BALO predators (Beninati *et al*., 2004; Sacchi *et al*., 2004) (Fig. 1E). IricES1 is part of a novel, separated clade within the Rickettsiales (Fig. 2), a group only consisting of obligate intracellular parasites or symbionts of eukaryotes.

Bdellovibrionales form the two distant and internally diverse families Bacteriovoracaceae and Bdellovibrionaceae, clustered within the δ-*proteobacteria* (Davidov and Jurkevitch, 2004). The latter is an early diverging lineage, distinct among the other members of this class. It contains SSU rRNA secondary structures motifs atypical to δ- but common in the α-*proteobacteria* (Davidov and Jurkevitch, 2004). Moreover, genome analysis of *Bdellovibrio bacteriovorus* 100 did not yield a particular phylogenetic relation to any other microbial genome (Rendulic *et al*., 2004), and the LexA protein of this strain was recently suggested to represent a primordial δ-*proteobacteria* LexA, prior to specialization seen in other proteobacteria (Campoy *et al*., 2005). Thus, *Bdellovibrio*-like predators seem to form deep branches within both the δ- and the α-*proteobacteria* classes. These two classes might have shared a distant ancestry exclusive of other bacteria (Gupta, 2005). Although, it is possible that *Bdellovibrio*-like predatory lifestyles evolved independently in these two classes, and predation is a derived character that appeared recently in the long *Micavibrio*-branch, it is equally possible that a *Bdellovibrio*-like bacterium was a common ancestor of some or even all of these lineages. Many BALOs are probably unculturable as they cannot utilize the prey used in the isolation procedures or depend on unculturable prey and on other non-standard growth conditions or do not form visible plaques under cultivation. As BALOs can show a wide range of predatory efficiency towards their laboratory hosts (Rogosky *et al*., 2006) and as they are isolated on lawns of potential prey, the more aggressive they are, the easier is their detection. A BALO that lyses its prey slowly or with low efficiency will thus probably remain undetected. It is therefore reasonable to assume that such interactions involving 'moderate' aggressiveness and unknown BALO clades exist.

Phylogenetic analyses indicate that mitochondria are derived from an α-*proteobacterium* ancestor. Among the known living α-proteobacterial groups, Rickettsiales seem to represent the closest group to mitochondria (Kurland and Andersson, 2000; Emelyanov, 2003; Gupta, 2005; Fitzpatrick *et al*., 2006) (Figs 3 and 4). While both mito-

chondria and Rickettsiales are obligate intracellular entities, they most probably evolved from free-living bacteria by independent reductive evolution (Gray *et al*., 1999; Burger and Lang, 2003; Gabaldón and Huynen, 2003; Boussau *et al*., 2004). What was the nature of these free living ancestors? Computational inference suggested an aerobic motile bacterium with pili and surface proteins for interactions with its host cells, as the ancestor of the α-*proteobacteria* (Boussau *et al*., 2004). A reconstruction of the proto-mitochondrial proteome revealed an abundance of metabolite transporters including lipid, glycerol and amino acid transporters, and generally pointed towards an aerobic organism with a host dependency for several compounds (Gabaldón and Huynen, 2003). These primordial bacteria probably preceded the origin of eukaryotes, suggesting their hosts were prokaryotes, i.e. they were predatory bacteria.

Predation as the mechanism for the mitochondrial endosymbiotic acquisition

The most accepted hypotheses concerning the origin of the eukaryotic cell suggest that it resulted from the integration of Bacteria and Archaea cells, probably through endosymbiotic acquisition (Gupta, 1998; Emelyanov, 2003; Embley and Martin, 2006; Martin and Koonin, 2006). However, prokaryotes were never observed to fuse their cytoplasms or to engulf other cells. In mealy bug bacteriocytes γ-proteobacterial endosymbionts are found within the cytoplasm of a β-proteobacterial host (von Dohlen *et al*., 2001). Yet, the means by which this association developed in these obligate endosymbionts is still unknown and its relevance for free living bacteria is questionable (Cavalier-Smith, 2002). Phagotrophy requires a complex internal cytoskeleton, which interacts with the plasma membrane that is absent in prokaryotes. Instead, prokaryotes normally possess a rigid cell wall acting as an exoskeleton that also prevents phagocytosis. Therefore, engulfment of a prokaryotic cell by another prokaryote is mechanistically problematic. Accumulating data suggest that all characterized amitochondrial eukaryotes, including hydrogenosome-bearing cells are derived from mitochondriate ancestors (van der Giezen *et al*., 2005; Gray, 2005; Embley and Martin, 2006). Furthermore, recent studies also suggest that mitochondrial endosymbiosis preceded nucleus formation and the ability to phagocytose (Mans *et al*., 2004; Staub *et al*., 2004). Consequently, and because predation does not call for hitherto unknown mechanisms to explain the entry of the endosymbiont into its host, we propose that a primeval micropredator exhibiting a *Bdellovibrio*-like feeding mode, could explain the acquisition mechanism at the origin of the mitochondrial endosymbiosis. *Bdellovibrio*-and-like organisms are known to parasite on Proteobacteria. Interestingly, a prokaryotic parasite of Archaea was recently discovered (Huber *et al*., 2002), suggesting that an ancestral predator or even modern BALOs may be able to attack archaeal hosts.

A possible scenario for the origin of the mitochondrion from predatory bacteria

Predation and parasitism appeared early during the evolution of prokaryotes (Guerrero *et al*., 1986; Maynard-Smith and Szathmary, 1995). In the absence of competition from eukaryotic predators, many lineages of predatory bacteria may have evolved. The drastic global increase of atmospheric O_2 levels about 2.45–2.2 billion years ago (Canfield, 2005) was likely to create a major crisis for anaerobes that could not adapt to the increasing oxygen levels. It has been proposed that an oxygen respiring bacterium reducing local oxygen concentration may have enabled anaerobic cells in its vicinity to survive, thereby providing the basis for the establishment of the endosymbiosis (Margulis, 1993; Andersson and Kurland, 1999). Accordingly, we propose that this relationship derived from predatory events involving a primordial aerobic α-*proteobacterium* predator that formed a stable association with an anaerobic archaeal host, thus providing the basis for their mutualistic symbiosis.

In non-obligate bacterial predators, predation can be modulated when nutrient resources increase (Casida, 1988). Nutrient supply by an anerobic host to its respiring predatory partner could have modulated the latter's aggressiveness. For example, host-independent mutants of BALOs that grow axenically in a rich medium attack and consume prey cells under nutrient-poor conditions (Barel and Jurkevitch, 2001). Moreover, stable bdelloplasts (an infected prey containing a BALO) can survive for months, and bdellocysts (cyst-like forms of *Bdellovibrio*) may form within invaded cells (Tudor and Conti, 1977; Sanchez-Amat and Torrella, 1990) further providing mechanisms for the development of stable endosymbiotic interactions between prokaryotes.

Processes needed for the evolution of successful interactions between eukaryotic hosts and parasitic or mutualistic endosymbiotic bacteria are largely similar (Ochman and Moran, 2001). Furthermore, it appears that mutualistic endosymbionts often arose from pathogenic bacteria as attenuated pathogens (Gil *et al*., 2004). However, in the pre-eukaryotic era prokaryotes could only parasitize other prokaryotes, maybe in a way similar to that observed with 'modern' BALOs. Along those lines, it can be suggested that endosymbiosis could have evolved from such parasites, ultimately giving rise to the most successful symbiosis interaction ever developed – the eukaryotic cell.

Experimental procedures

Bacterial strains isolation procedure and prey range assays

The predatory bacteria strains EPB, EPC2 and EPC4 were isolated using *P. corrugata* PC as prey, while strains ESA, ESB and ESC were isolated using *P. syringae* as prey. All isolates originated in a soil sampled from a spring bank in Kibbutz En-Hanaziv in the Beit-Shean valley of Israel. Isolation procedure, media and maintenance were as described previously (Jurkevitch *et al*., 2000). *Micavibrio aeruginosavorus* strain ARL-13, which was isolated from sewage, Pushchino, Russia using *P. aeruginosa* as prey (Lambina *et al*., 1983), was kindly donated by A. Afinogova (previously at the Russian Academy of Sciences, Pushchino, Russia). The prey range of strain EPB was tested on the following bacteria: *Agrobacterium tumefaciens* C58, *Azospirillum brasilense* Cd, *Bacillus megaterium*, *Pectobacterium carotovorum* ssp. *carotovorum, E. coli* ML35, *Pseudomonas aeruginosa, P. putida* ATCC 12633, *Stenotrophomonas maltophilia* according to Davidov and colleagues (2006).

Electron microscopy

Samples were negatively stained and supported on carbon-formvar-coated copper grids. Grids were inverted over a drop of 1% uranyl acetate. Thin sections were prepared by retrieving 5 ml of sample from a 50-ml co-culture of predator and prey cells at appropriate incubation times (as determined by phase contrast light microscopy). The samples were mixed with 0.5 ml of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, centrifuged and resuspended in 1 ml of the same solution for 3 h. Cells were washed in cacodylate buffer, fixed with 1% osmium tetroxide and enrobed in agar. After fixation in 2% uranyl acetate cells were dehydrated in an ethanol series and embedded in Epon resin. Thin sections were cut and stained with uranyl acetate and lead citrate. Specimens were examined with a Philips CM10 electron microscope operated at 80 kV.

Amplification and sequencing

To remove residual prey while retaining the predatory cells, late two-membered cultures were twice filtered through 0.45 μm filters. DNA was purified using a Qiagen Genomic DNA Kit (Qiagen, Hilden, Germany). Templates used for polymerase chain reaction were either 1–10 ng of purified DNA or suspensions of filtered two-membered cultures subjected to three cycles of freezing in liquid nitrogen followed by three min in boiling water with a final cooling on ice and the addition of 10% DMSO.

Amplification and sequencing of the 16S rRNA genes were as previously described (Jurkevitch *et al*., 2000). The *rpoB, atpD, cox2, cox3* and *cob* genes encoding for the RNA polymerase β-subunit, the ATPase β-subunit, cytochrome oxidase subunits 2 and 3, and apocytochrome *b*, respectively, are commonly used as markers for bacterial or bacterial-mitochondrial phylogeny (Gray *et al*., 1999; Ludwig and Klenk, 2001). Degenerative primers (see Table S2 in the *Supplementary material*) directed against conserved regions of these genes were designed, thereby enabling amplification and sequencing of parts of these genes in *Micavibrio* strain EPB. Polymerase chain reaction conditions were: 95°C for 4 min; 30 cycles of 94°C for 1 min, 54–65°C (Table S2) for 1 min and 72°C for 1–2 min; and a final elongation step at 72°C for 5 min. Additional internal custom-designed primers were used as needed for primer walking (data not shown).

16S rRNA phylogenetic analysis

Sequences were added to the rRNA gene sequence database of the ARB phylogenetic program package (Ludwig *et al*., 2004). Sequences appearing in BLASTN (Altschul *et al*., 1997) and FASTA (http://www.ebi.ac.uk/fasta33/) similarity searches using *Micavibrio* sequences were also included in the analysis. Trees were constructed as previously described (Davidov and Jurkevitch, 2004) with ML, parsimony and neighbour-joining analyses, excluding different degrees of variable positions and using different outgroups.

Retrieval of protein sequences and alignment

Bacterial and mitochondrial sequences with homology to the deduced *Micavibrio* proteins were searched using BLASTP, TBLASTN (Altschul *et al*., 1997), GOBASE (O'Brien *et al*., 2003) and GOLD (Bernal *et al*., 2001). Bacterial sequences mostly originated from complete or ongoing genomic projects. All α- and δ-proteobacterial taxa with available sequences for the five genes mentioned above were included in the analyses, except for strains of the same species and for closely related species of same genus. Sequence alignment of protein sequences were performed individually for each gene. Sequences were aligned with CLUSTAL_X (Thompson *et al*., 1997). Alignments were corrected manually. The final alignments are available upon request.

Phylogenetic analysis of protein sequences

Maximum likelihood and Bayesian methods were independently used for each gene and for concatenated sequences. The JTT model of amino acid substitution (Jones *et al*., 1992) and gamma distribution with eight categories for among site rate variation were used. Maximum likelihood analyses were conducted using PHYML v2.4.4 (Guindon and Gascuel, 2003) after preliminary analyses estimated the proportion of invariant sites as very small. Non-parametric BP were computed using 500 replicates.

Also, a super-network was reconstructed from the ML trees of the five protein-encoding gene using the Z-closure algorithm (Huson *et al*., 2004) included in SplitsTree 4beta23 (Huson, 1998) and using 1000 repeats.

A Bayesian analysis was performed with MrBayes3_0b4 (Ronquist and Huelsenbeck, 2003). The analysis was conducted on partitioned data with each of the five genes evolving with independent model parameters. One million generations of Metropolis-coupled Markov chain Monte Carlo (MCMCMC) were run with four chains sampled every 100 generation. Clade posterior probabilities were calculated after removal of a burning containing the first 5000 trees.

Alternative topologies were explored with MOLPHY 2.3b3 (Adachi and Hasegawa, 1996) that was used to write the 105 possible bifurcating topologies connecting the six following clades: 1-Rhizobiales + Rhodobacterales + Caulobacterales + Sphingomonadales; 2-Rickettsiales + mitochondria; 3-*Micavibrio*; 4-Rhodospirillales except for *Gluconobacter*; 5-*Gluconobacter oxydans*, and; 6-δ-*proteobacteria*. Additionally, the possibility that mitochondria could be related to Rhodospirillales (Esser *et al*., 2004) was also tested while placing the mitochondria with the Rhodospirillales excluding *Gluconobacter,* or as a sister clade of *Micavibrio* + *Gluconobacter* + other Rhodospirillales. Statistical comparisons were conducted with PAML v3.14 (Yang, 1997). A partition analysis on the combined data was conducted with each of the five genes evolving with independent model parameters. Partitioned log-likelihoods were then compared using the Shimodaira and Hasegawa test (Shimodaira and Hasegawa, 1999).

Nucleotide sequences accession numbers

The sequences reported in this study are deposited under GenBank (http://www.ncbi.nlm.nih.gov/GenBank) Accession numbers DQ186612–DQ186623.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Partial sequence alignment for rpoB protein from diverse α-proteobacterial species. A 25-amino-acid insertion is shown in *Micavibrio* and all *Rhodospirillales* but absent from all other α-*proteobacteria*. *Reclinomonas americana*, which is the only known mitochondrial sequence encoding for this gene, shows a shorter insertion at the same region. However, the alignment of this sequence is ambiguous and the insertion event possibly occurred independently.

Fig. S2. A phylogenetic super-network showing the affiliation of mitochondria, α- and δ-*proteobacteria*.

Fig. S3. Independent maximum likelihood amino acid trees for the *rpoB* (A), *atpD* (B), *cox2* (C), *cox3* (D) and *cob* (E) genes. Corresponding BP computed after 500 replicates are indicated. Only BP supports above 50% are indicated. The ML analysis was conducted with the program PHYML v2.4.4 using the JTT model of amino acid substitution. Among site rate variation was represented by a gamma distribution with eight categories. Full names are shown in Fig. 3. The network obtained by applying the Z-closure method to the five protein ML trees. The relationships among the α-*proteobacteria* indicated in the rectangle are detailed in Fig. 4.

Table S1. Primers used for amplification and sequencing.

Table S2. Primer-pairs and annealing temperatures used for amplification.

Table S3. Environmental clone sequences exhibiting similarity to *Micavibrio* 16S rRNA sequences.

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