

The versatile ϵ -proteobacteria: key players in sulphidic habitats

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Abstract | The ϵ -proteobacteria have recently been recognized as globally ubiquitous in modern marine and terrestrial ecosystems, and have had a significant role in biogeochemical and geological processes throughout Earth's history. To place this newly expanded group, which consists mainly of uncultured representatives, in an evolutionary context, we present an overview of the taxonomic classification for the class, review ecological and metabolic data in key sulphidic habitats and consider the ecological and geological potential of the ϵ -proteobacteria in modern and ancient systems. These integrated perspectives provide a framework for future culture- and genomic-based studies.

Although pathogenic species such as *Helicobacter pylori* have been well studied, the ϵ -proteobacteria, to which *H. pylori* is affiliated, is the most poorly characterized class within the Proteobacteria^{1–3}. In 2002, the International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Campylobacter* and related bacteria⁴ recognized the increasing number of unclassified and unaffiliated ϵ -proteobacterial 16S ribosomal RNA (rRNA) sequences deposited into the public databases and recommended that future investigations should deal with this growing problem. Despite recent culture-based investigations and descriptions for novel ϵ -proteobacterial groups, most lineages are still without cultured representatives or are known only from environmentally retrieved 16S rRNA gene sequences from PCR-based studies of anaerobic to microaerophilic, sulphur-rich marine and terrestrial aquatic environments, or from symbioses with metazoans. Many of these habitats are deemed 'extreme' environments — from the hydrothermal fluids of deep-sea vents to the cold darkness of sulphidic caves.

A taxonomic framework for the ϵ -proteobacteria is still lacking. For lineages without cultured representatives, this has made it difficult to fully assess the importance of any newly discovered bacteria. In this review, we evaluate class taxonomic structure as a frame of reference for placing new ϵ -proteobacterial sequences derived from 16S rRNA gene analyses in an evolutionary context. With this perspective, and to offer recommendations for future research directions, we explore major habitats and highlight ecophysiological diversity patterns based on phylogeny and current metabolic and genomic properties of cultured representatives.

Phylogenetic and ecophysiological diversity

Ideally, taxonomic classification should be performed through a polyphasic approach using more than one molecular marker and phenotypic information derived from cultured representatives^{5,6}. However, because of the widespread and almost exclusive use of the 16S rRNA gene for phylogenetic studies, and the dearth of cultures, we compiled 1,037 16S rRNA gene sequences (>1,200 bp) from public databases (RDPII, GenBank, EMBL and DDBJ) up to May 2005 and from published reports of clones, strains or sequences described as 'uncultured bacterium' with previously determined phylogenetic affinity to the ϵ -proteobacteria. To construct a phylogenetic foundation for more detailed analyses, a Neighbour Joining (NJ) tree was constructed in PAUP* (REF.7), calculating distances under the general time-reversible model incorporating invariable sites and rate heterogeneity. The analyses revealed that a few previously affiliated ϵ -proteobacterial 16S rRNA gene sequences were chimeric or misidentified (see **Supplementary information S1** (table)). The four clades that contain environmental sequences were then subjected to more rigorous maximum likelihood analyses using PHYML⁸ with the same model chosen for the NJ analysis. To estimate nodal supports, 100 bootstrap replicates were performed.

The ϵ -proteobacterial sequences currently belong to two valid orders, the Nautiliales (genera *Nautilia*, *Caminiobacter* and *Lebetimonas*)^{2,9–11} and the Campylobacteriales (families Campylobacteraceae, Helicobacteraceae and Hydrogenimonaceae)^{12,13}. Excluding clinical systems (such as infectious associations with humans) affiliated with the *Campylobacter* and *Helicobacter* genera, the remaining ϵ -proteobacterial sequences are diagnosed

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Thermophile

An organism that grows optimally at high temperatures, usually above 45°C.

Autotroph

An organism that can use carbon dioxide as the sole source of carbon for growth.

Heterotroph

An organism that uses organic compounds as nutrients to produce energy for growth.

Chemocline

A chemical gradient from high to low concentrations, often consisting of a relatively small stratum where the concentration changes rapidly between the two endpoints.

Mesophile

An organism that grows optimally at moderate temperatures, ranging between 20°C and 45°C.

into four robust phylogenetic clusters — classified here as the Nautiliales, *Arcobacter*, *Sulfurospirillum* and environmental sequence clusters — that consist of sequences retrieved from various marine systems (for example, deep-sea hydrothermal vents, vent fauna and deep-sea marine subsurfaces) and terrestrial systems (for example, groundwater, caves and springs) (FIG. 1).

With few exceptions, ϵ -proteobacterial sequence affinities strongly correlate with ecotype for each of the phylogenetic clusters (denoted as coloured lines in FIG. 2 and coloured text in **Supplementary information S2** (figure)) and metabolic capabilities (denoted as coloured symbols in **Supplementary information S2** (figure)). Within the deeply branching group of the Nautiliales, sequences have been retrieved exclusively from hydrothermal systems, and cultured representatives of the family are thermophilic, autotrophic and can reduce elemental sulphur with molecular hydrogen (see **Supplementary information S2** (figure), part a). Even within the *Sulfurospirillum* (FIG. 1; see **Supplementary information S2** (figure), part b) and *Arcobacter* (FIG. 1; see **Supplementary information S2** (figure), part c) clusters, nearly all of the sequences are grouped based on environmental setting and metabolism. For instance, although all characterized *Sulfurospirillum* spp. ferment

using fumarate and can reduce nitrate to ammonia, with the exception of *Sulfurospirillum multivorans*¹⁴, one feature that phylogenetically distinguishes the cultured sulfurospirilla is their ability to respire using alternative electron acceptors under heterotrophic conditions¹⁵ (TABLE 1; see **Supplementary information S2** (figure), part b). Sequences from different strains that respire using similar elements are more closely related to each other compared with other species within the family, despite strains originating from different geographical locations (for example, *Sulfurospirillum carboxydovorans*, *Sulfurospirillum arcachonense* and *Sulfurospirillum* sp. Am-N).

Although arcobacters have been implicated in human and animal enteric diseases¹⁶, few studies have combined isolation and molecular techniques to examine their habitat range¹⁷. The type species of the genus *Arcobacter nitrofigilis* was isolated from a salt-marsh plant root¹⁸, but there is still significant diversity among the arcobacters. Similar to the sulfurospirilla, *Arcobacter* sequences retrieved from marine and terrestrial habitats group together (FIG. 1) and with ecotype (see **Supplementary information S2** (figure), part c). Although the metabolic capabilities of most arcobacters have not been studied in detail, many of the cultured representatives originate from marine environments with a well defined geochemical interface between dissolved oxygen and sulphide concentrations¹⁷. For example, 'Candidatus *Arcobacter sulfidicus*' was isolated from coastal marine sediments with an oxygen–sulphide chemocline¹⁹. This bacterium undergoes mesophilic, chemolithoautotrophic growth, and produces filamentous sulphur with sulphide and oxygen as the electron donor and acceptor, respectively. Based on radio- and stable-isotopic experiments of carbon-fixation processes, Candidatus *A. sulfidicus* was the first ϵ -proteobacterium thought to assimilate inorganic carbon sources, not through the Calvin–Benson pathway, but by means of the reductive TCA cycle (rTCA cycle)¹⁹.

The phylogenetic assignment of the remaining sequences is problematic. Based on the bootstrap supported phylogenetic topology, there is a large group that is distinct from the other major clusters (FIGS 1,2; see **Supplementary information S2** (figure), part d). This cluster represents the largest increase in 16S rRNA gene-sequence diversity throughout the ϵ -proteobacteria and includes several recently described genera. Currently, this sequence cluster has no hierarchical taxonomic classification and future taxonomic revision is required to elucidate the possibility that the cluster might represent more than one hierarchical group.

We have provisionally named this clade Thiovolgaceae fam. nov. for ease of reference throughout this review^{20–22}. Thiovolgaceae is derived from *thio* meaning 'sulphur' and *vulgar* meaning 'of, pertaining to, common', forming Thiovolga meaning 'pertaining to sulphur';-aceae represents the ending to denote a family. The cultured genera that belong to the family are Gram-negative bacteria that have rod-, vibrio- or filamentous-shaped non-spore-forming cells. Organisms are found in mesophilic conditions, and cultured representatives are

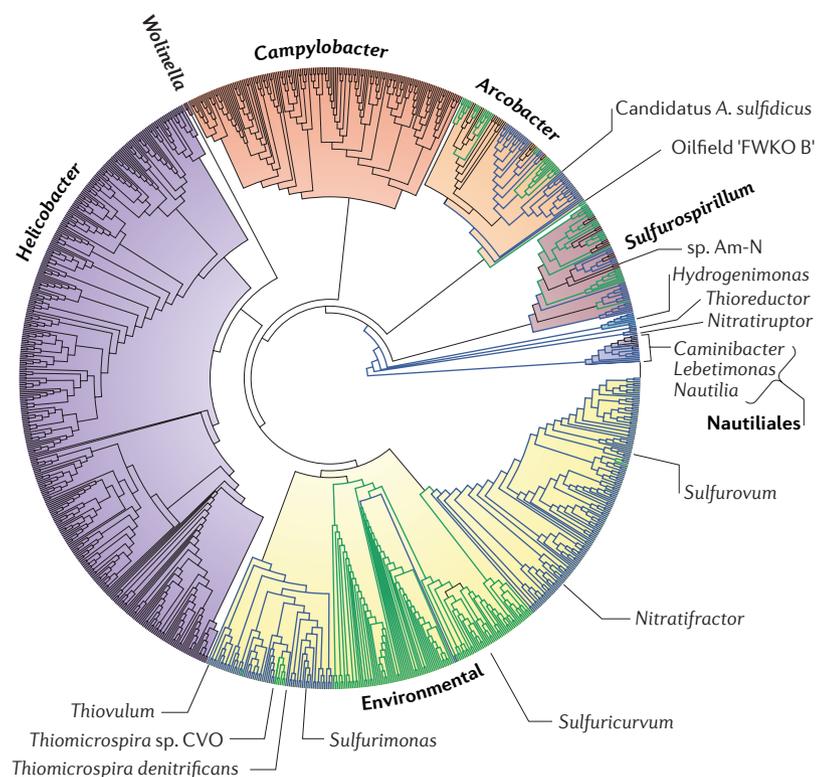


Figure 1 | Phylogeny of 1,037 near full-length (>1,200 bp) ϵ -proteobacterial sequences collected from public databases and published research. Sequences were aligned using Muscle v3.52 (REF. 116) followed by removal of highly divergent and ambiguous regions using Gblocks v0.91b (REF. 117). The phylogeny was reconstructed using Neighbour Joining under a general time-reversible model of evolution. Major taxonomic divisions, and all of the currently recognized genera, are indicated. Branches for environmental sequences are coloured to represent either marine (blue) or terrestrial (green) habitats.

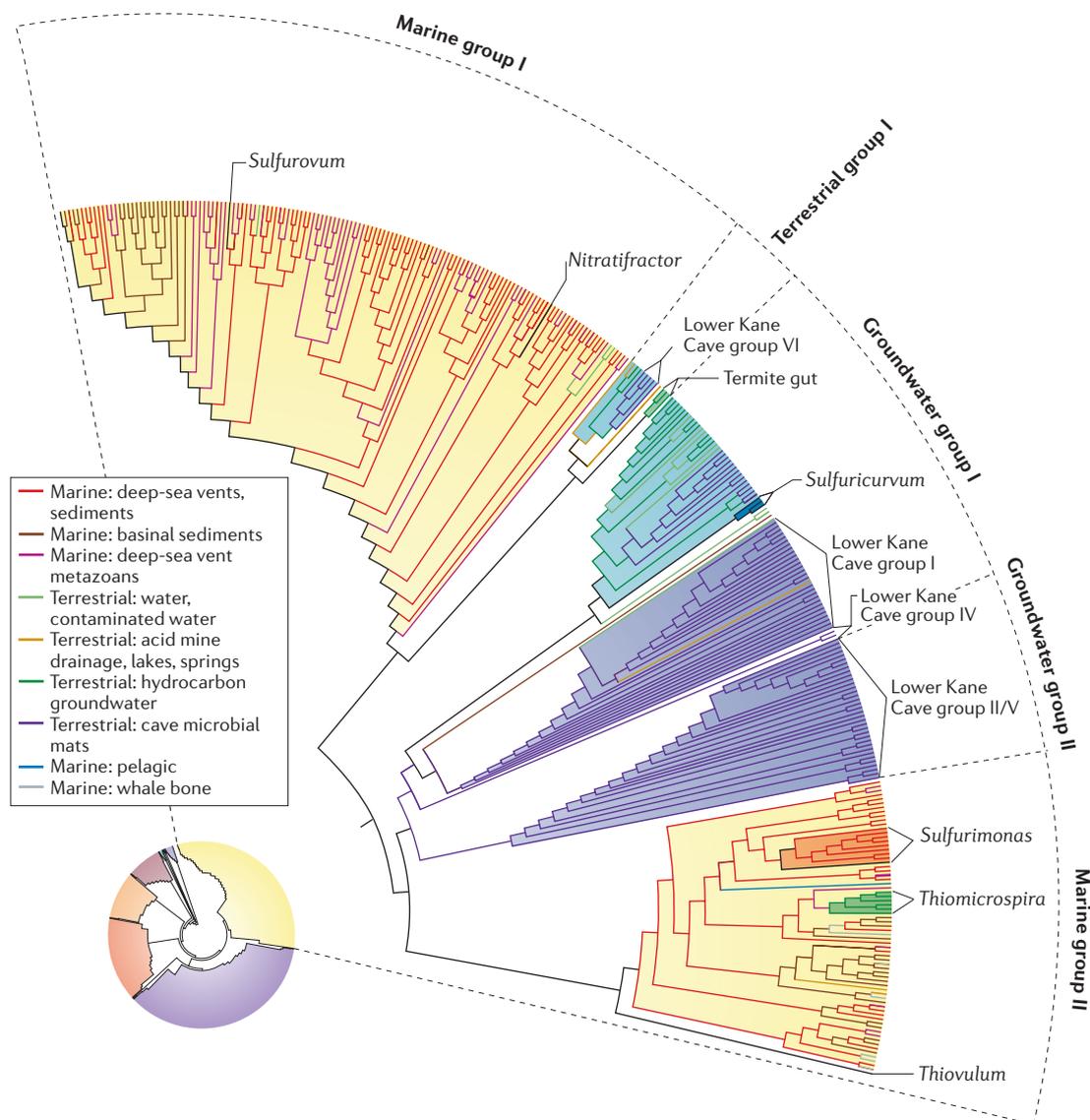


Figure 2 | The provisional Thiovulgaceae fam. nov. clade. This figure is expanded from FIG. 1. Branches are coloured to represent ecotype. Based on additional, more rigorous maximum likelihood analysis, terrestrial group I is placed outside of the Thiovulgaceae fam. nov. as shown in Supplementary information S2 (figure).

Chemolithoautotroph
An organism that obtains energy from inorganic compounds and carbon from CO₂.

Calvin–Benson pathway
Also known as the Calvin–Benson cycle. A series of biochemical, enzyme-mediated reactions in which CO₂ is reduced and incorporated into organic molecules.

Reductive TCA cycle
(rTCA cycle). The TCA cycle in reverse, leading to the fixation of CO₂. Represents a putatively ancient metabolic pathway in which autotrophic carbon fixation occurs under anaerobic conditions.

chemolithoautotrophic and can use molecular hydrogen and/or reduced sulphur compounds as electron donors. Members of the family have been isolated from both marine and freshwater habitats.

The Thiovulgaceae fam. nov. family is a member of the order Campylobacterales and comprises the genera *Thiovolulum*²³, *Nitratifactor*²⁴, *Sulfurovum*²⁵, *Sulfuricurvum*²⁶, *Thiomicrospira*^{27,28} and *Sulfurimonas*²⁹, which cluster into four main sequences groups, within which are two discrete ecological units — marine group (MG) and groundwater group (GG) (FIG. 2; see **Supplementary information S2** (figure), part d). Ecotype groups are closely related to each other (for example MG I and MG II), but relatedness is not supported by bootstrap values, which indicates that significant diversity has yet to be uncovered within the cluster. Unlike the phylogenetic and ecotype patterns within the *Sulfurospirillum* and *Arcobacter* clusters, there are little recognizable

fine-scale ecotype clade associations. Both MG I and MG II are composed of sequences retrieved from deep-sea vents and sediments, or are associated with vent fauna; however, MG II contains a slightly broader ecotype diversity than MG I, as MG II contains a clade of terrestrial wastewater (sludge) organisms and *Thiomicrospira* spp.

The newly described genera *Sulfurovum*²⁵ and *Nitratifactor*²⁴ are affiliated with MG I, and the sulphur-oxidizing genera *Sulfurimonas*²⁹, *Thiovolulum*²³ and *Thiomicrospira*^{27,28} are affiliated with MG II. A large group of sequences retrieved from groundwater is separated into two clusters, GG I and GG II. Whereas GG I includes the genus *Sulfuricurvum*²⁶, Lower Kane Cave groups I and IV³⁰, and sequences isolated from wastewater, sludge or groundwater contaminated with petroleum, uranium or trichloroethene, GG II consists of sequences only from Lower Kane Cave (FIG. 2; see **Supplementary information S2** (figure), part d).

Table 1 | Physiological characteristics of ϵ -proteobacteria from deep-sea hydrothermal habitats and other selected environments

Isolate/phylogenetic association	Isolation site	Growth temperature	Carbon metabolism	Electron donor	Electron acceptor	Sulphur/nitrate reduction to:	Ref.
Order Nautiliales, Family Nautiliaceae							
<i>Nautilia lithotrophica</i>	<i>Alvinella pompejana</i> tube, 13°N EPR	53 °C	Mixotroph	H ₂ , formate	Sulphite, elemental sulphur	H ₂ S	10
<i>Nautilia</i> sp. str. Am-H	<i>Alvinella pompejana</i> tube, 13°N EPR	45 °C	Mixotroph	H ₂ , formate	Elemental sulphur	H ₂ S	44
<i>Caminibacter hydrogeniphilus</i>	<i>Alvinella pompejana</i> tube, 13°N EPR	60 °C	Mixotroph	H ₂ , complex organic compounds	Nitrate, elemental sulphur	H ₂ S/NH ₃	9
<i>Caminibacter profundus</i>	Vent cap, Rainbow Field, MAR	55 °C	Autotroph	H ₂	Nitrate, oxygen (microaerobic) elemental sulphur	H ₂ S/NH ₃	2
<i>Caminibacter mediatlanticus</i>	Chimney, Rainbow Field, MAR	55 °C	Autotroph	H ₂	Nitrate, elemental sulphur	H ₂ S/NH ₃	46
<i>Lebetimonas acidiphila</i>	<i>In situ</i> colonization system, TOTO, MA	50 °C	Autotroph	H ₂	Elemental sulphur	H ₂ S	11
Order uncertain, Family Hydrogenimonaceae							
<i>Hydrogenimonas thermophila</i>	Chimney, Kairei Field, CIR	55 °C	Autotroph	H ₂	Nitrate, oxygen (microaerobic), elemental sulphur	H ₂ S/NH ₃	13
Order uncertain, Family Nitratiruptoraceae							
<i>Nitratiruptor tergaricus</i>	Chimney, Iheya North Field, OT	55 °C	Autotroph	H ₂	Nitrate, oxygen (microaerobic), elemental sulphur	H ₂ S/N ₂	24
Order uncertain, Family Thioreductoraceae							
<i>Thioreductor micantisoli</i>	Sediment, Iheya North Field, OT	32 °C	Autotroph	H ₂	Nitrate, elemental sulphur	H ₂ S/NH ₃	45
Order Campylobacterales, Family Campylobacteraceae							
<i>Sulfurospirillum</i> sp. str. Am-N	<i>Alvinella pompejana</i> , 13°N EPR	41 °C	Heterotroph	Formate, fumarate	Elemental sulphur	H ₂ S	44
<i>Arcobacter</i> sp. str. FWKO B	Production water, Coleville oil field	30 °C	Autotroph	H ₂ , formate, sulphide	Nitrate, oxygen (microaerobic), elemental sulphur	H ₂ S/NO ₂ ⁻	28
Order uncertain, Family Thiovulgaceae							
<i>Sulfurovum lithotrophicum</i>	Sediment, Iheya North Field, OT	30 °C	Autotroph	Elemental sulphur, thiosulphate	Nitrate, oxygen (microaerobic)	N ₂	25
<i>Nitratifractor salsuginis</i>	Chimney, Iheya North Field, OT	37 °C	Autotroph	H ₂	Nitrate, oxygen (microaerobic)	N ₂	24
<i>Sulfurimonas autotrophica</i>	Sediment, Hatoma Knoll, OT	25 °C	Autotroph	Elemental sulphur, thiosulphate	Oxygen (microaerobic)		29
<i>Sulfuricurvum kujiense</i>	Groundwater, Japan oil storage cavity	25 °C	Autotroph	H ₂ , sulphide, thiosulphate, elemental sulphur	Nitrate, oxygen (microaerobic)	NO ₂ ⁻	26
<i>Thiomicrospira</i> sp. str. CVO	Production water, Coleville oil field	30 °C	Mixotroph	Sulphide, elemental sulphur	Oxygen (microaerobic), nitrate, nitrite	N ₂ , N ₂ O	28

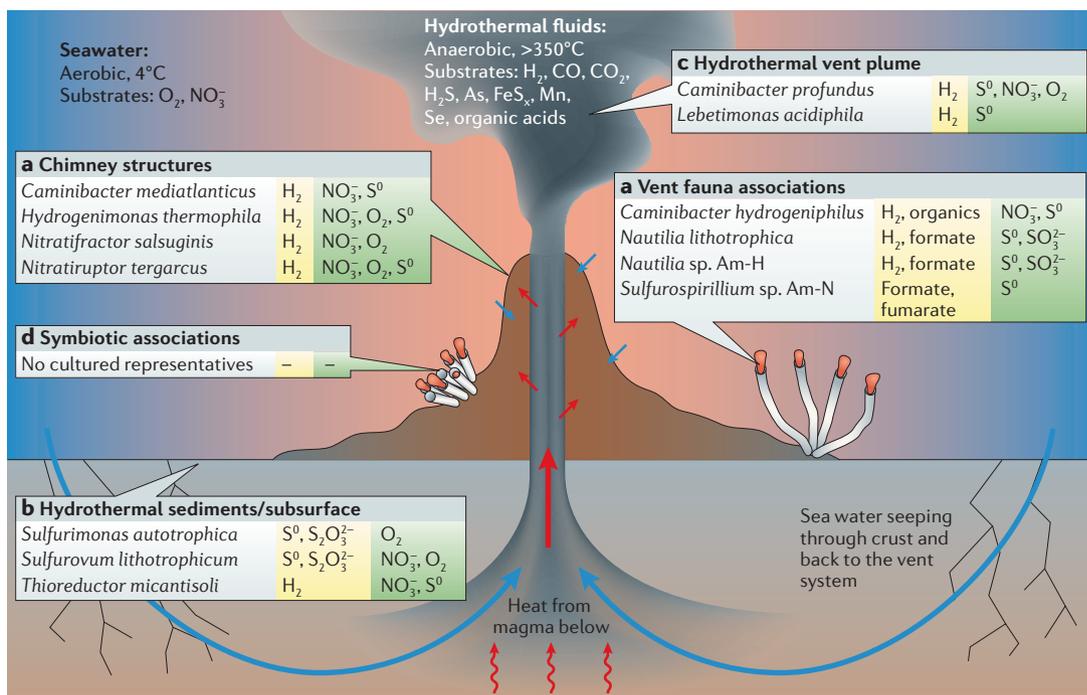
Location abbreviations: CIR, Central Indian Ridge; EPR, East Pacific Rise; MA, Mariana Volcanic Arc; MAR, Mid-Atlantic Ridge; OT, Okinawa Trough; TOTO, TOTO caldera deep-sea hydrothermal field. Chemical abbreviations: H₂S, hydrogen sulphide; NH₃, ammonia; N₂O, nitrous oxide; NO₂⁻, nitric oxide.

An additional sequence cluster, representing other terrestrial ecotypes (TG), is placed outside of the Thiovulgaceae fam. nov. and other families within the order Campylobacterales (see [Supplementary information S2](#) (figure), part d). The TG I sequences from acid mine drainage, Lower Kane Cave, contaminated groundwater and termite guts might represent greater

taxonomic diversity than previously hypothesized. Moreover, other than these few termite-gut sequences, virtually nothing is known about the potential of ϵ -proteobacterial symbioses with terrestrial organisms. Future work in these poorly investigated or unexplored terrestrial systems should increase the known diversity of ϵ -proteobacterial groups.

Box 1 | Integrating ecology and biogeochemistry: hydrothermal vents as a case study

The ϵ -proteobacteria have been found at, and sometimes dominate, four main deep-sea hydrothermal vent-specific habitats: mats on the surfaces of rocks, chimneys and animal surfaces (a in the figure); discharged vent fluids and sub-seafloor (b); within the hydrothermal vent plume (c); and symbiotic associations with vent animals such as *Alvinella pompejana*, *Alviniconcha* aff. *hessleri*, and *Rimicaris* spp.¹⁰³ (d). The metabolically versatile ϵ -proteobacteria are uniquely suited to thrive in deep-sea habitats and other extreme settings. These hydrothermal-vent habitats are all dynamic suboxic to anaerobic environments, and the ϵ -proteobacteria use many metabolic processes, including sulphur oxidation, sulphur/sulphite reduction, nitrate (NO_3^-) or nitric oxide (NO_2^-) reduction to ammonium or nitrogen, and hydrogen and formate oxidation (see figure). In the figure, known electron donors are shown in the yellow shaded blocks and electron acceptors in the green shaded blocks. Some of the ϵ -proteobacteria might use complex sulphur species for sulphur oxidation or they might biotically influence the formation of iron-sulphur minerals, such as pyrite, at vents^{104–106}. Carbon monoxide might also be used as an electron donor and metal(oid)s (iron, manganese, arsenic and selenium) might be used as electron acceptors, although these metabolisms have not been fully examined in vent habitats. These energy pathways can either be coupled with autotrophy (probably through the reductive TCA cycle), mixotrophy or heterotrophy (TABLE 1). The ϵ -proteobacterial groups establish themselves as the primary (and perhaps the first) colonizers in the dynamic diffuse flow vent environment because of their metabolic flexibility (TABLE 1), specialized gene assemblages⁸⁷, the possibility of special modes for attachment to surfaces⁴³, rapid colonization at O_2 – H_2S interfaces (possibly by formation of filamentous sulphur from hydrogen sulphide³⁹) and phylotypic diversification over time as new habitats are colonized following eruptions or with titanium ring for *Alvinella* colonization (TRAC) deployment^{34,43,48,70}. Ecological principles indicate that there is a tendency for the most productive species in an ecosystem to be the most dominant in a habitat, thereby pushing other species to comparatively lower densities. It is not surprising that the metabolically versatile ϵ -proteobacteria colonize extensive areas that are warmer (20–60°C) and have higher concentrations of sulphur species than locations where typical chemolithoautotrophic γ -proteobacteria are found.



In the figure, the cultured species from each hydrothermal-vent-specific habitat are shown. Coloured arrows indicate the flow of either hot hydrothermal fluids (red) or cold sea water (blue). S^0 , elemental sulphur; SO_3^{2-} , sulphite; $\text{S}_2\text{O}_3^{2-}$, thiosulphate.

ϵ -Proteobacteria from marine systems

Hydrothermal vents and vent-associated subsurfaces.

To interpret the possible ecological and geological significance of the uncultured ϵ -proteobacterial groups, we look to the exemplary hydrothermal vent system (BOX 1). Since the discovery of hydrothermal vents in 1977, the importance of microorganisms as the prevailing biological feature in these environments has been clearly established. Whereas early studies focused on the endosymbiotic microbial assemblages of the vent tube worm, *Riftia pachyptila*³¹, the accumulated

knowledge about the microbial diversity of vent sites now reveals that ϵ -proteobacteria are probably key players in the cycling of (at least) carbon, nitrogen and sulphur, and have important roles in symbiotic associations with vent metazoans (BOX 2). Moreover, deep-sea hydrothermal environments can be regarded as one of the largest reservoirs of diverse environmental ϵ -proteobacteria on Earth, ranging from the deeply-branching Nautiliales and *Nitratiruptor* groups to the *Sulfurospirillum*, *Arcobacter*, and the MG I and MG II of the Thiovulgaceae fam. nov.

Mixotroph

An organism that can use both heterotrophic and autotrophic metabolic processes.

Although PCR biases and differences in library construction and screening³² might skew interpretations, most 16S rRNA gene-based studies indicate an overwhelming dominance of ϵ -proteobacteria in the free-living populations in vent fluids or on, or near, the natural surfaces of vent chimney structures where the most intensive hydrogeochemical mixing occurs between ambient (1–4°C), oxygenated bottom sea water and high-temperature, anoxic and sulphide-enriched vent fluid diffusing from the interior portions of the chimney^{33–37}. The full-cycle rRNA approach (which includes 16S rRNA gene clone library construction and fluorescence *in situ* hybridization (FISH)) has unequivocally shown that up to 90% of the microbial communities found in these hydrothermal sites are composed of ϵ -proteobacteria, predominately associated with the *Nautilia* and *Sulfurimonas* genera^{33,37}.

Other lines of evidence also point to the importance of ϵ -proteobacteria at vents. Taylor and Wirsen³⁸ showed that the flocculent discharge emanating from diffuse flow vents was similar to the filamentous sulphur production by chemolithoautotrophic sulphur-oxidizing bacteria, which were later shown to belong to the genus *Arcobacter*¹⁹. Filamentous sulphur mats composed of both vibrioid and filamentous sulphide-oxidizers, many of which were probably ϵ -proteobacteria, have also been found on titanium devices deployed at vents³⁹. Several other research groups have retrieved ϵ -proteobacterial 16S rRNA gene sequences from vent caps or other *in situ* colonization devices^{34,40–43}. For instance, ϵ -proteobacteria comprised ~81% of the total microbial community from an *in situ* colonization device deployed into diffuse flow vent emissions for 4 days in the Mid-Okinawa Trough³⁴. In this study, water samples collected from 2 m and 10 m away from the chimney structure had dramatically different percentages of ϵ -proteobacteria, from 83% to 17.6%, respectively, indicating that the ϵ -proteobacteria tolerate the immediate and proximal vent conditions, probably owing to the increased availability of energy sources compared with more distal habitats or cold sea water (BOX 1).

Deep-sea hydrothermal-vent habitats have also been important for obtaining pure cultures of diverse phylogenetic groups. The first successful isolations were of hydrogen-oxidizing, sulphur-reducing, thermophilic chemolithoautotrophs from *Alvinella pompejana* symbiont-associated biomass and tube samples; these isolates belong to the Nautiliales^{10,44}. Recently, several previously uncultivated, phylogenetically diverse ϵ -proteobacterial groups were isolated from various geologically and geographically distinct deep-sea hydrothermal fields, all with a diverse range of physiological characteristics and utilization of electron donors (for example, hydrogen and sulphur) and acceptors (for example, sulphur and nitrogen) coupled to carbon fixation^{2,11,13,24,29,34,45–47} (FIG. 1; TABLE 1; see **Supplementary information S2** (figure)).

A sub-seafloor model proposed by Huber *et al.* indicates that ϵ -proteobacteria thrive in diffuse flow areas surrounding vent chimneys and heated crustal fields and sediments, where sea water mixes with hydrothermal fluids; ϵ -proteobacterial cells are discharged when eruptions at mid-ocean-ridge axes flush fluids and resident microorganisms within the seafloor crust and sediment to the seafloor surface and into sea water. Indeed, eruption plumes contain diverse microbial communities, but ϵ -proteobacteria make up ~20–60% of 16S rRNA gene clone libraries from these plumes, with more intergroup diversity occurring from particle-attached libraries than from free-living populations⁴⁸. Eventually, the particles and microorganisms fall to the ocean floor and again become part of the marine sediment and subsurface habitats. These hydrogeological processes have the potential to link microorganisms in all of the marine habitats, and might result in a homogenized genetic pool of microorganisms over time. This could be one explanation why members of ϵ -proteobacteria from the deep-sea hydrothermal vent and sediment systems are closely related to each other.

Box 2 | Episymbionts of *Alvinella pompejana*

At least two endemic hydrothermal vent fauna, *Alvinella pompejana* (East Pacific Rise) and *Rimicaris exoculata* (Mid-Atlantic Ridge), contain ϵ -proteobacterial episymbionts^{107,108}. *A. pompejana*, also known as the Pompeii worm because of its heat tolerance, builds paper-like tube colonies attached to hydrothermal-vent chimneys along the East Pacific Rise. The hydrothermal vent shrimp, *R. exoculata*, forms large clusters on the warmer sections of vents along the Mid-Atlantic Ridge. *A. pompejana*, the biology of which has been extensively reviewed¹⁰⁹, contains two closely related ϵ -proteobacterial phylotypes that comprise over 65% of a 16S rRNA gene library^{107,110} derived from episymbiont biomass. These two groups, both within Marine Group I, are filamentous and distinctly separated horizontally on individual dorsal expansions of *A. pompejana*, indicating niche specialization (M. T. Cottrell and S. C. Cary, unpublished data and REF. 110). More recently, the first endosymbiotic ϵ -proteobacterium, represented by a single ϵ -proteobacterial phylotype, was discovered in a deep-sea hydrothermal-vent-endemic gastropod *Alviniconcha* spp.^{111,112}

There have been few clues as to the role of the epibionts of *A. pompejana* because, despite many attempts, the filamentous ϵ -proteobacterial symbionts have not yet been cultured. Surveys of geochemical conditions within *A. pompejana* tubes revealed high temperatures (~20–80°C) and anoxia, exceeding that of any known metazoan habitat, surprisingly low or trace free hydrogen sulphide (<0.2–46.53 μ M), pH values between 5.3 and 6.4, high concentrations of potential electron acceptors (sulphate, nitrate and iron), and potentially lethal levels of heavy metals (zinc, nickel, vanadium, copper, lead, cadmium, cobalt and silver)^{113–115}. Preliminary analysis of a metagenomic library of the *A. pompejana* symbionts supports the hypothesis that at least a portion of the symbiotic ϵ -proteobacteria detoxify sulphide by rendering it biologically unavailable through metal-transport and sulphide-oxidation processes so that *A. pompejana* can thrive in this extreme microhabitat (S. C. Cary *et al.*, unpublished data). Also, sequence analysis of a fosmid library revealed the potential for these symbionts to use the reductive TCA cycle, owing to the presence of a key gene in the pathway, *aclBA* (ATP citrate lyase)⁸⁷.

Phylotype

A group of sequences that show some threshold of sequence similarity, usually >97%, and that also form a monophyletic clade.

Epibiont

An organism that lives attached to a host organism without apparent consequence (benefit or detriment) to the host.

Marine and subsurface environments. Several recent studies indicate that ϵ -proteobacteria, specifically those affiliated with the Thiovolgaceae fam. nov., have important roles in nutrient cycling and ecosystem function at other marine interfaces and the overall marine subsurface habitat⁴⁹. PCR-based studies reveal that ϵ -proteobacteria occur in high abundance at oxic–anoxic interfaces, such as the chemocline-transition zone where hydrogen sulphide from the sediment meets oxygenated sea water. This is particularly true in both the Black Sea and Cariaco Basin (southern Caribbean Sea), two of the largest anoxic basins on the planet^{50,51}. The ϵ -proteobacteria are also prevalent in deep-sea sediment push cores, the hydrocarbon-rich Guaymas Basin sediment cores, methane cold seeps, gas hydrates and deltaic mud^{52–56}. In many of these habitats, there is also a relatively high abundance of δ -proteobacteria. In one study of cold-seep sediments, δ - and ϵ -proteobacteria comprised >78% of the metabolically active fraction (RNA), with the ϵ -proteobacteria dominating the lower, sulphide-rich sediment fractions⁵⁶. The strong relationship between δ - and ϵ -proteobacteria could be due to their respective roles in the sulphur cycle (that is, sulphate reduction for the δ -proteobacteria and sulphur oxidation for the ϵ -proteobacteria).

ϵ -Proteobacteria in terrestrial systems

According to some researchers, an immense subsurface microbial biosphere might exist that is not just associated with marine sediments or deep-sea hydrothermal vent systems^{57,58}. On the basis of recent PCR-based investigations of terrestrial ecotypes, including naturally sulphur-rich environments such as oil-field brines^{15,27,28,59}, hydrocarbon-contaminated groundwater^{15,59–61}, uncontaminated groundwater⁶², sulphidic springs^{63–65} and limestone caves^{30,66–68}, we are beginning to discover the importance of ϵ -proteobacteria in these habitats. Although there are terrestrial sequences belonging to the *Sulfurospirillum* and *Arcobacter* clusters, generally, most of the recently acquired ϵ -proteobacterial sequences from natural, uncontaminated terrestrial habitats are affiliated with the Thiovolgaceae fam. nov. (FIG. 2; see **Supplementary information S2** (figure)). Several chemolithoautotrophic, nitrate-reducing, sulphur-oxidizing, microaerophilic ϵ -proteobacteria have been isolated from oil-field brines and oil-contaminated groundwater, including *Arcobacter* sp. strain FWKO B²⁸, *Thiomicrospira* sp. strain CVO^{27,28} and *Sulfuricurvum kujiense*^{26,61} of GG I (FIGS 1, 2; see **Supplementary information S2** (figure)). So far, *S. kujiense* (isolated from hydrocarbon-contaminated Japanese groundwater⁶⁰) is the only cultured terrestrial representative in the Thiovolgaceae fam. nov.

Sulphidic caves (limestone caves with discharging hydrogen-sulphide-rich groundwater) allow easy access to the subsurface and are currently one of the best-studied natural terrestrial sites for ϵ -proteobacteria^{30,63,66–68}. Lower Kane Cave serves as an ideal model system for understanding terrestrial ϵ -proteobacteria, particularly the terrestrial Thiovolgaceae fam. nov., because all of the sequences retrieved so far from Lower Kane Cave are affiliated with this evolutionary lineage (FIG. 2; see

Supplementary information S2 (figure))^{30,68}. Subsurface terrestrial habitats are geographically isolated from one another owing to geological structures, hydrostratigraphic connectivity and plate tectonics, and terrestrial organisms should have limited dispersal mechanisms and are not capable of atmospheric dispersal processes^{68,69}. Therefore, high sequence similarity for the GG I sequences retrieved from around the world indicates that the ancestral population giving rise to the modern group might have originated from one geographical location. However, because GG II consists of only Lower-Kane-Cave-derived sequences, this clade might be endemic only to Lower Kane Cave. More research is needed to validate these relationships.

Ecological significance of ϵ -proteobacteria

The ϵ -proteobacteria have significant roles in the habitats in which they thrive, as primary colonizers, primary producers or in symbiotic associations. Lopez-Garcia *et al.*⁷⁰ suggest that, in deep-sea habitats, the ϵ -proteobacteria maximize overall ecosystem function owing to their high biomass and growth rates, rapid adaptations to changing geochemical conditions and metabolic versatility. These factors all facilitate the colonization of new substrates and habitats^{39,43}. For *Candidatus A. sulfidicus*, the formation of filamentous sulphur might also stimulate colonization of surfaces in marine habitats³⁵.

Because nearly all of the microorganisms isolated from deep-sea hydrothermal-vent or marine-sediment ecotypes are chemolithoautotrophs (TABLE 1), these colonizers also serve as one of the crucial sources for organic carbon to the ecosystems (BOXES 1, 2), especially at oxic–anoxic interfaces^{50,71,72}. To assess the importance of chemolithoautotrophy in marine and terrestrial settings, however, *in situ* rates of carbon production or substrate use are needed. Carbon-fixation rates estimated for *Candidatus A. sulfidicus* were equal to, or exceeded, those of known sulphur-oxidizing bacteria that use the Calvin cycle¹⁹. Furthermore, based on previous work that describes the organic carbon-stable-isotope compositions for some marine-vent organisms^{73,74} and corresponding carbon-isotope fractionation patterns, organic carbon is probably supplied from primary producers that use the rTCA cycle⁷⁴. In Lower Kane Cave, M. L. P. estimated that the rate of chemolithoautotrophic primary productivity by H¹⁴CO₃ assimilation was 96.5 ± 6.0 mg carbon gram dry weight per hour for the ϵ -proteobacterial-dominated microbial mats²⁴, which is comparable to rates of other autotrophic organisms⁷⁵. Stable-carbon-isotope analyses in Lower Kane Cave corroborate that chemolithoautotrophically produced carbon supports the otherwise nutrient-poor system³⁰. Although the autotrophic carbon-fixation pathways were not evident from the study, the Calvin–Benson cycle was implicated in carbon fixation.

In addition to cycling carbon, we know that ϵ -proteobacteria metabolically convert various forms of reduced and oxidized sulphur and nitrogen compounds (TABLE 1), which has important bearing on the speciation of sulphur/nitrogen within a habitat and on global sulphur/nitrogen cycling, as well as on geochemical and geological processes. Few studies have measured rates

Push cores

Soft sediment collected using a hollow plastic collection tube that is pushed into the sediment, after which the ends are closed.

Methane cold seeps

Areas of the deep ocean floor where oil and methane gas bubble up from under sea-sediment layers at ambient temperatures, providing an energy source that can sustain deep-sea microbial communities.

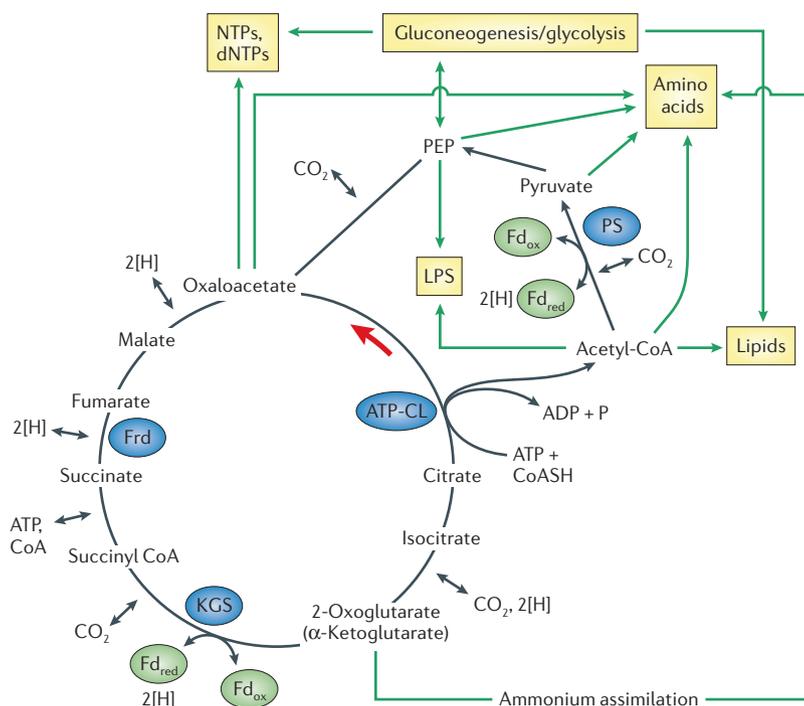


Figure 3 | The reductive or reverse TCA (rTCA) cycle of carbon fixation. The two ferredoxin-linked (Fd) CO_2 -fixation reactions (green) are oxygen sensitive; therefore, this cycle is generally found in anaerobic to microaerophilic microorganisms. The net product of the cycle is one molecule of acetyl-coenzyme A (CoA) synthesized from two molecules of CO_2 . Acetyl-CoA can be converted to pyruvate and phosphoenolpyruvate (PEP), which can either regenerate the intermediates of the cycle or be used for gluconeogenesis. Many rTCA-cycle intermediates are used in the generation of other cellular components, as indicated by the green arrows. Key enzymes ATP citrate lyase (ATP-CL), pyruvate synthase (PS, also known as pyruvate:ferredoxin oxidoreductase), ketoglutarate synthase (KGS, also known as 2-oxoglutarate:ferredoxin oxidoreductase) and fumarate reductase (Frd) are shown in blue ovals. ATP-CL, KGS and Frd allow the TCA cycle to operate in reverse (red arrow indicates reverse direction). A shared feature of the Calvin–Benson and rTCA cycles is their bidirectionality; in the presence of small organic compounds, microorganisms can use the rTCA cycle in the forward, oxidizing direction.

of sulphur/nitrogen oxidation/reduction in cultured organisms, much less in the environments in which they dominate. In Lower Kane Cave, an assessment of sulphide-consumption rates in the cave revealed that, under microaerophilic conditions, the chemolithoautotrophic ϵ -proteobacterial Lower Kane Cave group II (GG II of the Thiovolgaceae fam. nov.) consumed sulphide more rapidly than abiotic hydrogen sulphide loss mechanisms, and were consequently found to be responsible for sulphuric-acid dissolution of the cave host limestone⁷⁶. These results not only linked the biogeochemical carbon and sulphur cycles but also provided evidence for the geological importance of the ϵ -proteobacteria to processes such as cave development⁷⁶.

ϵ -Proteobacteria and the rTCA cycle

Many of the ϵ -proteobacteria studied so far are chemolithoautotrophs, and it is relevant to the evolutionary history of this group that chemolithoautotrophy is thought to be the first type of metabolic pathway to have evolved^{77,78}. One of two extant autotrophic pathways, the acetyl-coenzyme A (CoA) pathway (also called the

Wood–Ljungdahl pathway) or the rTCA (or Arnon) cycle, most closely resembles the first known autotrophic pathway^{79–83} (FIG. 3). Until the latest studies on chemolithoautotrophic ϵ -proteobacteria, the rTCA cycle had been described in only a few microorganisms, including the green sulphur bacterium *Chlorobium limicola* (Chlorobiaceae), a few members of the δ -proteobacteria (for example *Desulfobacter hydrogenophilus*) and some members of the thermophilic Aquificales and archaeal Thermoproteaceae groups^{84–86}. Within the ϵ -proteobacteria, the rTCA cycle was initially thought to be a potential CO_2 fixation pathway in Candidatus *A. sulfidicus*¹⁹. Subsequently, two fosmids were sequenced from fosmid libraries linked to the dominant ϵ -proteobacterial episymbionts of *A. pompejana*⁸⁷. Both fosmids contained the key indicator gene in the rTCA cycle, ATP citrate lyase (*aclBA*). Evidence for the potential presence and significance of the rTCA cycle for autotrophic carbon fixation at deep-sea vents has accumulated from phylogenetic analysis of rTCA genes amplified directly from hydrothermal vent chimney samples, from enzymatic expression analyses of *aclB*^{88–90}, and from genetic analyses of the cultures of Candidatus *A. sulfidicus* and the chemolithoautotrophic *Nautilia* sp. strain AmH^{19,87}.

Phylogenetic evidence points to the close evolutionary relatedness among the *acl* gene-encoded ATP citrate lyases (ATP-CLs) of *Persephonella marina* (Aquificales), the ϵ -proteobacteria, and plants and animals^{87,90}. The plant and bacterial ATP-CLs are encoded by two subunits, *aclB* (the small subunit) and *aclA* (the large subunit). Sections of the *acl* subunits have significant homologies to the large subunit of succinyl-CoA synthetase and the small subunit of succinyl-CoA synthetase and citrate synthetase⁹¹. The *acl* gene of *C. limicola* is more distantly related and might be an ancestral form⁹². The phylogenetic relationships point to the possibility of transfer of the *acl* gene to the eukaryotic population somewhere between the *Chlorobium* and Aquificales split. However, there is evidence of two types of citrate-cleaving systems within the Aquificales themselves, with the citrate-cleaving citryl-CoA lyase/synthetase enzymes found in both *Hydrogenobacter thermophilus* and *Aquifex aeolicus*, and ATP citrate lyase in *P. marina*^{87,91}.

ϵ -Proteobacteria throughout Earth's history

Although more data are needed to resolve the evolution of the citrate-cleaving system in relation to the evolution of the rTCA cycle and the early evolution of life, the presence and use of ATP-CL in some ϵ -proteobacteria clearly incite some interesting questions with respect to the overall evolutionary history of the ϵ -proteobacteria. How old is the subdivision? What role did these organisms have in Early Earth habitats? Because marine deep-sea vents are thought to be some of the most ancient colonized habitats on Earth⁹³, it is not far-reaching to hypothesize that, as the ϵ -proteobacteria are dominant and important organisms in modern vents and similar extreme habitats, this group has been significant to ecological and biogeochemical processes throughout much of Earth's history.

Wood–Ljungdahl pathway
Also known as the acetyl-coenzyme A pathway. An ancient carbon-fixation pathway found in bacteria and archaea in which CO_2 is converted to acetate; the key enzyme is acetyl-coenzyme A synthase/CO dehydrogenase.

Aphotic

Receiving no light or energy from the sun.

Research to reconstruct the evolutionary relationships among prokaryotic genomes using comparative 16S rRNA phylogeny and protein-sequence analyses has resulted in mixed information regarding the origin of non-photosynthetic sulphur-oxidizing bacteria and the ϵ -proteobacteria^{94–98}. However, there is sufficient evidence to indicate that the ϵ -proteobacteria, along with the δ -proteobacteria, have closer genetic ancestry with the Chlorobiaceae and Aquificales than with the other Proteobacteria^{94,97}. Sheridan *et al.* estimated that the divergence of 16S rRNA gene sequences of ϵ -proteobacteria dated to 1.37 billion years ago (Ga), which also corresponds to work by Brocks *et al.*, who detected the presence of the Chlorobiaceae, along with the purple sulphur bacteria (Chromatiaceae) in rocks from 1.64 Ga based on unique hydrocarbon biomarkers (molecular fossils). However, much remains to be explored about the evolution of the ϵ -proteobacteria because, unlike the Chlorobiaceae and Chromatiaceae⁹⁹, no molecular fossils have yet been identified in the rock record for the ϵ -proteobacteria.

On the basis of biological and isotopic evidence^{98–102}, the time period when ϵ -proteobacteria might have arisen on Early Earth, as far back as ~2 Ga, is marked by a shift from a reducing to an oxidizing ocean and global atmosphere owing to cyanobacterial photosynthesis^{98,100}. Although considerable debate surrounds the nature of the geochemical conditions on Early Earth, and the relative timing of this transition is hotly deliberated¹⁰¹, recent sulphur-isotope data from ancient mineral deposits indicate that free oxygen was present in the atmosphere and surface environments as early as ~2.2 Ga¹⁰², which has implications for the evolution of oxygen-dependent sulphur-oxidation pathways. For present-day sulphur-oxidizing bacteria that require aerobic or even micro-aerophilic conditions (for instance, those affiliated with the γ -proteobacteria), the availability of free oxygen would have been crucial for growth. However, oxygen is not essential for many of the isolated sulphur-reducing or sulphur-oxidizing ϵ -proteobacteria, especially for the deeply-branching Nautiliales, which are obligate anaerobes, or for other ϵ -proteobacteria that use various alternative electron acceptors (TABLE 1). Because the metabolic characteristics and ecotype preferences of the modern ϵ -proteobacteria, including thermophilic growth, anaerobic metabolism and autotrophy through the rTCA cycle, are similar to the Chlorobiaceae and Aquificales, the evolution and significance of the ϵ -proteobacteria throughout Earth's history are provocative avenues to pursue in future research.

Future prospects

The ϵ -proteobacteria represent a unique assemblage of microorganisms that, despite the attention given to the pathogenic members, have had little defined taxonomic or ecological consideration. Our taxonomic considerations of the ϵ -proteobacteria reveal that there is a large group of environmentally relevant ϵ -proteobacteria known mainly from phylogenetic studies of 16S rRNA genes, which we have provisionally termed Thiovolgaceae fam. nov. Our phylogenetic analysis indicates that there are several clades of ϵ -proteobacteria (for example, Thiovolgaceae fam. nov., *Thioreductor* and *Nitratiruptor*) that will most likely reveal more diversity in the future.

In many sulphidic habitats, especially at oxic-anoxic interfaces, ϵ -proteobacteria are not only present in the microbial communities, but might be the dominant microorganisms involved in the cycling and recycling of carbon, nitrogen and sulphur compounds. Quantitative measurements of ϵ -proteobacteria (for example, using FISH) and biogeochemical cycling (by measuring uptake or consumption rates) are few, and more studies are needed to correlate the roles of ϵ -proteobacteria with their dominance in aphotic, sulphur-rich environments. Certainly, cave and terrestrial spring environments are more suited to these types of measurements than deep-sea hydrothermal vents or the deep subsurface because these are readily accessible sites where phototrophic productivity can be eliminated.

Molecular methods, including FISH, metabolic gene presence/expression quantification and genome sequencing, hold promise for understanding the biogeochemical roles of ϵ -proteobacteria in remote extreme environments. At present, genomes from at least six environmentally relevant chemolithoautotrophic ϵ -proteobacteria from the *Nautilia*, *Caminibacter*, *Arcobacter*, *Thiomicrospira*, *Sulfurovum* and *Nitratiruptor* genera are being sequenced. More projects are needed, however, to understand the metabolic flexibility of this group, and to better characterize the pathogenic ϵ -proteobacteria. Genome projects will also further our understanding of ϵ -proteobacterial phylogenetic diversity and ecophysiology, and will undoubtedly allow for the identification of molecular markers to elucidate the evolutionary history of the entire class, including the Thiovolgaceae fam. nov. Another major challenge is to integrate this extensive molecular information and *in situ* biogeochemical culture-based strategies to improve our ability to isolate diverse metabolic groups.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

The following terms in this article are linked online to: Entrez Genome Project: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomemrj> *Aquifex aeolicus* | *Chlorobium limicola* | *Helicobacter pylori* | *Nautilia* sp. strain AmH | *Persephonella marina*

FURTHER INFORMATION

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Annette Summers Engel's laboratory: <http://www.geol.lsu.edu/Faculty/Engel/profile.html>
A metagenome of *Alvinella pompejana* symbiont database: <http://ocean.dbi.udel.edu/index.php>
DDBJ: <http://www.ddbj.nig.ac.jp>
EMBL: <http://www.ebi.ac.uk/embl>
ε-Proteobacteria sequence data files and alignments: <http://geol.lsu.edu/Faculty/Engel/epsilon.htm>
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