

The hierarchical system of the 'Alphaproteobacteria': description of *Hyphomonadaceae* fam. nov., *Xanthobacteraceae* fam. nov. and *Erythrobacteraceae* fam. nov.

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Phylogenetic analysis of the class 'Alphaproteobacteria', including physiologically diverse species, was conducted by using small-subunit rRNA gene sequences. The 16S rRNA gene sequences of 261 species in the class 'Alphaproteobacteria' were obtained from GenBank/EMBL/DBJ for constructing a phylogenetic tree by using maximum-likelihood analysis. In the resulting tree, members of the class 'Alphaproteobacteria' were subdivided into five major clusters, which were compared with the taxonomic outline of *Bergey's Manual of Systematic Biology* and the ARB tree. Based on this phylogenetic tree, three novel families are proposed: *Hyphomonadaceae* fam. nov. to accommodate the bacterial genera *Hyphomonas*, *Hirschia*, *Maricaulis* and *Oceanicaulis*, *Xanthobacteraceae* fam. nov. to include the genera *Xanthobacter*, *Azorhizobium*, *Ancylobacter*, *Labrys* and *Starkeya*, and *Erythrobacteraceae* fam. nov. to accommodate the genera *Erythrobacter*, *Porphyrobacter* and *Erythromicrobium*. The phylogenetic tree of 16S rRNA gene sequences established in this study may provide a sound basis for future taxonomic reconstruction of the class 'Alphaproteobacteria'.

INTRODUCTION

Molecular phylogenetic methods were first developed and applied to the practical taxonomic study of various kinds of organism in the 1970s. The taxonomy of bacteria became clear mainly from the partial sequencing of small-subunit (SSU) rRNA genes described by Woese and coworkers (Woese *et al.*, 1975, 1978; Zablen & Woese, 1975; Fox *et al.*, 1980). In the 1980s, the sequences of SSU rRNA genes were determined for various kinds of organism, mainly by Woese (1987) and coworkers, and they proposed the generally accepted hierarchical structure of life, including eukaryotes, archaea and eubacteria. The system proposed by Woese was supported by various phylogenetic analyses that used other molecular techniques (Ludwig & Klenk, 2001). In *Bergey's Manual of Systematic Bacteriology* (BMSB), 2nd edn (Garrity

& Holt, 2001), a first attempt was made to establish a hierarchical system for the domains *Bacteria* and *Archaea*. Twenty-three phyla were proposed for these domains. In the first description of the *Proteobacteria* by Stackebrandt *et al.* (1988), the *Proteobacteria* were defined as a class. However, in BMSB (2nd edn), the *Proteobacteria* were categorized as one of 23 phyla of bacteria and the five classes 'Alphaproteobacteria', 'Betaproteobacteria', 'Gammaproteobacteria', 'Deltaproteobacteria' and 'Epsilonproteobacteria' were proposed in the phylum. The phylum *Proteobacteria* is one of the largest phyla in the domain *Bacteria*, including more than 200 genera.

The alpha, beta, gamma and delta subclasses of the class *Proteobacteria* were defined by Stackebrandt *et al.* (1988) and the epsilon subclass was defined by Olsen *et al.* (1994). The monophyletism of the classes 'Alphaproteobacteria', 'Betaproteobacteria' and 'Gammaproteobacteria' was strongly supported by many phylogenetic analyses (Ludwig & Klenk, 2001). The classes 'Deltaproteobacteria' and 'Epsilonproteobacteria' are considered to have separated very early from the other proteobacterial classes (Olsen *et al.*, 1994; Trust *et al.*, 1994; Ludwig & Klenk, 2001). Species of the classes 'Alphaproteobacteria', 'Betaproteobacteria' and

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Abbreviations: BMSB, *Bergey's Manual of Systematic Bacteriology*; GSL, glycosphingolipid; LSU, large-subunit; ML, maximum-likelihood; SSU, small-subunit.

A full version of Fig. 1 is available as supplementary material in IJSEM Online.

'*Gammaproteobacteria*' are very heterogeneous in their physiological characteristics. Each of the three classes includes aerobes and anaerobes, photosynthetic and non-photosynthetic organisms, and is distributed ubiquitously in terrestrial and aquatic environments in very high abundance.

For construction of a phylogenetic tree, the first priority should be selection of an appropriate molecule. The most important factor in this selection is to choose molecules that are found ubiquitously in the living world, and SSU rRNA, large-subunit (LSU) rRNA (De Rijk *et al.*, 1995; Ludwig *et al.*, 1998), elongation factor EF-Tu/ α (Ludwig *et al.*, 1998), RNA polymerases (Klenk & Zillig, 1994), F₁F₀ ATPase β -subunit (Ludwig *et al.*, 1993, 1998; Ludwig & Schleifer, 1994), RecA protein (Wetmur *et al.*, 1994; Eisen, 1995; Karlin *et al.*, 1995) and Hsp60 heat-shock protein (Viale *et al.*, 1994; Gupta, 1998) have, for this reason, proved to be good molecules for the phylogenetic inference of prokaryote taxonomy. SSU rRNA gene sequences have advantages over the other molecules. LSU rRNA is considered to be as good as SSU rRNA, but not enough sequences are available in the databases. The above-mentioned proteins also have the prerequisites for phylogenetic inference, but there are critical drawbacks (Ludwig & Klenk, 2001), as most of the genes for these proteins were produced by gene duplications. There is also a critical problem in the alignment of SSU rRNA, which has many helices and loops. The helices, together with ribosomal proteins, maintain the three-dimensional structure of SSU rRNA and the loops are related to the function of the ribosome. Significant differences in mutational rate are found among these structures. The rapidly evolving parts of SSU rRNA are called variable regions; however, because of the variable secondary structure of these variable regions, it is impossible to align the SSU rRNA gene sequences of phylogenetically diverse lineages and any kinds of alignment for such sequences include artificial inferences. These artificial alignments easily lead to the distortion of phylogenetic trees.

The aim of this study was to use SSU (16S) rRNA gene sequences available in databases to clarify the phylogenetic relationships for the class '*Alphaproteobacteria*' of the phylum *Proteobacteria*.

METHODS

Analysis of sequence data. 16S rRNA gene sequences of members of the class '*Alphaproteobacteria*' were obtained from GenBank/EMBL/DDBJ. Sequences over 1350 bp in length were used for the analyses. Maximum-likelihood (ML) analysis was carried out by using the program package MOLPHY (version 2.3b2) (Adachi & Hasegawa, 1996). The ML distance matrix was calculated by using NUCML, and NJDIST in the MOLPHY package reconstructed the initial neighbour-joining tree. The ML tree was finally produced by using NUCML with the R (local rearrangement search) option based on the HKY model (Hasegawa *et al.*, 1985). Local bootstrap probabilities were estimated by the resampling of the estimated log-likelihood (RELL) method (Kishino *et al.*, 1990; Hasegawa & Kishino, 1994). Variable regions in the 16S rRNA gene sequences were eliminated

from the comparison of sequences. Six regions (positions 70–100, 181–219, 447–487, 1004–1036, 1133–1141 and 1446–1456 in the *Escherichia coli* numbering system) were eliminated from the comparison because the secondary structures of these regions differed between strains.

The ARB tree. We obtained the ARB program package and database (developed by Wolfgang Ludwig, Oliver Strunk and colleagues in Munich, Germany) at <http://www.arb.de.vu/> and installed it on a LINUX system. The phylogenetic tree of the ARB database from the ssujun02.arb version was used for comparison with the tree constructed in this study.

RESULTS AND DISCUSSION

Phylogenetic analysis of 261 species of the class '*Alphaproteobacteria*' was carried out. In total, 903 nt of the 16S rRNA gene sequences were compared. In this study, we found that the phylogenetic tree was affected by incomplete and relatively short sequences; therefore, to obtain a robust phylogenetic tree, the following strains were removed: *Agromonas oligotrophica* JCM 1494^T (GenBank accession no. D78366), *Albibacter methylovorans* DM10^T (AF273213), *Ensifer adhaerens* ATCC 33212^T (AF191739), *Jannaschia helgolandensis* Hel 10^T (AJ438157), *Methylophila capsulata* IM1^T (AF004844), *Methylorhabdus multivorans* DM13^T (AF004845), *Neorickettsia helminthoeca* (U12457), *Rhodospseudomonas rosea* DSM 5909^T (D14429), *Rhodovibrio sodomensis* SG3105 (AJ318524), *Roseinatronobacter thiooxidans* ALG 1^T (AF249749), *Rubrimonas cliftonensis* OCH 317^T (D85834) and *Tistrella mobilis* (AB071665). By removing these strains, a phylogenetic tree with much higher confidence limits was obtained (Fig. 1). Within this study, the nomenclature of the orders and families follows the taxonomic outline of BMSB (Garrity & Holt, 2001; Garrity *et al.*, 2004).

Within our tree, the class '*Alphaproteobacteria*' was subdivided into five major clades: the orders *Caulobacterales*, '*Rhizobiales*', *Rickettsiales*, '*Sphingomonadales*' and *Rhodospirillales*.

The cluster of the order *Caulobacterales*

In our classification, the cluster of the order *Caulobacterales* Henrici and Johnson 1935 has been subdivided into three clades: the families *Caulobacteraceae*, *Hyphomonadaceae* fam. nov. and '*Rhodobacteriaceae*'.

The cluster of the family *Caulobacteraceae*. The genera *Brevundimonas*, *Caulobacter*, *Asticcacaulis*, *Mycoplasma* and *Phenylobacterium* comprised this cluster with high bootstrap values. The cluster contained two sub-clusters. The first consisted of members of the genus *Brevundimonas*, including *Brevundimonas diminuta* (the type species of the genus), *Brevundimonas vesicularis*, [*Caulobacter*] *intermedius*, *Brevundimonas aurantiaca*, *Brevundimonas intermedia*, *Brevundimonas variabilis*, *Brevundimonas alba* and *Brevundimonas subvibrioides*. The second subcluster contained members of the genus *Caulobacter*, including *Caulobacter fusiformis*, *Caulobacter*

bacteroides, *Caulobacter crescentus* (the type species) and *Caulobacter henricii*.

In this study, *Asticcacaulis excentricus* ATCC 15261^T (GenBank accession no. AB016610), the type species of the genus *Asticcacaulis*, belonged to this cluster and was related closely to the genera *Brevundimonas* and *Caulobacter*, in agreement with Abraham *et al.* (2001). However, *Asticcacaulis biprosthecium* ATCC 27554^T (GenBank accession no. AJ007799), used in this study, is related closely to the genus *Sphingomonas*, which concurs with Sly *et al.* (1999). Abraham *et al.* (2001) stated, however, the possibility that the *Asticcacaulis* strains studied by Sly *et al.* (1999) were misidentified on the basis of analysis of 16S rRNA gene sequences and fatty acid data. Therefore, the 16S rRNA gene sequences of *Asticcacaulis* species in the databases should be re-evaluated to avoid taxonomic confusion.

The cluster of the family *Hyphomonadaceae* fam. nov. This cluster consisted of the genera *Hyphomonas*, *Maricaulis*, *Hirschia* and *Oceanicaulis*, and was supported by high bootstrap values. The members of this cluster, mainly isolated from marine habitats, formed a robust clade and have similar morphological, physiological and biological features (Moore *et al.*, 1984; Schlesner *et al.*, 1990; Abraham *et al.*, 1999; Strömpl *et al.*, 2003).

In our classification, the phylogenetic position of the family *Hyphomonadaceae* differed from that in the hierarchical system of BMSB, but was similar to that of the ARB tree. Phylogenetically, this cluster was related closely to the family *Caulobacteraceae*, which was consistent with the ARB tree. Garrity & Holt (2001) stated, however, that this cluster was included in the family '*Rhodobacteraceae*'. We also noticed that the phylogenetic topology of this cluster was affected by intervening taxa, which were removed in our study. Hence, it might be difficult to classify this cluster as part of the family '*Rhodobacteraceae*'.

Consequently, we propose a novel family, *Hyphomonadaceae* fam. nov., to accommodate the bacterial genera *Hyphomonas*, *Hirschia*, *Maricaulis* and *Oceanicaulis*.

The cluster of the family '*Rhodobacteraceae*'. In our classification, this cluster consisted of five main subclusters. The first subcluster consisted of the genus *Paracoccus*, the second included the genera *Rhodobacter* and *Pseudorhodobacter*, the third contained the genus *Rhodovulum*, the fourth included the genus *Amaricoccus* and the fifth comprised the genera *Antarctobacter*, *Ketogulonicigenium*, *Leisingera*, *Octadecabacter*, *Roseivivax*, *Roseobacter*, *Roseovarius*, *Ruegeria*, *Sagittula*, *Silicibacter*, *Staley* and *Sulfitobacter*.

The cluster of the order '*Sphingomonadales*'

The order '*Sphingomonadales*' in our classification consisted of two subclusters: the families *Sphingomonadaceae* and *Erythrobacteraceae* fam. nov. (newly proposed).

BMSB's hierarchical system proposed the order '*Sphingomonadales*' to include only the family *Sphingomonadaceae*. The ARB tree also showed that five *Sphingomonas* subgroups and the *Porphyrobacter*/*Erythrobacter*/*Erythromicrobium* groups were included in the *Sphingomonas* cluster. However, we found that the order '*Sphingomonadales*' was separated into two clades: the families *Sphingomonadaceae* and *Erythrobacteraceae* fam. nov. Our tree showed that the phylogenetic relationships between the two clusters had enough distance and high enough bootstrap values to separate them into two families.

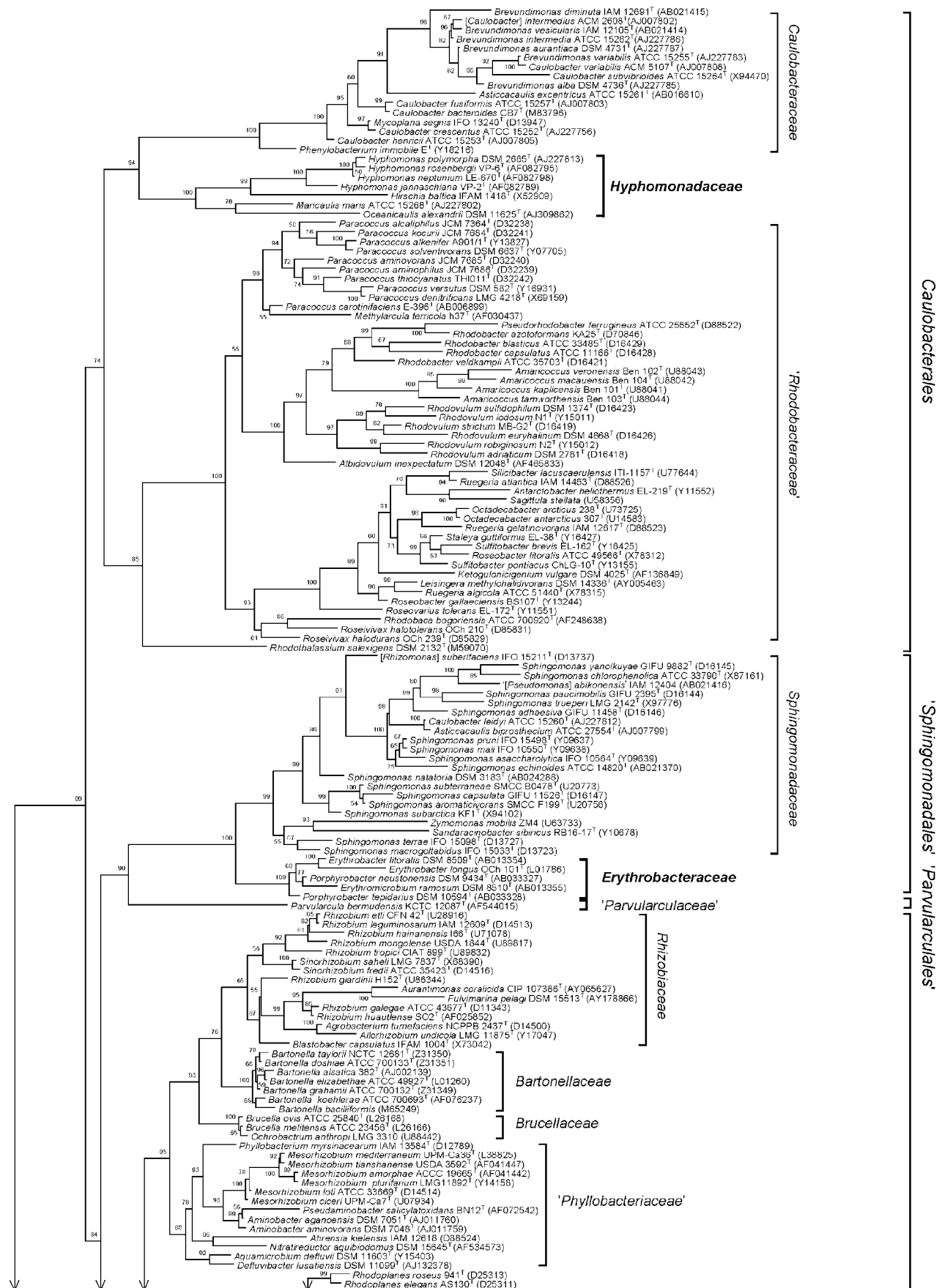
The cluster of the family *Sphingomonadaceae*. Within our classification, the family *Sphingomonadaceae* contained the genera *Sphingomonas*, *Zymomonas* and *Sandaracinobacter* and the species *Caulobacter leidy* and *Asticcacaulis biprosthecium*, as well as the misnamed '[*Pseudomonas*] *abikonensis*' and [*Rhizomonas*] *suberifaciens*.

The family *Sphingomonadaceae* was established by Kosako *et al.* (2000), based on the results of 16S rRNA gene sequence and cellular lipid analyses. Takeuchi *et al.* (2001) divided the genus *Sphingomonas* into four genera, *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic analysis of 16S rRNA gene sequences and chemotaxonomic and phenotypic differences.

In this study, the genus *Sphingomonas* contained three main subclusters: the first included *Sphingomonas paucimobilis* (the type species of the genus), *Sphingomonas adhaesiva*, *Sphingomonas trueperi*, *Sphingomonas chlorophenolica*, *Sphingomonas yanoikuyae*, *Sphingomonas asaccharolytica*, *Sphingomonas pruni*, *Sphingomonas mali* and *Sphingomonas echinoides*; the second consisted of *Sphingomonas suberifaciens*, *Sphingomonas subterranea*, *Sphingomonas aromaticivorans*, *Sphingomonas capsulata* and *Sphingomonas subarctica*; and the third contained *Sphingomonas macrogoltabidus* and *Sphingomonas terrae*. This differed slightly from the tree proposed by Takeuchi *et al.* (2001) and the ARB tree, which divided the genus *Sphingomonas* into four and five groups, respectively.

Caulobacter leidy ATCC 15260^T (GenBank accession no. AJ227812) was included in this cluster and was related to *Sphingomonas trueperi*, in agreement with Abraham *et al.* (1999). Therefore, *Caulobacter leidy* should be transferred to the genus *Sphingomonas* following further taxonomic studies. '[*Pseudomonas*] *abikonensis*' was also included in the *Sphingomonas* rRNA lineage and was related closely to *Sphingomonas chlorophenolica*, in accordance with Kersters *et al.* (1996) and Anzai *et al.* (2000). Therefore, '[*Pseudomonas*] *abikonensis*' should be transferred to the genus *Sphingomonas* following further taxonomic studies.

The cluster of the family *Erythrobacteraceae* fam. nov. In our study, the cluster that accommodated the genera *Erythrobacter*, *Porphyrobacter* and *Erythromicrobium* was clearly separated with high bootstrap support.



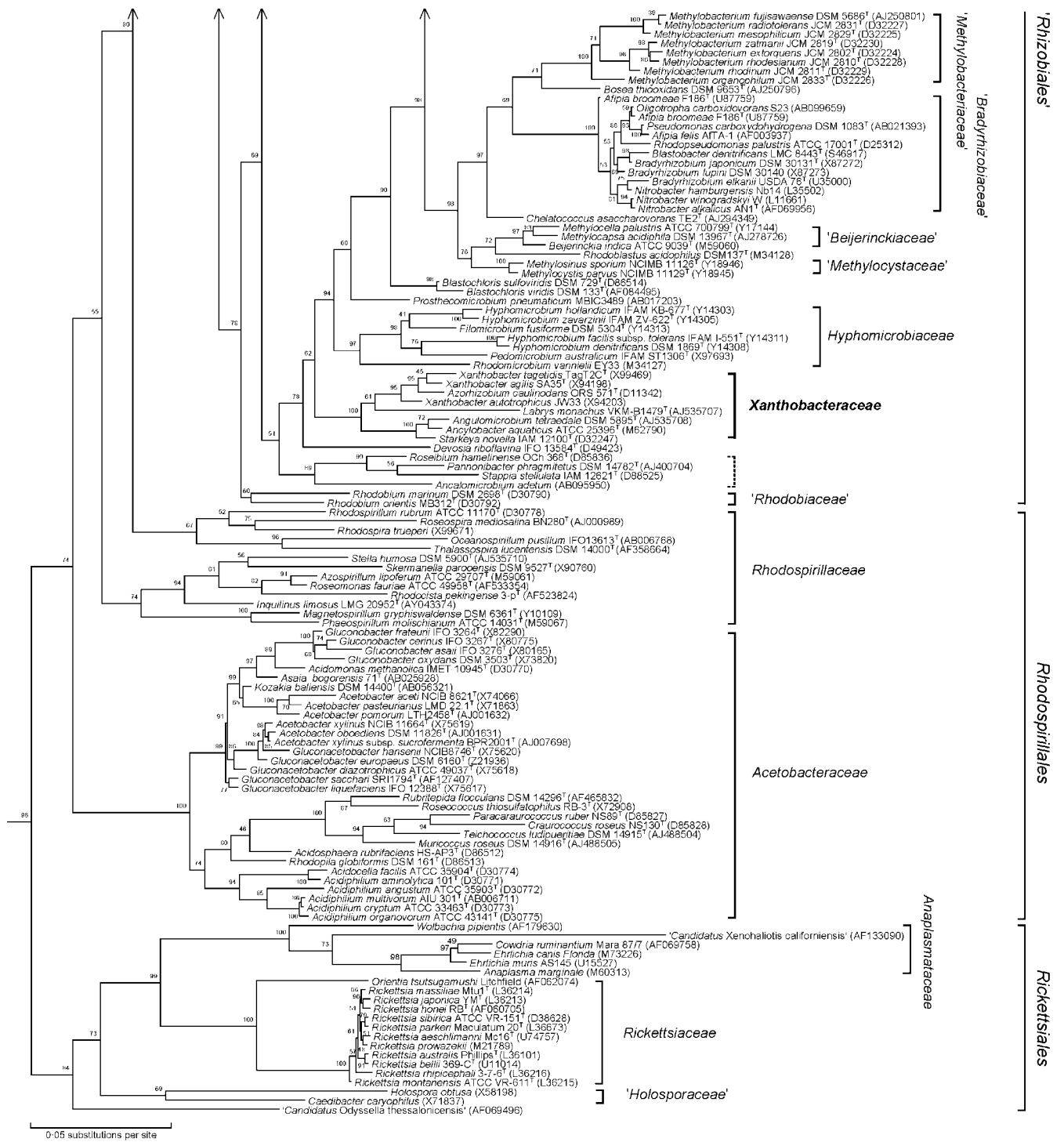


Fig. 1. Phylogenetic tree of the 'Alphaproteobacteria' based on 16S rRNA gene sequences. *Pseudomonas aeruginosa* LMG 1242^T (GenBank accession no. Z76651) was used as the root organism. New taxa proposed in this study are shown in bold type. The dotted line indicates a potential phylogenetic group. A full version of the figure is available as supplementary material in IJSEM Online.

This cluster has previously been shown to belong to the lineage of the genus *Sphingomonas*. We have observed, however, that this cluster is separated from the main lineage

of the family *Sphingomonadaceae*, which was strongly supported by the bootstrap value. The topology of this cluster differed from that in the ARB and BMSB trees, both

of which showed this cluster to be included in the lineage of the genus *Sphingomonas*. The members of this cluster produce pigment (yellow, orange or pink) and mainly contain bacteriochlorophyll *a*, whereas members of the genus *Sphingomonas* do not (Shiba & Simidu, 1982; Fuerst *et al.*, 1993; Takeuchi *et al.*, 1994; Yurkov *et al.*, 1994; Denner *et al.*, 2002). Takeuchi *et al.* (2001) also showed that the genus *Sphingomonas* was related only distantly to the genera *Erythrobacter*, *Erythromicrobium* and *Porphyrobacter* based on 16S rRNA gene sequence similarity, which was 92.9–94.8%, and the genus *Sphingomonas* had the presence of one or more oligosaccharide-type glycosphingolipid(s) (GSL) as one of its most characteristic features, whereas the genera *Erythrobacter*, *Porphyrobacter* and *Erythromicrobium* contained monosaccharide-type GSLs only. On the basis of this work and previous polyphasic taxonomic studies, we propose this cluster as a novel family, *Erythrobacteraceae* fam. nov., to accommodate the bacterial genera *Erythrobacter*, *Porphyrobacter* and *Erythromicrobium*.

The cluster of the order 'Rhizobiales'

The members of this cluster are the most heterogeneous in the class 'Alphaproteobacteria'. The order 'Rhizobiales' includes members with a variety of morphological, physiological and biological features, which may impede their taxonomic definition.

Garrity *et al.* (2004) proposed that the order 'Rhizobiales' comprises 11 families: *Rhizobiaceae*, 'Aurantimonadaceae', *Bartonellaceae*, *Brucellaceae*, 'Phyllobacteriaceae', 'Methlocystaceae', 'Beijerinckiaceae', 'Bradyrhizobiaceae', *Hyphomicrobiaceae*, 'Methylobacteriaceae' and 'Rhodobiaceae', based on 16S rRNA gene sequence analysis. In our phylogenetic tree, the order 'Rhizobiales' is clearly separated into two main clusters, which is consistent with the ARB tree. The first main cluster constituted the families *Rhizobiaceae*, *Brucellaceae*, *Bartonellaceae* and 'Phyllobacteriaceae' and the second contained the families 'Methylobacteriaceae', 'Bradyrhizobiaceae', 'Methlocystaceae', 'Beijerinckiaceae', *Hyphomicrobiaceae*, 'Rhodobiaceae' and the newly proposed *Xanthobacteraceae* fam. nov.

The cluster of the family *Brucellaceae*. In this study, the cluster of the family *Brucellaceae*, proposed by Breed *et al.* (1957), consisted of the genera *Brucella* and *Ochrobactrum*, isolated from human clinical specimens (Holmes *et al.*, 1988), and was supported by very high bootstrap values.

The cluster of the family *Bartonellaceae*. Within our classification, the cluster of the family *Bartonellaceae* consisted of the genus *Bartonella* only. *Bartonella bacilliformis* (GenBank accession no. M65249), the type species of the genus *Bartonella*, was related to *Bartonella koehlerae* ATCC 700693^T (AF076237).

The cluster of the family *Rhizobiaceae*. The cluster of the family *Rhizobiaceae* consisted of the genera

Agrobacterium, *Allorhizobium*, *Rhizobium* and *Sinorhizobium* and the species *Blastobacter capsulatus*.

Blastobacter capsulatus IFAM 1004^T (GenBank accession no. X73042), isolated from freshwater habitats (Hirsch & Müller, 1985), was included in the cluster of the family *Rhizobiaceae* and was related closely to the genus *Rhizobium*, which was consistent with the ARB tree. Garrity & Holt (2001), however, showed the genus *Blastobacter* to be included in the family 'Bradyrhizobiaceae'. We also found that *Blastobacter denitrificans* LMG 8443^T (GenBank accession no. S46917) was included in the cluster of the family 'Bradyrhizobiaceae'. However, *Blastobacter henricii*, the type species of the genus, was not available from any culture collection. Thus, we speculate that the genus *Blastobacter* is heterogeneous; further taxonomic studies are required for clarification.

Within our tree, the following members of the genus *Sinorhizobium* [transferred from the genus *Rhizobium* by Chen *et al.* (1988)] were related closely to the genus *Rhizobium*: *Sinorhizobium fredii* (the type species of the genus) and *Sinorhizobium saheli*.

Aurantimonas coralicida, a coral pathogen (Denner *et al.*, 2003), and *Fulvimarina pelagi*, a marine bacterium (Cho & Giovannoni, 2003b), formed a distinct and deep evolutionary lineage in the cluster of the family *Rhizobiaceae* with high bootstrap support, and were proposed as the members of the family 'Aurantimonadaceae' by Garrity *et al.* (2004). The neighbours of this lineage were the genera *Agrobacterium*, *Allorhizobium* and *Rhizobium*, which was in accordance with the 16S rRNA gene sequence comparison of Cho & Giovannoni (2003b). However, evolutionary relationships between the neighbours were distant, with a deep branch. In our phylogenetic analysis, we observed an important point: this cluster, with a distinct and deep lineage, was placed in the family *Rhizobiaceae* with very high bootstrap values, although Garrity *et al.* (2004) proposed this cluster as a family. Therefore, it may be ambiguous to define the boundary of a family based on 16S rRNA gene sequence analysis. Further taxonomic studies should be carried out for this cluster to allow definite taxonomic conclusions to be made.

The cluster of the family 'Phyllobacteriaceae'. In our classification, the family 'Phyllobacteriaceae' was composed of three main subclusters with high bootstrap support, which concurred with Garrity *et al.* (2004). The first subcluster contained the genera *Aminobacter*, *Mesorhizobium*, *Phyllobacterium* and *Pseudaminobacter*, the second included the genera *Ahrensia* and *Nitratireductor* and the third contained the genera *Aquamicrobium* and *Defluviobacter*.

The cluster of the family 'Bradyrhizobiaceae'. This family, proposed by Garrity *et al.* (2004), consisted of the genera *Afipia*, *Bosea*, *Bradyrhizobium*, *Nitrobacter*, *Oligotropha* and *Rhodopseudomonas* and the species *Blastobacter*

denitrificans, as well as the phylogenetically misnamed [*Pseudomonas*] *carboxydohydrogena*.

[*Pseudomonas*] *carboxydohydrogena* DSM 1083^T (GenBank accession no. AB021393), established by Meyer *et al.* (1980), was a close neighbour of *Afipia felis*, isolated from human wound and respiratory sources (Brenner *et al.*, 1991), which was in accordance with Anzai *et al.* (2000). Therefore, for appropriate positioning, [*Pseudomonas*] *carboxydohydrogena* should be transferred to the genus *Afipia* following further taxonomic studies.

The cluster of the family 'Methylobacteriaceae'. The cluster consisting of the genus *Methylobacterium*, established by Patt *et al.* (1976), was separated from the other clusters of the order 'Rhizobiales' with high bootstrap values. Garrity & Holt (2001) proposed this cluster as the novel family 'Methylobacteriaceae' to accommodate the genus *Methylobacterium* only, which concurs with our data.

The cluster of the family Hyphomicrobiaceae. Within our classification, the cluster consisting of the genera *Hyphomicrobium*, *Pedomicrobium*, *Filomicrobium* and *Rhodomicrobium* was separated from the other clusters with high bootstrap values. The genus *Hyphomicrobium* comprised two subclusters: the first contained *Hyphomicrobium hollandicum*, *Hyphomicrobium zavarzinii* and *Filomicrobium fusiforme* from brackish water (Schlesner, 1987) and the second comprised *Hyphomicrobium denitrificans* and *Hyphomicrobium facile*. *Pedomicrobium australicum* from aquatic habitats (Gebbers & Beese, 1988) was related closely to the second subcluster of the genus *Hyphomicrobium*, in agreement with Rainey *et al.* (1998). Rainey *et al.* (1998) also reported that the genus *Hyphomicrobium* should be separated into two genera on the basis of phylogenetic analysis, but the possibility that two separate genera exist was excluded because of the lack of distinguishing phenotypic properties. Our results also indicated that the two clusters are phylogenetically distant enough to be separated into two genera. Therefore, further taxonomic studies of the genus *Hyphomicrobium* are required to allow definite taxonomic conclusions to be made.

The cluster of the family Xanthobacteraceae fam. nov. Within our study, the cluster that consisted of the genera *Xanthobacter*, *Azorhizobium*, *Ancylobacter*, *Labrys* and *Starkeya* was separated from the other clusters of the order 'Rhizobiales' with very high bootstrap values. This cluster contained two subclusters: the first included species of the genus *Xanthobacter*, *Azorhizobium caulino-dans* and *Labrys monachus*, and the second contained *Ancylobacter aquaticus* [transferred from the bacterial genus *Microcyclus* Ørskov 1928 (Raj, 1983)] and *Starkeya novella*, reclassified from *Thiobacillus novellus* (Kelly *et al.*, 2000).

The phylogenetic topology of the family *Xanthobacteraceae* was similar to that in the ARB tree, but differed from that of BMSB, which showed that this cluster was a subdivision of the family *Hyphomicrobiaceae* Babudieri 1950, including 20 species. We have found, in agreement with the ARB tree, that this cluster was placed separately from the cluster of the family *Hyphomicrobiaceae* Babudieri 1950.

It was difficult to find common features among the members of the family *Hyphomicrobiaceae* Babudieri 1950, which show a variety of characteristics. In DNA G+C content, members of the newly established family *Xanthobacteraceae* are highly similar (66–69.1 mol%), whereas the species of the family *Hyphomicrobiaceae* Babudieri 1950 have high heterogeneity (59–71.4 mol%) (Raj, 1983; Dreyfus *et al.*, 1988; Schlesner *et al.*, 1990; Rainey *et al.*, 1998; Kelly *et al.*, 2000; Fritz *et al.*, 2004). Based on the deep branching observed in 16S rRNA gene sequence-based phylogenetic analysis, we propose a novel family, *Xanthobacteraceae* fam. nov., to include the genera *Xanthobacter*, *Azorhizobium*, *Ancylobacter*, *Labrys* and *Starkeya*.

The cluster of the family 'Rhodobiaceae'. The family 'Rhodobiaceae', accommodating the genera *Rhodobium* and *Roseosprillum* (not used in this study because of short sequence data), was proposed by Garrity *et al.* (2004) and was located out of the main cluster of the order 'Rhizobiales' in this study.

Intervening strains and a potential cluster as a taxon in the order 'Rhizobiales'

Within our phylogenetic tree, we observed several bacteria located in an intermediate position within the order 'Rhizobiales'. The genera *Blastochloris*, transferred from the genus *Rhodopseudomonas* (Hiraishi, 1997), *Bosea*, capable of oxidizing reduced inorganic sulfur compounds (Das *et al.*, 1996), *Chelatococcus*, capable of utilizing nitrilotriacetate (Auling *et al.*, 1993), *Devosia*, created to accommodate '*Pseudomonas riboflavina*' (Nakagawa *et al.*, 1996), and *Prosthecomicrobium*, a prosthecate freshwater bacterium (Staley, 1968), and the species *Rhodoblastus acidophilus*, transferred from *Rhodopseudomonas acidophila* (Imhoff, 2001), were located in the outline of various clusters, at the family level. Thus, it was difficult to define their taxonomic hierarchy in our phylogenetic tree. Therefore, further taxonomic studies should be carried out to allow definite taxonomic conclusions about these six taxa to be made.

In this study, *Roseibium hamelinense*, an aerobic, bacteriochlorophyll-containing bacterium (Suzuki *et al.*, 2000), *Pannonibacter phragmitetus*, an alkali-tolerant bacterium (Borsodi *et al.*, 2003), *Stappia stellulata*, a marine species reclassified from the genus *Agrobacterium* (Uchino *et al.*, 1998), and *Ancalomicrobium adetum*, a prosthecate freshwater bacterium (Staley, 1968), formed a distinct branch with high bootstrap values. Phenotypic and chemotaxonomic characteristics shared commonly among the strains did not clearly define the taxonomy of this cluster. Garrity

et al. (2004) proposed that the genera *Pannonibacter*, *Roseibium* and *Stappia* and the genus *Ancalomicrobium* were classified into the families 'Rhodobacteriaceae' and *Hyphomicrobiaceae*, respectively. However, in our data, we observed that this cluster, accommodating the four genera, is robustly formed with a distinct branch. Suzuki *et al.* (2000) and Borsodi *et al.* (2003) also stated that *Roseibium hamelinense* and *Pannonibacter phragmitetus* were related closely to the members of this branch on the basis of 16S rRNA gene sequence analysis. The 16S rRNA gene sequence similarity for *Pannonibacter phragmitetus* supported the observation that its closest neighbours were members of the genera *Roseibium* and *Stappia*. The physiological and molecular features of the genus *Ancalomicrobium* were not discriminatory enough to support this cluster. Further taxonomic study of this cluster, including the four genera, may establish a novel family-level taxon.

The cluster of the order *Rhodospirillales*

Garrity & Holt (2001) showed that the order *Rhodospirillales* [established by Pfennig & Trüper (1971)] includes two families, *Rhodospirillaceae* and *Acetobacteraceae*, and the ARB tree also indicated that the order *Rhodospirillales* comprised a cluster with the two families. Within our tree, the order *Rhodospirillales* contained three distinct subclusters, which did not branch from an origin cluster. Thus, it was difficult to classify the order *Rhodospirillales* including the two families within our tree. To enable precise taxonomic conclusions to be made about the order *Rhodospirillales*, further taxonomic studies must be carried out.

The cluster of the family *Rhodospirillaceae*. In this study, the topology of the family *Rhodospirillaceae* differs from that proposed in BMSB and the ARB tree. In the BMSB system, the family *Rhodospirillaceae* includes the genera *Azospirillum*, '*Dechlorospirillum*', '*Defluvicoccus*', *Inquilius*, *Magnetospirillum*, *Phaeospirillum*, *Rhodocista*, *Rhodospira*, *Rhodospirillum*, *Rhodovibrio*, *Roseospira*, *Skermanella*, *Thalassospira* and *Tistrella*. The classification of *Rhodospirillaceae* as a family is ambiguous, because the members of the family *Rhodospirillaceae* proposed by BMSB were separated into two distinct phylogenetic lineages. *Rhodospirillum rubrum*, *Rhodospira trueperi*, *Roseospira mediosalina*, *Oceanospirillum pusillum* and *Thalassospira lucentensis* formed a cluster with relatively low bootstrap values. The genera *Azospirillum*, *Inquilius*, *Phaeospirillum*, *Magnetospirillum*, *Rhodocista*, *Roseomonas*, *Skermanella* and *Stella* also formed a distinct lineage. Each strain had a deep branch and it was therefore problematic to define a family-level taxon based on 16S rRNA gene sequence analysis. On the basis of our results, the family *Rhodospirillaceae* should be re-evaluated to allow definite taxonomic conclusions to be reached.

The cluster of the family *Acetobacteraceae*. Within our classification, the family *Acetobacteraceae*, proposed by Gillis & De Ley (1980), was subdivided into two

subclusters with very high bootstrap values: the first cluster included the genera *Gluconobacter*, *Acidomonas*, *Asaia*, *Kozakia*, *Acetobacter* and *Gluconacetobacter*, and the second contained the genera *Acidiphilium*, *Acidisphaera*, *Rhodopila*, *Rubritepida*, *Roseococcus*, *Paracraurococcus*, *Craurococcus*, *Teichococcus* and *Muricoccus*. In our tree, we observe that the genera *Rubritepida*, *Roseococcus*, *Paracraurococcus*, *Craurococcus*, *Teichococcus* and *Muricoccus* form a distinct subcluster with very high bootstrap values. According to several studies (Saitoh *et al.*, 1998; Alarico *et al.*, 2002; Kämpfer *et al.*, 2003), phylogenetic relationships between each strain were close on the basis of 16S rRNA gene sequence analysis. However, their physiological and biochemical features have impeded their phylogenetic consolidation: some strains contain bacteriochlorophyll *a* and some do not, and either Q-9 or Q-10 may be present. Based on the phylogenetic results, this subcluster may be classified as a novel taxon at the family level; therefore, further taxonomic studies should be carried out to determine whether a novel family is supported.

The cluster of the order *Rickettsiales*

Dumler *et al.* (2001) described the order *Rickettsiales*, including the families *Anaplasmataceae* and *Rickettsiaceae*, on the basis of genetic analyses of 16S rRNA, *groESL* and surface-protein genes.

Within our classification, the order *Rickettsiales* contained two main subclusters: the families *Anaplasmataceae* and *Rickettsiaceae*, which was consistent with Dumler *et al.* (2001). The family *Anaplasmataceae* comprised the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Wolbachia* and '*Candidatus Xenohalictis*', and the family *Rickettsiaceae* comprised the genera *Rickettsia* and *Orientia*.

The protozoan endosymbionts *Holospora obtusa* and '*Candidatus Odysseella thessalonicensis*' (Birtles *et al.*, 2000) formed a cluster with low bootstrap values in our study. The two strains also formed distinct lineages with a deep branch. The endosymbionts in the class '*Alphaproteobacteria*' were classified into a novel family, '*Holosporaceae*', by Garrity *et al.* (2004). However, we found that it was ambiguous to classify these endosymbionts as a taxon based on 16S rRNA gene sequence analysis, because of the lack of consistency in defining the boundaries of a taxon with low bootstrap values (69%). Therefore, an extensive study for endosymbionts should be carried out to allow definite taxonomic conclusions to be made. In this study, '*Candidatus Xenohalictis californiensis*', an obligately intracellular, pleomorphic bacterium (Friedman *et al.*, 2000), formed a distinct lineage outside the clusters of the families *Anaplasmataceae* and *Rickettsiaceae*, which did not concur with Garrity *et al.* (2004), who proposed that this strain should be included in the family *Anaplasmataceae*. We also found that '*Candidatus Xenohalictis californiensis*' formed a deep enough branch to be classified as another taxon with very high bootstrap values. Therefore, further taxonomic

studies should be carried out to define the phylogenetic hierarchy of 'Candidatus Xenohaliotis californiensis'.

Intervening clusters and strains within this phylogenetic tree

Rhodobium orientis MB312^T (GenBank accession no. D30792) and *Rhodobium marinum* DSM 2698^T (D30790), purple, non-sulfur, phototrophic bacteria, were isolated and transferred to this genus by Hiraishi *et al.* (1995). The genus *Rhodobium* formed a cluster with relatively low bootstrap values and was related to the order 'Rhizobiales' in our phylogenetic tree, in which the genus *Roseospirillum* was not included because of short 16S rRNA gene sequences. Garrity *et al.* (2004) proposed the novel family 'Rhodobacteraceae', including the genera *Rhodobium* and *Roseospirillum*, in the order 'Rhizobiales'. However, we have found a general lack of consistency in defining the boundaries of such a family based on our phylogenetic analysis. Therefore, further taxonomic studies should be carried out to determine the phylogenetic relationships of the genera *Rhodobium* and *Roseospirillum*.

Rhodothalassium salexigens DSM 2132^T (GenBank accession no. M59070), transferred from the genus *Rhodospirillum* by Imhoff *et al.* (1998), clustered with the family 'Rhodobacteraceae' in this study. We have observed a lack of consistency in defining the phylogenetic hierarchy of *Rhodothalassium salexigens*, even though Garrity *et al.* (2004) classified it as a member of the family 'Rhodobacteraceae', because its topology was changed by adding further strains. Therefore, further taxonomic studies should be carried out to define the phylogenetic hierarchy of *Rhodothalassium salexigens*.

Parvularcula bermudensis HTCC2503^T (GenBank accession no. AF544015), isolated from a marine environment by Cho & Giovannoni (2003a), formed a deep branch in the 'Alphaproteobacteria' according to our phylogenetic data, which concur with the results of Cho & Giovannoni (2003a). *Parvularcula bermudensis* clustered with the order 'Sphingomonadales' with relatively high bootstrap values. However, phylogenetic relationships between *Parvularcula bermudensis* and the order 'Sphingomonadales' were not close. Garrity *et al.* (2004) proposed this branch as the order 'Parvularculales', including *Parvularcula bermudensis* only.

Approach to establish a more reliable phylogenetic hierarchical system

The tree (version ssujun02.arb) in the ARB database, maintained by W. Ludwig and O. Strunk at the Technical University of Munich, was used for comparison with our tree, which included 2154 SSU 16S rRNA sequences. In this study, we have found some discordant points between our phylogenetic tree and the ARB tree. The phylogenetic topology of some clusters in the two trees was slightly different. In our tree, the branch length of each cluster was long enough to classify it as a taxon.

We speculate that the main reason for the differences between phylogenetic hierarchical systems based on 16S rRNA gene sequences is their alignment, which has some variable regions. Ludwig & Klenk (2001) also pointed out the critical drawbacks of 16S rRNA gene sequences for phylogenetic inference. The secondary structures of the variable regions show variability, making it impossible to align the SSU rRNA sequences of phylogenetically diverse lineages. Thus, any kinds of alignment for such sequences include artificial inferences. These artificial alignments easily lead to the production of distorted phylogenetic trees. In this study, we excluded the variable regions from the alignment. Hence, our alignment includes fewer inferences and is of higher reliability for the topology. As a result, the bootstrap values of the branches were very high and the interconnecting branches between the major clusters were long.

In BMSB, Garrity & Holt (2001) proposed the establishment of a new hierarchical system for the domains *Bacteria* and *Archaea* based on 16S rRNA gene sequences. They elevated the *Proteobacteria* from the rank of class, making them one of 23 newly established phyla. Recently, Garrity *et al.* (2004) have updated the BMSB hierarchical system with newly isolated strains. In this system, the hierarchical system that we propose is for the class 'Alphaproteobacteria'. The hierarchical system for the 'Alphaproteobacteria' was slightly different between our results and those of BMSB. They proposed the class 'Alphaproteobacteria' to be subdivided into seven orders and 20 families (Table 1). The order 'Rhodobacterales' (of BMSB) is eliminated and the family 'Rhodobacteraceae', of the order 'Rhodobacterales' in BMSB, is included in the order *Caulobacterales* in our proposed system, because the name of the order *Caulobacterales* has been validly published, but that of the order 'Rhodobacterales' has not. The family 'Aurantimonadaceae' in BMSB is not included in our proposed system because of the lack of consistency in defining the boundaries of this taxon based on 16S rRNA gene sequence analysis. The families *Hyphomonadaceae*, *Xanthobacteraceae* and *Erythrobacteraceae* are newly proposed here, because these groups were separated very clearly from other taxa in our phylogenetic tree (Fig. 1).

With the development of various molecular methods, the hierarchical system will be revised and changed. However, it is necessary to recognize the drawbacks of the molecules used for phylogenetic inference. We hope that this current approach will provide the basis for a more meaningful and reliable classification of the proteobacteria.

Description of *Hyphomonadaceae* fam. nov.

Hyphomonadaceae (Hy.pho.mo.na.da'ceae. N.L. fem. n. *Hyphomonas* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Hyphomonadaceae* the *Hyphomonas* family).

Gram-negative, rod-shaped bacteria. Motile. Do not form spores. Chemo-organotrophic. Some species require

Table 1. Comparison of the two hierarchical systems

Quotation marks indicate names that have not been validly published. Bold type indicates novel taxa proposed in this study.

This study	Taxonomic outline of BMSB
Order <i>Caulobacterales</i>	Order <i>Caulobacterales</i>
Family <i>Caulobacteraceae</i>	Family <i>Caulobacteraceae</i>
Family <i>Hyphomonadaceae</i>	Order 'Rhodobacterales'*
Family 'Rhodobacteraceae'	Family 'Rhodobacteraceae'
Order 'Sphingomonadales'	Order 'Sphingomonadales'
Family <i>Sphingomonadaceae</i>	Family <i>Sphingomonadaceae</i>
Family <i>Erythrobacteraceae</i>	
Order 'Rhizobiales'	Order 'Rhizobiales'
Family <i>Rhizobiaceae</i>	Family <i>Rhizobiaceae</i>
Family <i>Brucellaceae</i>	Family <i>Brucellaceae</i>
Family <i>Bartonellaceae</i>	Family <i>Bartonellaceae</i>
Family 'Phyllobacteriaceae'	Family 'Phyllobacteriaceae'
Family 'Bradyrhizobiaceae'	Family 'Bradyrhizobiaceae'
Family 'Methylobacteriaceae'	Family 'Methylobacteriaceae'
Family 'Methylocystaceae'	Family 'Methylocystaceae'
Family 'Beijerinckiaceae'	Family 'Beijerinckiaceae'
Family <i>Hyphomicrobiaceae</i>	Family <i>Hyphomicrobiaceae</i>
Family 'Rhodobiaceae'	Family 'Rhodobiaceae'
Family <i>Xanthobacteraceae</i>	Family 'Aurantimonadaceae'*
Order <i>Rickettsiales</i>	Order <i>Rickettsiales</i>
Family <i>Rickettsiaceae</i>	Family <i>Rickettsiaceae</i>
Family <i>Anaplasmataceae</i>	Family <i>Anaplasmataceae</i>
	Family 'Holosporaceae'*
Order <i>Rhodospirillales</i>	Order <i>Rhodospirillales</i>
Family <i>Rhodospirillaceae</i>	Family <i>Rhodospirillaceae</i>
Family <i>Acetobacteriaceae</i>	Family <i>Acetobacteriaceae</i>
Order 'Parvularculales'	Order 'Parvularculales'
Family 'Parvularculaceae'	Family 'Parvularculaceae'

*Taxa with uncertain taxonomic status in this study.

peptone or B vitamins and amino acids. Aerobic or facultatively anaerobic. Some species denitrify. In most species, the major isoprenoid quinone is Q-10. Members of the family have been isolated from sea water. The family is a member of the 'Alphaproteobacteria'. The family comprises the type genus *Hyphomonas* Pongratz 1957 emend. Moore *et al.* 1984 and the genera *Hirschia* Schlesner *et al.* 1990, *Maricaulis* Abraham *et al.* 1999 and *Oceanicaulis* Strömpl *et al.* 2003.

Description of *Xanthobacteraceae* fam. nov.

Xanthobacteraceae (Xan.tho.bac.te.ra'ceae. N.L. masc. n. *Xanthobacter* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Xanthobacteraceae* the *Xanthobacter* family).

Gram-negative, rod-shaped bacteria. Motile or (some species) non-motile. Do not form spores. Chemo-organotrophic. Aerobic. Some species fix N₂. In most species, the major

isoprenoid quinone is Q-10. Members of the family have been isolated from plant roots and stems, freshwater and lake silt. The family is a member of the 'Alphaproteobacteria'. The family comprises the type genus *Xanthobacter* Wiegel *et al.* 1978 and the genera *Ancylobacter* Raj 1983, *Angulomicrobium* Vasil'eva *et al.* 1986, *Azorhizobium* Dreyfus *et al.* 1988, *Labrys* Vasilyeva and Semenov 1985 and *Starkeya* Kelly *et al.* 2000.

Description of *Erythrobacteraceae* fam. nov.

Erythrobacteraceae (E.ry.thro.bac.te.ra'ceae. N.L. masc. n. *Erythrobacter* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Erythrobacteraceae* the *Erythrobacter* family).

Gram-negative, rod-shaped bacteria. Motile or (some species) non-motile. Do not form spores. Chemo-organotrophic. Some species require biotin. Aerobic. Cells contain bacteriochlorophyll *a* and carotenoids. Members of the family have been isolated from freshwater. The family is a member of the 'Alphaproteobacteria'. The family comprises the type genus *Erythrobacter* Shiba and Simidu 1982 and the genera *Erythromicrobium* Yurkov *et al.* 1994 and *Porphyrobacter* Fuerst *et al.* 1993.

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- salinarum* to *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomensis* comb. nov., of *Rhodospirillum salexigens* to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum mediosalinum* to *Roseospira mediosalina* comb. nov. *Int J Syst Bacteriol* **48**, 793–798.
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