

Description of ‘*Synergistetes*’ phyl. nov. and emended description of the phylum ‘*Deferribacteres*’ and of the family *Syntrophomonadaceae*, phylum ‘*Firmicutes*’

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The number of bacterial phyla has greatly increased in the past decade. Among them, a candidate division named ‘*Synergistes*’ was proposed in a phylogenetic study on the global diversity of bacteria. We previously described the genus *Jonquetella* and suggested that it belonged to this not yet well-delineated candidate phylum. 16S rRNA gene based-phylogeny studies were conducted using four reconstruction methods and 599 sequences forming five datasets were used in an alternative treeing approach. These analyses indicated that the genera *Aminiphilus*, *Aminobacterium*, *Aminomonas*, *Anaerobaculum*, *Dethiosulfovibrio*, *Jonquetella*, *Synergistes*, *Thermanaerovibrio* and *Thermovirga* should be grouped in the same high-level taxon. This taxon was shown to be a phylum-rank lineage in the domain *Bacteria* and, because of the prior use of the name *Synergistes* for a genus, the name ‘*Synergistetes*’ is proposed for this candidate phylum. We also propose an emended delineation of the phylum ‘*Deferribacteres*’, which is now only represented by the family *Deferribacteriaceae*. The emended family *Syntrophomonadaceae* is limited to the genera *Pelospora*, *Syntrophomonas*, *Syntrophothermus* and *Thermosyntropha*.

The phylum or division is the highest taxonomic rank in the three-domains tree of life. Consequently, the only phylogenetic character shared between members of two phyla is ‘belonging to the domain *Bacteria*’. A phylum is formed to accommodate a group of bacteria that cannot be aggregated to any taxon, except *Bacteria*. According to this definition, molecular phylogeny can easily delineate a phylum avoiding the use of controversial similarity thresholds, as done for species delineation. Despite this robust definition and the increasing number of candidate phyla, descriptions or emendation of phyla are published rarely, resulting in the misclassification of species or genera even in recent descriptions. This is the case for bacteria and clones phylogenetically related to *Synergistes jonesii* and

classified either in the phylum ‘*Deferribacteres*’ or in the family *Syntrophomonadaceae* (phylum ‘*Firmicutes*’).

Synergistes jonesii is a Gram-negative, anaerobic, non-spore-forming bacterium isolated from goat rumen that degraded dihydroxypyridone compounds (Allison *et al.*, 1992). When described, *Synergistes jonesii* was not related to any characterized bacteria, but this initial analysis was compromised by insufficient 16S rRNA gene sequences being available for outgrouping. At the same time, the moderate thermophile *Flexistipes sinuarabici* was described in the domain *Bacteria* but no relationship to any known phylum was detected (Fiala *et al.*, 1990). Later, the metal-reducing genera *Geovibrio* (Caccavo *et al.*, 1996) and *Deferribacter* (Greene *et al.*, 1997), as well as the nitrate-reducing bacteria of the genus *Denitrovibrio* (Myhr & Torsvik, 2000), were grouped together with *Flexistipes sinuarabici*, to form a separate clade in *Bacteria*. Both *Flexistipes*-like and *Synergistes*-like organisms have been proposed as new phyla in the domain *Bacteria* by Hugenholtz *et al.* (1998). Despite their independent deep branching, these two lineages were grouped in the phylum ‘*Deferribacteres*’ in the Taxonomic Outline of the Prokaryotes (Garrity *et al.*, 2004) and in the NCBI taxonomy database (www.ncbi.nlm.nih.gov). According

Abbreviations: ED, evolutionary distance; ML, maximum-likelihood; MP, maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences used in this study are given in Supplementary Table S1 in IJSEM Online.

A table of sequences used for the construction of datasets 1–5 and complete maximum-likelihood 16S rRNA gene-based phylogenetic trees reconstructed from the five datasets are available as supplementary material with the online version of this paper.

to both classifications, *Synergistes jonesii* remained unclassified as *incertae sedis* among the 'Deferribacteres'.

The family *Syntrophomonadaceae* was created by Zhao *et al.* (1993) to group anaerobic, saturated fatty acid- β -oxidizing syntrophic bacteria isolated from anaerobic digester sludge (genera *Syntrophomonas* and *Syntrophospora*, now reclassified as *Syntrophomonas*). Subsequently, bacteria from various environments such as a wastewater treatment lagoon (Menes & Muxi, 2002), activated sludge (Baena *et al.*, 1999; Sekiguchi *et al.*, 2000; Díaz *et al.*, 2007), hot springs (Gorlenko *et al.*, 2004; Sokolova *et al.*, 2002), sulfur mats in a saline environment (Surkov *et al.*, 2001) and a deep marine trench (Takai *et al.*, 1999) were aggregated to the family *Syntrophomonadaceae* in the phylum 'Firmicutes'. The family contained 16 genera (Garrity *et al.*, 2004), but most of them did not match the initial description of the family; in particular, syntrophic metabolism with hydrogenotrophic bacteria has only been observed for some of them (Svetlitschnyi *et al.*, 1996; Wu *et al.*, 2006a). In addition, their deep branching in the 16S rRNA gene tree suggested that they might represent a taxon of higher rank than the family.

The phylogenetic analyses conducted to describe most of the genera currently classified in *Syntrophomonadaceae*, such as *Dethiosulfovibrio* (Magot *et al.* 1997), *Aminomonas* (Baena *et al.*, 1999), *Anaerobaculum* (Menes & Muxi, 2002), *Aminobacterium* (Baena *et al.*, 2000), *Thermovirga* (Dahle & Birkeland, 2006) and *Aminiphilus* (Díaz *et al.*, 2007), did not include *Synergistes jonesii*. The first report that related *Synergistes*-like organisms to members of the family *Syntrophomonadaceae* was the descriptions of *Dethiosulfovibrio russensis* and *Dethiosulfovibrio marinus* (Surkov *et al.*, 2001), showing that *Aminobacterium*, *Aminomonas*, *Anaerobaculum*, *Dethiosulfovibrio*, *Thermanaerovibrio* and *Synergistes* formed a monophyletic cluster. Recently, the genus *Jonquetella* was included in this cluster (Jumas-Bilak *et al.*, 2007).

In this study, based on a comparative analysis of 16S rRNA genes, we propose that the genera *Aminiphilus*, *Aminobacterium*, *Aminomonas*, *Anaerobaculum*, *Dethiosulfovibrio*, *Jonquetella*, *Synergistes*, *Thermanaerovibrio* and *Thermovirga* should be assembled in the same high-level taxon. We also show that this taxon represents a new phylum in the domain *Bacteria*, for which the name 'Synergistetes' is proposed.

Paraphyly of the family *Syntrophomonadaceae* in the 16S rRNA gene-based phylogeny

Sequences of reference strains of genera representative of the family *Syntrophomonadaceae*, the phylum 'Deferribacteres', and the main orders of the phylum 'Firmicutes', according to the Taxonomic Outline of the Prokaryotes (Garrity *et al.*, 2004) and NCBI taxonomy in the greengenes database (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>) (DeSantis *et al.*, 2006), were compared in order to analyse their phylogenetic relationships. The

sequences downloaded from the greengenes database were NAST aligned based on the secondary structure and were checked for chimeras using Bellorophon v. 3 (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>). The sequences were also aligned using the CLUSTAL_X program (Thompson *et al.*, 1997) and the alignments were corrected manually to exclude ambiguously aligned regions corresponding to loops variable in length. The most appropriate substitution model determined according to the Akaike information criterion calculated with MODELTEST (v. 3.7) (Posada & Crandall, 1998) was TN93. Evolutionary distances (ED) were analysed by using neighbour-joining with MEGA 4.0 software (Tamura *et al.*, 2007). Maximum-likelihood (ML) phylogenetic analysis was performed using PHYML v. 2.4.6 (Guindon & Gascuel, 2003), the gamma shape parameter being estimated from the dataset. ML bootstrap support was computed using PHYML. Trees based on bayesian inference were reconstructed with MrBayes (v. 3.1.2) (Ronquist & Huelsenbeck, 2003). Four Markov chains were run for 200 000 generations and were sampled every 100 generations. Based on convergence of likelihood scores, the first 500 trees were discarded and a consensus tree was generated using the remaining trees, including posterior probability of clades and branch lengths. Maximum-parsimony (MP) trees were reconstructed and their robustness was evaluated by using bootstrap methods with DNAPARS of the PHYLIP package (Felsenstein, 1984). The phylogenies generated based on the four methods and the two types of alignments were congruent. The ML phylogenetic tree is shown in Fig. 1. Type strains of species of the genera *Aminiphilus*, *Aminobacterium*, *Aminomonas*, *Anaerobaculum*, *Dethiosulfovibrio*, *Jonquetella*, *Synergistes*, *Thermanaerovibrio* and *Thermovirga* formed the taxonomic framework of a well-separated, deep-branched group supported by high bootstrap values in ED, ML and MP trees and by high posterior probabilities in a Bayesian tree and was named 'Synergistes' clade. *S. jonesii* within this clade clearly branched out of the phylum 'Deferribacteres' where it was classified (Garrity *et al.*, 2004). The 'Synergistes' clade appeared to be subdivided into five main robust lineages (named A–E; Fig. 1). In order to evaluate the diversity of the 'Synergistes' clade, phylogenetic trees were reconstructed as described previously using a set of 81 sequences representative of the clade. The tree confirmed that the 'Synergistes' clade was subdivided into five deep branches, forming five subdivisions (data not shown).

The unclassified 'Deferribacteres' and *Caldithrix abyssi* in association with uncultured organisms formed a deep-branched lineage of 'Deferribacteres' and 'Synergistes' clade. The phylum 'Deferribacteres' appeared to be limited to the current family *Deferribacteriaceae*.

The genera *Anaerobranca*, *Caldicellulosiruptor*, *Carboxydocella*, *Pelospora*, *Syntrophomonas*, *Syntrophothermus*, *Thermaerobacter* and *Thermosyntropha*, which currently belong to the family *Syntrophomonadaceae*, were remote from the 'Synergistes' clade, whatever the treeing method and alignment

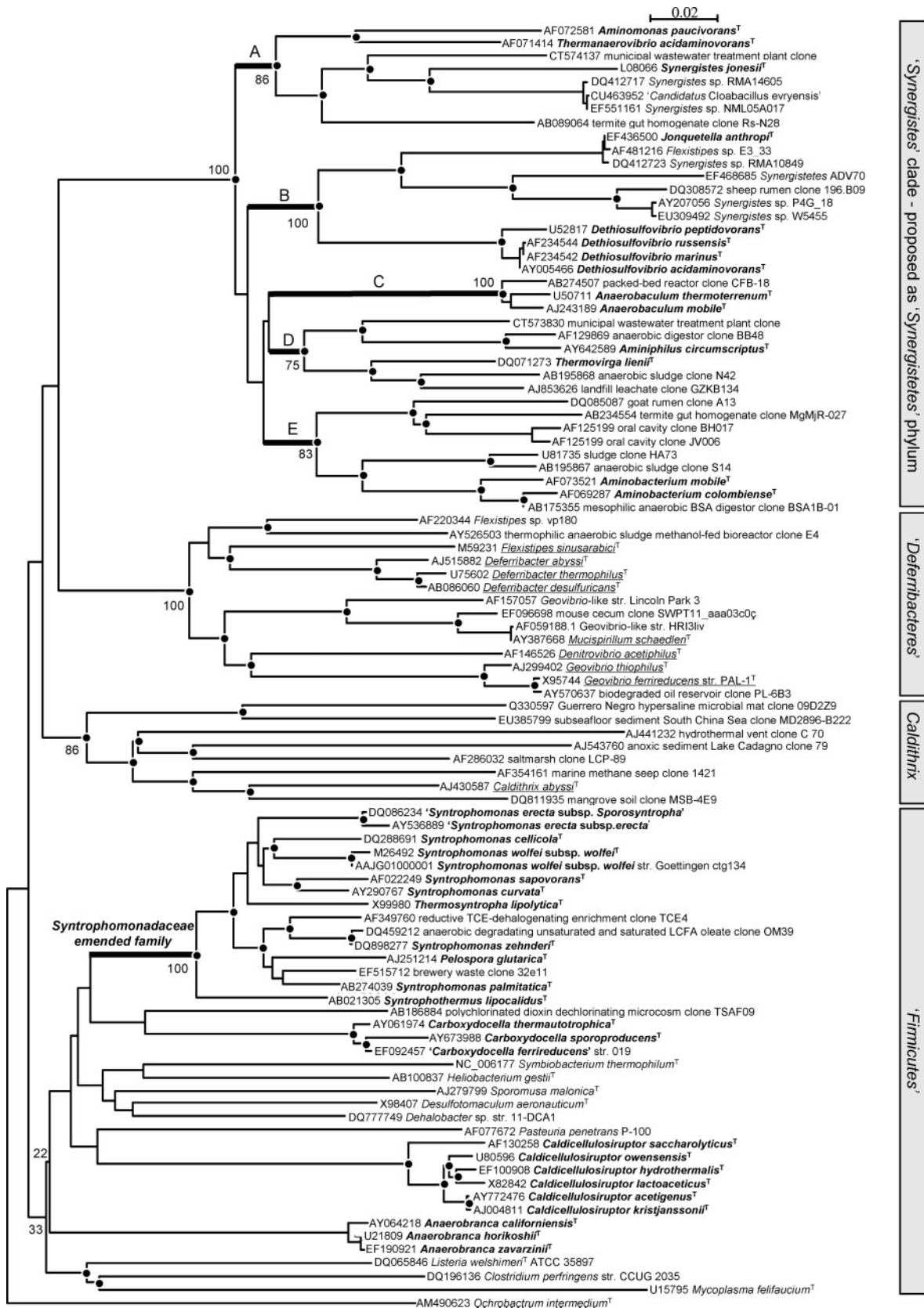


Fig. 1. ML 16S rRNA gene-based phylogenetic tree. Numbers at nodes supporting phylum level clades are bootstrap percentages based on 100 bootstrap replicates. Solid circles indicate nodes supported by bootstrap values of >75% and conserved whatever the phylogenetic method used. GenBank accession numbers are given before the name of the organism. Members of the current family *Syntrophomonadaceae* according to the Taxonomic Outline of the Prokaryotes (Garrity *et al.*, 2004) are in bold type. Members of the current phylum 'Deferribacteres' are underlined. The root of the emended family *Syntrophomonadaceae* is indicated in bold. The superscript Ts indicate that the type strain of the species was used. The roots of robust subdivisions of the phylum 'Synergistetes' are in bold type and named A–E. Subdivision A corresponded to the class *Synergistia*. Bar, 0.02 substitutions per nucleotide position.

used. However, the relative branching of these genera was supported by low bootstrap values and varied according to the method used. Therefore, the 16S rRNA gene-based phylogeny clearly split the members of the current family *Syntrophomonadaceae* into at least two unrelated groups.

A major ultrastructural character, cell-wall structure, supported the separation of the 'Synergistes' clade from other members of the current family *Syntrophomonadaceae*. The members of the 'Synergistes' clade had a typical Gram-negative cell-wall structure with an outer membrane, as observed using electron microscopy (Allison *et al.*, 1992; Baena *et al.*, 1999, 2000; Dahle & Birkeland, 2006; Magot *et al.*, 1997; Menes & Muxi, 2002; Surkov *et al.*, 2001; Zavarzina *et al.*, 2000). In contrast, other members of *Syntrophomonadaceae* possessed a Gram-positive type cell-wall structure without an outer membrane but with a thin peptidoglycan layer (Cayol *et al.*, 1995; Gorlenko *et al.*, 2004; Huang *et al.*, 1998; Sokolova *et al.*, 2002; Sekiguchi *et al.*, 2000; Svetlitsnyi *et al.*, 1996; Takai *et al.*, 1999). The thinness of this layer could explain the negative reaction to Gram stain observed for these bacteria. Among them, members of the genus *Syntrophomonas* showed variable responses to Gram staining and presented with Gram-positive type or unusual cell-wall ultrastructure (Wu *et al.*, 2006a, b; McInerney *et al.*, 1981).

Finally, the cell-wall structure, which was considered to be a character of high significance in bacterial evolution (Gupta, 1998; Cavalier-Smith, 2002), supported the argument for the paraphyly of the current family *Syntrophomonadaceae*. Current members of the family *Syntrophomonadaceae* other than those belonging to the 'Synergistes' clade also formed a deep-branched clade, but was supported by low bootstrap values. The family should be probably elevated to a higher rank than that of the family and also split into several taxa, for which the relationships with members of the phylum *Firmicutes* remain to be explored. The genera *Syntrophomonas*, *Syntrophothermus* and *Thermosyntropha* shared syntrophy as a functional character and, together with the genus *Pelospora*, formed a robust phylogenetic group (Fig. 1) and might represent the 'core family' *Syntrophomonadaceae*.

The 'Synergistes' clade is a phylum-level taxon for which the name 'Synergistetes' is proposed

The typical inter-phyla 16S rRNA gene sequence differences ranged from 20 to 25% (Hugenholtz *et al.*, 1998).

The 'Synergistes' clade displayed less than 75% 16S rRNA gene similarity with the sequences of representative type strains of genera in the 'Firmicutes', 'Deferribacteres' and other phyla. We used a phylogenetic approach in order to confirm the phylum rank of the 'Synergistes' clade. A bacterial phylum was defined from a phylogenetic point of view by Hugenholtz *et al.* (1998) as a group of sequences that are reproducibly monophyletic and are not affiliated to other groups at the phylum level. Approaches that include various methods and datasets have been recommended for the description of phylogenetic relationships between high-level taxa (Dalevi *et al.*, 2001). In particular, multiple sets of outgroup sequences have to be used to test the monophyly of the ingroup. This approach was used in this study and five datasets were analysed as follows.

The sequences used for the construction of datasets 1–5 are given in Supplementary Table S1 (available in IJSEM Online). Each set of sequences contained a constant ingroup, formed by 29 sequences from cultured or uncultured organisms of the candidate phylum. Twenty-one sequences of cultured or uncultured members of the phylum 'Deferribacteres' formed constant outgroups in each dataset. Each dataset displayed other division-level outgroups that varied according to the dataset. The aligned sequences were downloaded from the greengenes database. The most appropriate substitution model for all datasets was general time-reversible (GTR) plus gamma-distribution, plus invariant sites. For each dataset, ED, ML, MP and Bayesian phylogenies were reconstructed as described above except for the ED trees, which were reconstructed by using neighbour-joining in the ARB environment (Ludwig *et al.*, 2004). The ML trees corresponding to the five datasets are shown with collapsed branches in Fig. 2(a–e). The complete ML trees are shown in Supplementary Fig. S1(a–e) in IJSEM Online. In all of the phylogenetic reconstructions, the ingroup sequences representing the 'Synergistes' clade formed a cluster at the phylum level, as its branching was as deep as those of recognized phyla. The cluster was monophyletic and could not be related to a previously described phylum, particularly to 'Deferribacteres' or 'Firmicutes'. The ingroup tested here corresponded to a phylum-level clade, supported by bootstrap values of >95% or Bayesian posterior probability values of 1.0, independent of the composition of the dataset. Each clade corresponding to a known phylum was supported by good confidence values. Lower clade confidence values were observed at the higher nodes,

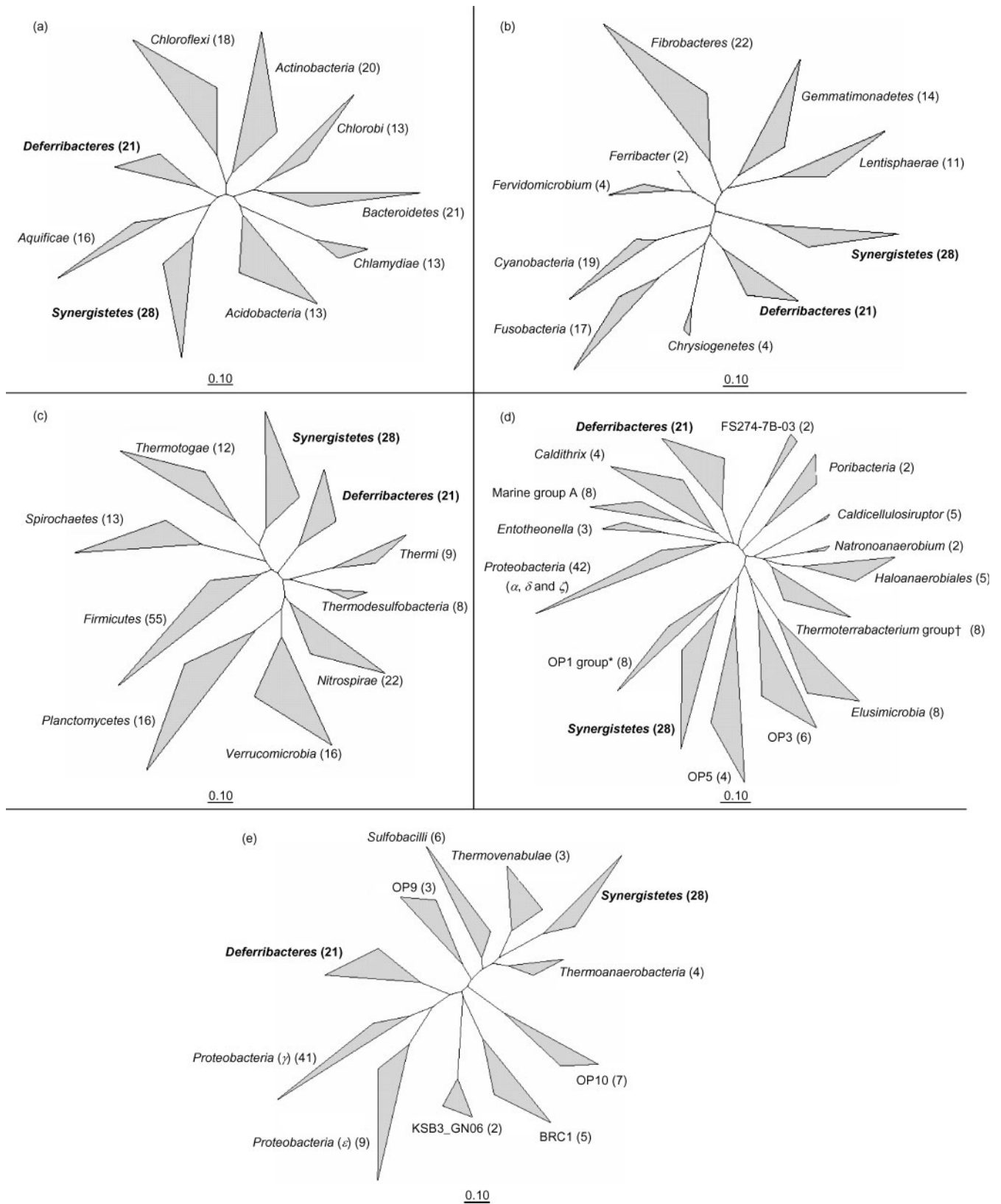


Fig. 2. ML 16S rRNA gene-based phylogenetic trees with collapsed branches reconstructed from the five datasets given in Supplementary Table S1 (in IJSEM Online): (a) dataset 1; (b) dataset 2; (c) dataset 3; (d) dataset 4; (e) dataset 5. The corresponding complete ML trees with bootstrap values are shown in Supplementary Fig. S1(a–e) in IJSEM Online. Numbers in parentheses are the number of sequences in each of the collapsed branches. Constant groups are indicated in bold type. The recognized or non-recognized names of the phylum-level taxa are indicated according to the Hugenholtz classification in the greengenes database. *Contains OP1, *Dictyoglomi* and *Coprothermobacteria*. †Contains *Moorella*, *Thermoacetogenium* and *Thermoterrabacterium*. Bars, number of substitutions per nucleotide position.

indicating lack of statistical significance of the inter-phyla branching order. The branching order also varied according to the phylogenetic method used. As described previously (Hugenholtz *et al.*, 1998), this unsolved deep multifurcation suggested the limits of the 16S rRNA-based phylogeny resolution but could also reflect a true biological explosive radiation corresponding to the phylum radiation in *Bacteria*. The ‘*Synergistes*’ clade was rooted at this radiation confirming its phylum rank.

The name proposed for the new phylum is *Synergistetes* because of the precedence of the genus *Synergistes* as proposed by Jumas-Bilak *et al.* (2007). It includes *Synergistia* classis nov., which corresponds to subdivision A, the first robust subdivision after the phylum root in the lineage of the genus *Synergistes*. The order *Synergistales* ord. nov. and the family *Synergistaceae* fam. nov. correspond to the next two internal nodes to the species *Synergistes jonesii*. In the current family *Syntrophomonadaceae* (Garrity *et al.*, 2004), only the representatives of the genera *Aminobacterium*, *Anaerobaculum*, *Aminiphilus*, *Dethiosulfovibrio*, *Thermovirga*, *Thermanaerovibrio* and *Aminomonas* were related to the phylum ‘*Synergistetes*’. The multi-dataset approach also confirmed the monophyly and the phylum rank of the family *Deferribacteriaceae*, the sole representative of the phylum ‘*Deferribacteres*’.

Description of *Synergistetes* phyl. nov.

Synergistetes (Syn.er.gist.et'es. N.L. masc. n. *Synergistes* -is a genus included in the phylum; N.L. fem. pl. n. *Synergistetes* the phylum of the genus *Synergistes*).

The phylum is defined on the basis of 16S rRNA gene phylogeny of 11 type strains corresponding to eight recognized genera (*Aminobacterium*, *Aminomonas*, *Anaerobaculum*, *Dethiosulfovibrio*, *Jonquetella*, *Synergistes*, *Thermovirga*, *Thermanaerovibrio*). Includes anaerobic, Gram-negative, rod-shaped bacteria isolated from humans, animals and terrestrial and oceanic habitats. Amino-acid degrading bacteria.

Type order: *Synergistales* ord. nov.

Description of *Synergistia* classis nov.

Synergistia (Syn.er.gis'ti.a. N.L. n. *Synergistes* type genus of the type order of the class; suff. -ia ending proposed by Gibbons and Murray and by Stackebrandt *et al.* to denote a class; N.L. fem. pl. n. *Synergistia* the *Synergistales* class).

The description is the same as that for the phylum *Synergistetes*.

Type order: *Synergistales* ord. nov.

Description of *Synergistales* ord. nov.

Synergistales (Sy.ner.gist'al.es. N.L. masc. n. *Synergistes* type genus of the order; -ales ending to denote an order; N.L. fem. pl. n. *Synergistales* the order of the genus *Synergistes*).

The description is the same as for the genus *Synergistes*.

Type family: *Synergistaceae*.

Description of *Synergistaceae* fam. nov.

Synergistaceae (Sy.ner.gist.a'ce.ae. N.L. fem. n. *Synergistes* type genus of the family; -aceae ending to denote a family; N.L. fem. n. *Synergistaceae* family of the genus *Synergistes*).

The description is the same as for the genus *Synergistes*.

Type genus: *Synergistes*

Emended description of the phylum *Deferribacteres* Garrity and Holt 2001

Includes the genera *Deferribacter*, *Denitrovibrio*, *Flexistipes*, *Geovibrio* and *Mucispirillum*.

Emended description of the family *Syntrophomonadaceae* Zhao *et al.* 1993

Includes the genera *Pelospora*, *Syntrophomonas*, *Syntrophothermus* and *Thermosyntrophia*.

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