

# Proposal for a new classification of a deep branching bacterial phylogenetic lineage: transfer of *Coprothermobacter proteolyticus* and *Coprothermobacter platensis* to *Coprothermobacteraceae* fam. nov., within *Coprothermobacterales* ord. nov., *Coprothermobacteria* classis nov. and *Coprothermobacterota* phyl. nov. and emended description of the family *Thermodesulfobiaceae*

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## Abstract

The genus *Coprothermobacter* (initially named *Thermobacteroides*) is currently placed within the phylum *Firmicutes*. Early 16S rRNA gene based phylogenetic studies pointed out the great differences between *Coprothermobacter* and other members of the *Firmicutes*, revealing that it constitutes a new deep branching lineage. Over the years, several studies based on 16S rRNA gene and whole genome sequences have indicated that *Coprothermobacter* is very distant phylogenetically to all other bacteria, supporting its placement in a distinct deeply rooted novel phylum. In view of this, we propose its allocation to the new family *Coprothermobacteraceae* within the novel order *Coprothermobacterales*, the new class *Coprothermobacteria*, and the new phylum *Coprothermobacterota*, and an emended description of the family *Thermodesulfobiaceae*.

*Coprothermobacter* is a bacterial genus that includes anaerobic thermophilic members that are proteolytic and produce acetate, H<sub>2</sub> and CO<sub>2</sub> [1]. This genus currently comprises only two species with validly published names: *Coprothermobacter proteolyticus* and *Coprothermobacter platensis*.

*C. proteolyticus*, initially named *Thermobacteroides proteolyticus* by Ollivier *et al.* in 1985 [2], was isolated from a thermophilic digester fed with tannery wastes and cattle manure. Interestingly this bacterium was isolated together with the archaeon *Methanothermobacter thermautotrophicus* from a thermophilic methanogenic enrichment culture, in which they were the predominant micro-organisms [2]. The first 16S rRNA gene phylogenetic analysis of the former genus *Thermobacteroides* revealed that *T. proteolyticus* differed from the other member of the genus, and indicated an ancient origin of this micro-organism that was considered to be a representative of a novel deep bacterial lineage close to members of the *Thermotogales* [3]. Based on this analysis,

along with phenotypic characterization, Rainey and Stackebrandt [1] reclassified this bacterium as *Coprothermobacter proteolyticus* in 1993. The genome sequence of *C. proteolyticus* DSM 5265<sup>T</sup> was published in 2014 [4] (accession number CP001145.1) as part of the ‘Assembling the Tree of Life’ project. The second species of the genus *Coprothermobacter*, namely *C. platensis*, was isolated from a methanogenic mesophilic reactor treating protein-rich wastewater by Etchebehere *et al.* in 1998 [5]. The 16S rRNA gene phylogeny presented in that work placed *Coprothermobacter* as sister to *Fervidobacterium* and *Thermotoga* (both within the order *Thermotogales*), in accordance with Rainey and Stackebrandt [1]. The genome accession number of *C. platensis* DSM 11748<sup>T</sup> is ARJK00000000.1 (unpublished). In addition, several *Coprothermobacter* species were recently isolated from different sources [6] and the genome sequence of *Coprothermobacter* sp. EBM-25 was obtained (accession number MPOZ00000000.1). A comprehensive revision of the genus *Coprothermobacter*, including an ecological

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perspective and biotechnological potential, was given by Gagliano *et al.* [6].

In the current bacterial taxonomic system, the genus *Coprothermobacter* is classified within the phylum *Firmicutes*, class *Clostridia*, as belonging to the order *Thermoanaerobacterales* and the family *Thermodesulfobiaceae* [7]. This family was described by Mori *et al.* [8] to include the genus *Thermodesulfobium* that along with *Coprothermobacter* constitute the only members of the family [7, 9]. *Thermodesulfobium* had a single species, *Thermodesulfobium narugense* [8], until the recent addition of *Thermodesulfobium acidiphilum* [10]. We tried to trace back the history of the taxonomic assignment of *Coprothermobacter* to this family but it seems that this has never been done formally, as the description of the *Thermodesulfobiaceae* family [8] does not mention the genus *Coprothermobacter*. Paradoxically, the road map to the phylum *Firmicutes* in *Bergey's Manual of Systematic Bacteriology* states that although *Coprothermobacter* and *Thermodesulfobium* are not particularly closely related to each other, both of them are more distantly related to other members of the phylum *Firmicutes* [11]. Moreover, the taxonomic status of many bacteria in that phylum currently remains controversial.

Early studies performed on the two species of the genus *Coprothermobacter* already recognized it as a deep rooting novel phylum [1, 5]. This observation was supported by several studies that were not specially focused on this genus, but nonetheless provided conclusive evidences that *Coprothermobacter* is misclassified within the phylum *Firmicutes*.

The aim of the present study is to formally resolve the taxonomic position of *Coprothermobacter* and to propose a new classification for this deep branching bacterial phylogenetic lineage.

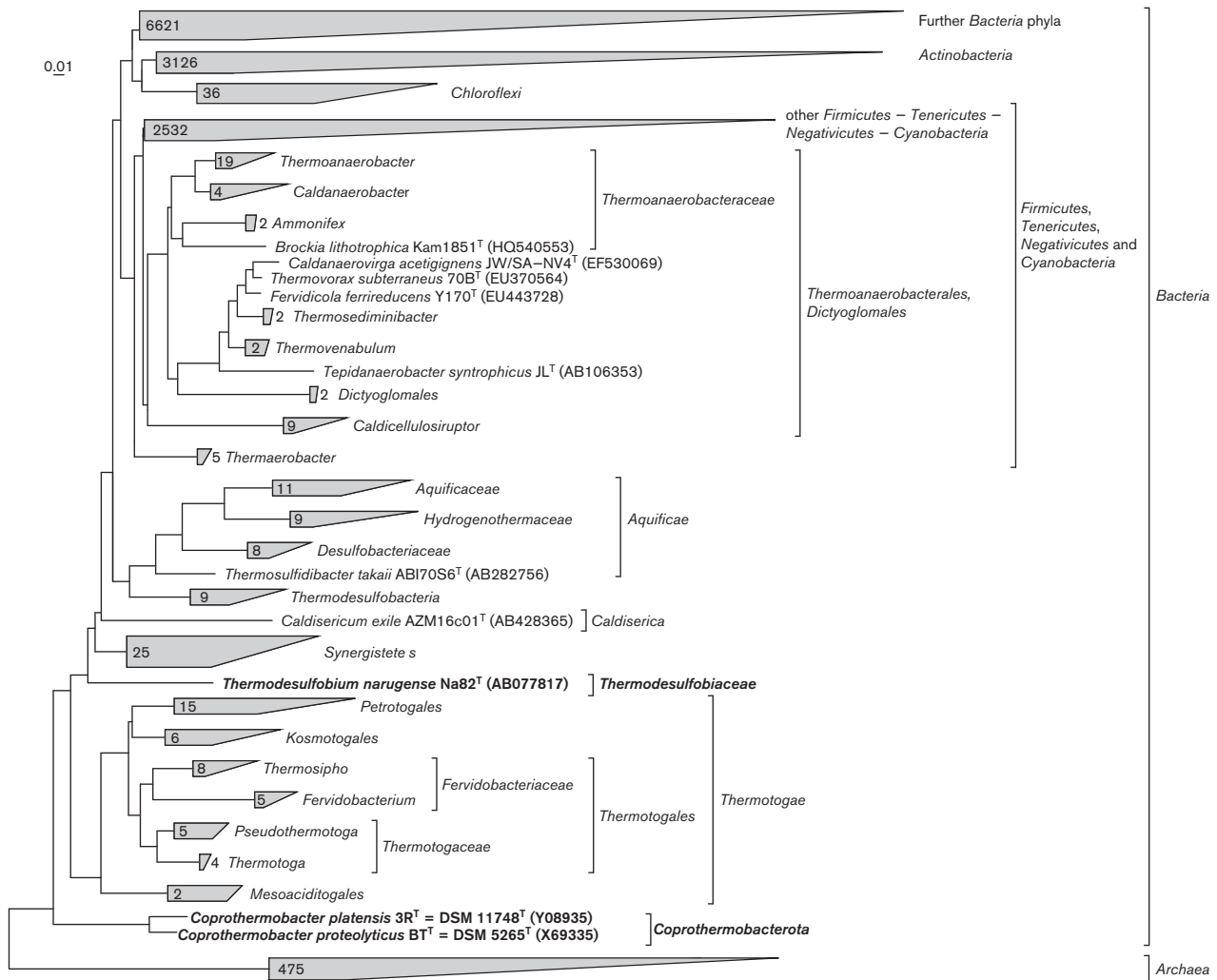
In an evolutionary tree reconstructed to assess bacterial diversity from a phylogenetic point of view that used the 8000 bacterial 16S rRNA gene sequences available two decades ago, *Coprothermobacter* constituted a distinct deeply rooted branch [12]. In the 2004 microbial census, *Coprothermobacter* was considered an 'established phylum' [13] and the 16S rRNA gene phylogenetic tree of the All-Species Living Tree showed a close relationship between *C. proteolyticus* and the phylum *Thermotogae* [14]. The distinct branching of the type strains of both *Coprothermobacter* species is clearly appreciated in the current Living Tree Project (LTP) database tree, version LTPs 128 (Fig. 1).

In the first wide phylogenomic analysis that included *C. proteolyticus*, Beiko [15] highlighted the unusual position of its genome that branched with the other major thermophile-containing phyla *Dictyoglomi*, *Synergistetes* and *Thermotogae*, near the base of the bacterial tree and apart from the *Firmicutes*. This was also supported by Nishida *et al.* [16] that, during the analysis of the close phylogenetic relationship between *Dictyoglomi* and *Thermotogae* through whole-genome comparisons, clearly showed that *C. proteolyticus* did not belong to the phylum *Firmicutes*. Both gene content

and alignment trees reconstructed using 44 concatenated orthologous proteins indicated that *C. proteolyticus* formed a monophyletic group with *Dictyoglomus* and *Thermotogae*, although this study did not define which of them could be closest to *Coprothermobacter*. Another phylogenomic approach, based on the concatenated alignment of 50 ribosomal proteins from circa 1000 prokaryotic genomes performed by Yutin *et al.* [17], showed a tree topology compatible with the commonly accepted bacterial taxonomy, but with some evident deviations. One of them was that *C. proteolyticus* was sister to *Dictyoglomia* within the *Dictyoglomia-Thermotogae-Aquificae* group, and not within *Firmicutes*. *Thermodesulfobium* was not included in these studies.

Supertree methods, that reconcile in a single tree the topological information contained in a set of individual trees, were used to study the extent of lateral gene transfer and to identify highways of gene sharing between prokaryotic lineages [18]. Although lateral gene transfer is a widespread natural phenomenon, ribosomal proteins are less prone to it. The phylogeny of roughly 800 bacterial and archaeal genomes was reconstructed by Lang *et al.* [19] using 24 single copy genes, most of them coding for ribosomal proteins. The authors compared a supertree generated through a Bayesian concordance analysis with a maximum-likelihood tree for the concatenated gene sequences inferred using RAxML (Randomized Axelerated Maximum Likelihood) and found that both trees were similar to the phylogenetic tree inferred from the 16S rRNA gene. Although the supertree was unable to resolve the relationships among deep bacterial lineages, the large scale phylogenetic tree obtained using the concatenated genes showed that both *Coprothermobacter* and *Thermotogae* were sisters to *Dictyoglomus* and *Thermodesulfobium*, with moderate bootstrap support (53%). On the other hand, the clade formed by the pairs *Coprothermobacter* plus *Thermotogae* and *Thermodesulfobium* plus *Dictyoglomus* had a strongly supported (83%) sister relationship to a clade containing *Deinococcus*, *Thermus* and *Aquificaceae* [19]. Whidden *et al.* [20] developed another method to build supertrees based on subtree prune-and-regraft distances using a dataset of  $\approx 40\,000$  gene trees that included 244 genomes representing the major bacterial phyla. The inferred supertree recovered the major bacterial classes as monophyletic groups with some exceptions, one of which was that *C. proteolyticus* was grouped with *Thermotogae* rather than *Clostridia*.

The analysis performed with PhyloPhlAn, a novel high-throughput method for accurate microbial phylogeny reconstruction and taxonomy assignment, developed by Segata *et al.* [21], also indicated a deep phylogeny for *Coprothermobacter*. The method, that allowed the inference of phylogenies with high consistency and could resolve deep-branching lineages, was used to build a complete high-resolution microbial tree of life, that was shown to be robust even to high levels of lateral gene transfer. The final phylogeny obtained showed a deeply branching clade containing



**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences from all type strains available in the Living Tree Project database [45] (LTPs128, released in February 2017). The tree was exported from the LTP database using ARB [46]. Numbers in clusters represent the number of type strain sequences included in each cluster. Bar, 0.01 substitutions per nucleotide position.

*Coprothermobacter* grouped with the phylum *Caldiserica* (formerly candidate division OP5 [22]) with a consistent (85 %) bootstrapping support, both of them sisters to *Thermotogae* (45 % bootstrap value). This deep clade also included *Thermodesulfobium* plus *Dictyoglomi* with low bootstrap support (30 %) [21].

Trees reconstructed using the recently developed composition vector (CV) approaches also supported the status of a separate phylum for *Coprothermobacter* [23]. These methods conduct alignment-free whole-genome comparisons for phylogenetic reconstruction avoiding the selection of orthologous genes, circumventing the problem of lateral gene transfer [24]. The CVTree presented by Zuo and Hao [23] showed *Coprothermobacter* and *Caldiserica* within the branch that also clustered *Thermotogae* and *Dictyoglomi*. On the other hand, *T. narugense* appeared to be separated

from *Coprothermobacter*, and related to the phylum *Thermodesulfobacteria*. Another whole-genome alignment-free method, the SlopeTree (ST), was developed by Bromberg *et al.* [25] and tested on 495 bacterial genomes, filtered for lateral gene transfer to produce high quality trees. All ST trees revealed that the *Firmicutes* were polyphyletic, and showed that *C. proteolyticus* maintained a stable position alongside *Dictyoglomus turgidum*. Both *C. proteolyticus* and *D. turgidum* were neighbours to *Thermotogae*, and this group to *Synergistetes*. Once again, *T. narugense* was not close to *C. proteolyticus*.

The evolutionary relationships among *Clostridia* species and close relatives were analysed by Kunisawa *et al.* [26] through gene arrangement comparison and a Bayesian tree built using 21 concatenated ribosomal protein sequences of 141 bacterial genomes. This work placed *C. proteolyticus*

and *T. narugense* at the boundary between the *Firmicutes* and *Actinobacteria*, but it did not include deep branch taxa that are now known to be related to *C. proteolyticus* and *T. narugense*. Additionally, a gene arrangement that is unique to the *Firmicutes* was not found in these micro-organisms. For these reasons the authors concluded that *C. proteolyticus* and *T. narugense* should be placed outside the phylum *Firmicutes*.

In order to clarify the ambiguous phylogenetic relationships among a number of taxa in the phylum *Firmicutes*, Zhang et al. [27] conducted a wide phylogenetic analysis using the concatenated sequences of 81 conserved proteins from whole genome data, in addition to 16S rRNA gene and 44 concatenated ribosomal protein trees, for 105 species within this phylum. In these trees *Coprothermobacter* either formed a deep branch of its own or clustered along with *Thermotoga* and *Hydrogenobacter*, that were chosen as outgroups due to their closeness to the archaeal–bacterial branch point. *Thermodesulfobium* formed an independent branch near the base of all trees. This work proposed that *Coprothermobacter* and *Thermodesulfobium* should be elevated to phyla status, as both were only distantly related to other members of the phylum *Firmicutes*.

Recently, Mukherjee et al. [28] analysed 1003 reference genomes that were sequenced as part of the Genomic Encyclopedia of Bacteria and Archaea (GEBA) initiative to fill phylogenetic gaps. The maximum-likelihood tree obtained from the concatenated alignment of 56 conserved protein markers from the representative genomes of all cultivated phyla showed *C. platensis* as sister to *Caldiserica* and both of them clustering with *Thermotogae*. On the other hand, *T. narugense* grouped with *Dictyoglomi*.

All the phylogenetic studies mentioned above clearly support the need for the reclassification of both *Coprothermobacter* and *Thermodesulfobium*, but the taxonomic status of the latter is not addressed in this work.

As an additional approach we performed an exploratory genome analysis of *C. platensis*. The annotated genome sequence of *C. platensis* DSM 11748<sup>T</sup> (D890DRAFT\_scaffold00001.1) obtained from the Integrated Microbial Genomes (IMG) database [29] was analysed searching for the top-scoring BLAST hit for each of the 1406 proteins. *C. platensis* was observed to share 5.4 % proteins with *Archaea*, mainly *Euryarchaeota* (4.3 %) and *Crenarchaeota* (0.85 %). Moreover, 7.4 % proteins of *C. platensis* were related to *Thermotogae*, 4.8 % to *Caldiserica* and 4.7 % to *Dictyoglomi*, while only five proteins (0.35 %) were related to *Thermodesulfobium*. These results reveal that *Coprothermobacter* shares a great deal of genetic information with *Archaea* and the deep branching bacteria. This remarkable composition suggests a chimeric genomic nature, similar to what has been observed for the *Thermotogales* [30].

The phenotypic traits described for *Coprothermobacter* also point out that it belongs to the primitive bacteria group,

close to the archaeal–bacterial branch point. Among the scarce known characteristics of the genus *Coprothermobacter* are the capability to reduce thiosulfate to sulfide on carbohydrate and protein substrates and the production of L-alanine during glucose and pyruvate fermentation [31]. The addition of thiosulfate to the carbohydrate-containing medium improves growth both of *Coprothermobacter* and several members of the order *Thermotogales* [32]. On the other hand, Etchebehere and Muxí [31] suggested that L-alanine formation in *Coprothermobacter* could be an electron sink mechanism as observed for *Thermotogales*, that also produce L-alanine during carbohydrate utilization. This trait is shared with the archaea *Pyrococcus furiosus* and *Thermococcus profundus* that balance their metabolism by the formation of this compound [33–35]. Ravot et al. [35] proposed that L-alanine production from sugar fermentation can be regarded as an ancestral metabolic characteristic.

In conclusion, overall phenotypic properties and phylogenetic analysis along with the genome comparisons mentioned above strongly support the need for the taxonomic reclassification of *Coprothermobacter*. Based on this evidence we propose a novel family, *Coprothermobacteraceae* fam. nov., and a novel order, *Coprothermobacterales* ord. nov., to accommodate the genus *Coprothermobacter*, and create a novel class, *Coprothermobacteria* classis nov., and a novel phylum, *Coprothermobacterota* phyl. nov., with *C. proteolyticus* and *C. platensis* as the sole cultured species whose names have been validly published up to date. At present, ranks higher than order are not covered by the International Code of Nomenclature of Prokaryotes [36] so the recent proposal for rule 8 [37] was followed to name the class and phylum.

This reclassification proposal recognizes a general consensus and formalizes what some authors have already begun to use informally. For example, Hugenholtz [38] mentioned in a footnote that *Coprothermobacter* was misclassified as part of the phylum *Firmicutes*, Kunisawa [39] treated ‘*Coprothermobacter*’ as a phylum apart in his study on gene order in bacterial genomes and Wrighton et al. [40] called *Coprothermobacteria* to the *Coprothermobacter* clade obtained in their phylogenetic tree.

Recent environmental studies are revealing a great abundance of representatives of this deep lineage in uncultured complex populations [41–43]. Additionally, the syntrophic association of *Coprothermobacter* with methanogenic archaea, a consequence of its ability to degrade proteins linked to hydrogen production [44], could be relevant for the improvement of both renewable energy generation and sustainable waste disposal [42, 43]. Three decades after the isolation of the first *Coprothermobacter* from a thermophilic anaerobic digester, the discovery of new isolates and potential biotechnological applications highlight the importance to give these micro-organisms an accurate phylogeny-based taxonomic identity.

## DESCRIPTION OF *COPROTHERMOBACTERACEAE* FAM. NOV.

*Coprothermobacteraceae* (Co.pro.ther.mo.bac.te.ra.ce'ae. N. L. masc. n. *Coprothermobacter* type genus of the family; L. suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Coprothermobacteraceae* the family of the genus *Coprothermobacter*).

The description of the family is based on the shared phenotypic and genotypic characteristics of the type strains of the two species of the genus *Coprothermobacter* with validly published names [1, 2, 5, 31].

Obligately anaerobic, thermophilic and proteolytic bacteria. Gram-negative, non-spore-forming, non-motile rods. Chemorganotroph fermenters that produce acetic acid, H<sub>2</sub> and CO<sub>2</sub> as the principal end products. Able to reduce thiosulfate to sulfide but do not reduce sulfate. Usually in syntrophic associations with methanogenic archaea. Separation of *Coprothermobacter* into a new family is justified by its distinct phylogenetic lineage.

The type genus of the family is *Coprothermobacter* Rainey and Stackebrandt 1993.

## DESCRIPTION OF *COPROTHERMOBACTERALES* ORD. NOV.

*Coprothermobacterales* (Co.pro.ther.mo.bac.te.ra'les. N.L. masc. n. *Coprothermobacter* type genus of the order; L. suff. *-ales* ending to denote an order; N.L. fem. pl. n. *Coprothermobacterales* the order of the genus *Coprothermobacter*).

The description is the same as for the family *Coprothermobacteraceae*. The order constitutes one of the earliest diverging lineages of the bacterial phylogenetic tree as supported by analysis of 16S rRNA gene and whole genome sequences. The order contains the family *Coprothermobacteraceae*.

The type genus of the order is *Coprothermobacter* Rainey and Stackebrandt 1993.

## DESCRIPTION OF *COPROTHERMOBACTERIA* CLASSIS NOV.

*Coprothermobacteria* (Co.pro.ther.mo.bac.te'ri.a. N.L. masc. n. *Coprothermobacter* type genus of the type order of the class; L. suff. *-ia* ending to denote a class; N.L. neut. pl. n. *Coprothermobacteria* the class of the order *Coprothermobacterales*).

The description is the same as for the family *Coprothermobacteraceae*. The class constitutes one of the earliest diverging lineages of the bacterial phylogenetic tree as supported by analysis of 16S rRNA gene and whole genome sequences. The class contains the order *Coprothermobacterales*.

The type order of the class is the order *Coprothermobacterales*.

## DESCRIPTION OF *COPROTHERMOBACTEROTA* PHYL. NOV.

*Coprothermobacterota* (Co.pro.ther.mo.bac.te.ro'ta. N.L. masc. n. *Coprothermobacter* type genus of the type class of the phylum; L. suff. *-ota* ending to denote phylum; N.L. neut. pl. n. *Coprothermobacterota* the phylum of the class *Coprothermobacteria*).

The description is the same as for the family *Coprothermobacteraceae*. The phylum constitutes one of the earliest diverging lineages of the bacterial phylogenetic tree as supported by analysis of 16S rRNA gene and whole genome sequences. The phylum contains the class *Coprothermobacteria*.

## EMENDED DESCRIPTION OF THE FAMILY *THERMODESULFOBIACEAE* MORI ET AL. 2004

The family *Thermodesulfobiaceae* as designated by Mori et al. [8] needs to be emended by deletion of the genus *Coprothermobacter* from the list of the constituent genera. The genus *Thermodesulfobium* remains the type genus of the family.

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### Conflicts of interest

The authors declare that there is no conflicts of interest.

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