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 *Thermodesulfobiales*

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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 *Thermodesulfobiales*.

*Coprothermobacter* is a bacterial genus that includes anaerobic thermophilic members that are proteolytic and produce acetate, H₂ and CO₂ [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 archaean *Methanotrichobacter thermotrophicus* 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 5265T 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 based 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 11748T is ARJK0000000.1 (unpublished). In addition, several *Coprothermobacter* species were recently isolated from different sources [6] and the genome sequence of

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**Keywords:** *Coprothermobacter proteolyticus*; *Coprothermobacter platensis*; *Coprothermobacterales*; *Coprothermobacterota*; deep branch lineage.

**Abbreviations:** CV, composition vector; GEBA, genomic encyclopedia of bacteria and archaea; IMG, integrated microbial genomes; LTP, living tree project; RAxML, randomized accelerated maximum likelihood; ST, slope tree.
Coprothermobacter sp. EBM-25 was obtained (accession number MPOZ00000000.1). A comprehensive revision of the genus Coprothermobacter, including an ecological 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 Thermoclostridiales 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 so 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 phyllogenetic lineage.

In an evolutionary tree reconstructed to assess bacterial diversity from a phyllogenetic 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 phyllogenetic 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 phyllogenetic 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 phyllogenomic 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 phyllogenetic 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 Aquificaeae [19]. Whidden et al. [20] developed another method to build supertrees based on subtree prune-and-regraft distances using a dataset of ~ 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 PhyloPhIAn, a novel high-throughput method for accurate microbial phyllogeny reconstruction and taxonomy assignment, developed by Segata et al. [21], also indicated a deep phylogeny for Coprothermobacter. The method, that allowed the inference of phyllogenies with high consistency and could resolve deep-branched 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 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 [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 and Lu [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 (GEBBA) 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 11748T (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 Eurysarchaeota (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 Muxi [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 digestor, 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.thor.mo.bac.te.ra.ce.ae. N.L. masc. n. Coprotthermobacter type genus of the family; L. suff. -acea ending to denote a family; N.L. fem. pl. n. Coprotthermobacteraceae the family of the genus Coprotthermobacter).

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 Coprotthermobacter with validly published names [1, 2, 5, 31].

Obligately anaerobic, thermophilic and proteolytic bacteria. Gram-negative, non-spore-forming, non-motile rods. Chemical organotroph fermenters that produce acetic acid, H2 and CO2 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 Coprotthermobacter into a new family is justified by its distinct phylogenetic lineage.

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

DESCRIPTION OF COPROTHERMOBACTERALES ORD. NOV.

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

The description is the same as for the family Coprotthermobacteraceae. 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 Coprotthermobacteraceae.

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

DESCRIPTION OF COPROTHERMOBACTERIA CLASSIS NOV.

Coprothermobacteria (Co.pro.thor.mo.bac.te.ri.a. N.L. masc. n. Coprotthermobacter type genus of the type order of the class; L. suff. -i.a ending to denote a class; N.L. neut. pl. n. Coprotthermobacteria the class of the order Coprotthermobacterales).

The description is the same as for the family Coprotthermobacteraceae. 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 Coprotthermobacterales.

The type order of the class is the order Coprotthermobacteriales.

DESCRIPTION OF COPROTHERMOBACTEROTA PHYL. NOV.

Coprotthermobacteraota (Co.pro.thor.mo.bac.te.ro.ota. N.L. masc. n. Coprotthermobacter type genus of the type class of the phylum; L. suff. -ota ending to denote phylum; N.L. neut. pl. n. Coprotthermobacteraota the phylum of the class Coprotthermobacteria).

The description is the same as for the family Coprotthermobacteraceae. 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 Coprotthermobacteria.

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 Coprotthermobacter 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 are no conflicts of interest.

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