Published in final edited form as:
Environ Microbiol. 2013 October ; 15(10): 2631–2641. doi:10.1111/1462-2920.12173.

A genomic update on clostridial phylogeny: Gram-negative spore-formers and other misplaced clostridia

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Summary

The class *Clostridia* in the phylum *Firmicutes* (formerly low-G+C Gram-positive bacteria) includes diverse bacteria of medical, environmental, and biotechnological importance. The *Selenomonas-Megasphaera-Sporomusa* branch, which unifies members of the *Firmicutes* with Gram-negative-type cell envelopes, was recently moved from *Clostridia* to a separate class *Negativicutes*. However, draft genome sequences of the spore-forming members of the *Negativicutes* revealed typically clostridial sets of sporulation genes. To address this and other questions in clostridial phylogeny, we have compared a phylogenetic tree for a concatenated set of 50 widespread ribosomal proteins with the trees for beta subunits of the RNA polymerase (RpoB) and DNA gyrase (GyrB) and with the 16S rRNA-based phylogeny. The results obtained by these methods showed remarkable consistency, suggesting that they reflect the true evolutionary history of these bacteria. These data put the *Selenomonas-Megasphaera-Sporomusa* group back within the *Clostridia*. They also support placement of *Clostridium difficile* and its close relatives within the family *Peptostreptococcaceae*; we suggest resolving the long-standing naming conundrum by renaming it *Peptoclostridium difficile*. These data also indicate the existence of a group of cellulolytic clostridia that belong to the family *Ruminococcaceae*. As a tentative solution to resolve the current taxonomical problems, we propose assigning 78 validly described *Clostridium* species that clearly fall outside the family *Clostridiaceae* to six new genera: *Peptoclostridium*, *Lachnoclostridium*, *Ruminiclostridium*, *Erysipelatoclostridium*, *Gottschalkia*, and *Tyzzerella*. This work reaffirms that 16S rRNA and ribosomal protein sequences are better indicators of evolutionary proximity than phenotypic traits, even such key ones as the structure of the cell envelope and Gram-staining pattern.

Keywords

Sporulation; taxonomy; Gram staining; cellulose; xylan; *Clostridium difficile*

History of the Gram-negative members of the *Firmicutes*

Gram-negative, low-G+C Gram-positive bacteria may sound like an oxymoron. Nevertheless, the phylum *Firmicutes* (low-G+C Gram-positive bacteria) includes a number of organisms whose cells are surrounded by two membranes, which are separated by a

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relatively thin layer of peptidoglycan, and, accordingly, stain Gram-negative. Historically, a representative of this group may have been one of the first bacteria observed by Antonie van Leeuwenhoek in his own sputum back in 1683 (Kingsley and Hoeniger, 1973). Two hundred years later it was named *Spirillum sputigenum*, and later renamed *Selenomonas sputigena*. While *S. sputigena* and the closely related *Selenomonas ruminantium* could be easily identified based on the characteristic curved-rod cell shape and flagellar tuft protruding from the concave side, their taxonomic position long remained obscure, being assigned to spirilla, vibrios, and even protozoa. With the advent of the 16S rRNA sequencing, selenomonads were shown to belong to a large group of bacteria, whose representatives had been initially assigned to various Gram-negative bacterial lineages, primarily to *Bacteroidaceae* (*Dialister pneumosintes*, *Megamonas hypermegale*, *Pectinatus cerevisiiphilus*, *Propionispira arboris* and others), but also to *Neisseriaceae* (*Veillonella parvula*, *Acidaminococcus fermentas*), or remained without a clear assignment (Quin’s oval, later renamed *Quinella ovalis*). The overall phylogenetic position of *Selenomonas*-related bacteria remained unresolved until 1984, when scientists from the Deutsche Sammlung von Mikroorganismen (Braunschweig) and the University of Göttingen described a very unusual genus, *Sporomusa*. Its two members, *Sporomusa sphaeroides* and *Sporomusa ovata*, stained Gram-negative but were able to form typical round- or oval-shaped endospores that could survive heating to 80°C for 10 min (Möller *et al*., 1984), identifying them as legitimate members of the *Firmicutes*. This observation prompted a detailed analysis of their phylogenetic position using the 16S rRNA oligonucleotide catalogs and the assignment of the entire *Selenomonas-Megasphaera-Sporomusa* group to the “*Clostridium-Bacillus* cluster” (Stackebrandt *et al*., 1985), i.e. to the current phylum *Firmicutes*. Their strictly anaerobic lifestyle, as well as 16S rRNA sequence data, supported assignment of these bacteria to the class *Clostridia* (Willems and Collins, 1995a, b).

In the 2nd edition of Bergey’s Manual of Systematic Bacteriology (Ludwig *et al*., 2009), all 26 genera of the *Selenomonas-Megasphaera-Sporomusa* group were assigned to a single family *Veillonellaceae* within the class *Clostridia*. However, a year later, based on the Gram-negative type of cell wall and a revised 16S rRNA tree, Marchandin and colleagues (2010) suggested assigning this group to the new order *Selenomonadales* in the new class *Negativicutes* and dividing it into two families, *Acidaminococcaceae* and the emended *Veillonellaceae.* In addition, the family designation for many genera of the new order were unassigned, or *Selenomonadales* Insertae Sedis. This new taxonomy has been adopted by such key resources as the List of Prokaryotic Names with Standing in Nomenclature [LPSN, http://www.bacterio.net/ (Euzéby, 1997)], the Ribosomal Database Project [RDP, http://rdp.cme.msu.edu/ (Cole *et al*., 2009)], and the NCBI Taxonomy database [http://www.ncbi.nlm.nih.gov/taxonomy (Federhen, 2012)] and used for taxonomic assignments by the International Nucleotide Sequence Database Collaboration which includes GenBank, the European Nucleotide Archive and the DNA Data Bank of Japan (Nakamura *et al*., 2013). In contrast, SILVA rRNA gene database [http://www.arb-silva.de/ (Quast *et al*., 2013)] and Greengenes database [http://greengenes.lbl.gov/ (McDonald *et al*., 2012)] still list these organisms as members of the family *Veillonellaceae* in the order *Clostridiales*, class *Clostridia*.

In the past years, the Gram-negative cell walls of these bacteria have been characterized in some detail. The integrity of the cell envelope, with its relatively thin layer of peptidoglycan, has been attributed to the presence of cadaverine, which provides links between peptidoglycan and outer membrane proteins (Kojima *et al*., 2011). A recent detailed study of *Acetonema longum* membranes using electron cryo-tomographic imaging showed that in the course of sporulation, the inner membrane of the mother cell engulfs the prespore, becomes included into the growing spore and, upon its germination, forms the outer membrane of the daughter cell (Tocheva *et al*., 2011). This work put forward an intriguing hypothesis that the same process could have been responsible for the rise of the bacterial outer membrane and, more generally, for the origin of Gram-negative bacteria from Gram-positive ones, whereby the loss of sporulation in the relatives of *Acetonema* resulted in permanently Gram-negative bacteria, such as *S. sputigena*. While similar ideas on the evolutionary primacy of Gram-positive (single-membrane or “monoderm”) bacteria (and/or archaea) giving rise to the Gram-negative (two-membrane or “diderm”) bacteria have been proposed previously (Gupta, 2000, Sutcliffe, 2010), the work by Tocheva and colleagues (2011) provided the first experimental observation in support of that concept and proposed a realistic mechanism of how that could have happened.

Sporulation and phylogeny of the *Negativicutes*

The 16S rRNA tree constructed by Tocheva and colleagues (2011) put *A. longum* squarely within the clostridial clade, in accordance with the earlier data (Willems and Collins, 1995a, b, Ludwig *et al*., 2009). The tree built from the SpoIVA sequences had essentially the same topology (see Fig. 6 and S7 in Tocheva *et al*., 2011). As of April 1st, 2013, there were no complete genome sequences for any spore-forming members of the *Negativicutes*, which is why these bacteria were outside the scope of our recent comparative-genomics analysis of the sporulation proteins in *Bacilli* and *Clostridia* (Galperin *et al*., 2012). However, draft genome sequences have recently become available for *A. longum* and for six different strains of *Pelosinus fermentans*, another spore-forming member of *Negativicutes* (Tables 1 and S1). One more member of that group, *Thermosinus carboxydivorans*, has been reported not to form spores (Sokolova *et al*., 2004), but its draft genome encoded a substantial number of sporulation genes. These sequence data offered a possibility of analyzing sporulation genes in eight different genomes from three species of *Negativicutes*.

Previous studies have shown that *Bacilli* and *Clostridia* share a common set of key sporulation genes but exhibit substantial differences in the regulation of the onset of sporulation, the engulfment process, and the assembly and protein content of the spore coat (Stragier, 2002, Onyenwoke *et al*., 2004, de Hoon *et al*., 2010, Galperin *et al*., 2012). A recent analysis identified ~60 sporulation genes that were found in all spore-former genomes and ~60 genes that were found in all spore-forming bacilli but absent in all spore-forming clostridia (Galperin *et al*., 2012). A comparison of these gene sets against draft genomes of *A. longum*, *T. carboxydivorans*, and six strains of *P. fermentans* revealed a surprisingly consistent pattern. Of 56 genes that were present in all bacillar and clostridial spore-formers, 52 were also present in all eight spore-forming members of the *Negativicutes*, and 3 more (all except for *spoVG*) were only absent in *T. carboxydivorans* (Tables S2 and S3). This result was consistent with the classification of these bacteria within the *Firmicutes*. Further, out of 61 bacillar sporulation genes that were never found in clostridia, at least 57 were missing in all eight *Negativicutes* genomes (Table S4). Only few clostridia-specific sporulation genes have been identified so far (Lawley *et al*., 2009), and several of them had orthologs in *A. longum*, *P. fermentans*, and/or *T. carboxydivorans* (Table S5). Thus, genomes of spore-forming members of the *Negativicutes* displayed essentially the same distribution of sporulation genes as genomes of spore-forming members of the *Clostridia*. Given the previous 16S rRNA-based assignment of these bacteria (and the entire *Sporomusa*-*Selenomonas*-*Megasphaera* group) to the class *Clostridia* (Stackebrandt *et al*., 1985, Willems and Collins, 1995a, b, Ludwig *et al*., 2009), we have decided to re-examine their systematic position using protein-based phylogenetic trees.

The availability of complete or draft genomes for several members of the *Negativicutes* allowed us to collect the necessary protein sequences and construct phylogenetic trees from (i) a concatenated alignment of 50 ribosomal proteins and alignments of (ii) β-subunit of the DNA-directed RNA polymerase (RpoB) and (iii) β-subunit of the DNA gyrase (GyrB), see Fig. 1. Analysis of the phylogenetic trees for 3 spore-forming and 6 non-spore-forming species of the *Negativicutes* showed that they formed a well-defined separate group within the clostridial lineage (Fig. 1A). The pairwise groupings of *Acetonema* with *Thermosinus*, *Acidaminococcus* with *Phascolarctobacterium*, *Centipeda* with *Selenomonas*, and *Veillonella* with *Megasphaera* were fully consistent with the 16S rRNA-based trees of Ludwig *et al*. (2009), Rainey (2009), and Marchandin *et al*. (2010). However, protein-based trees did not fully support the division of this group proposed by Marchandin and colleagues (2010), as *Acetonema*, *Pelosinus* and *Thermosinus* clearly grouped together and separately from both *Acidaminococcus*/*Phascolarctobacterium* and *Veillonella*/*Megasphaera* branches (Fig. 1A). Therefore, if the original family *Veillonellaceae* (*Selenomonas-Megasphaera-Sporomusa* group), with all 26 genera that are listed by Ludwig *et al*. (2009) and Rainey (2009) and up to 8 recently described genera (see Table S6), is to be divided, *A. longum*, *P. fermentans* and *T. carboxydivorans* should be put into a separate family. Thus, *Veillonellaceae* would need to be split into at least four different families, including the *Acidaminococcaceae* with its 4 genera and *Veillonellaceae sensu stricto* with 6 genera as emended by Marchandin *et al*. (2010). The remaining members could be divided between families *Selenomonadaceae* (up to 10 genera) and *Sporomusaceae* (up to 14 genera of mostly spore-forming bacteria), in accordance with the 16S rRNA trees of Ludwig *et al*. (2009) and Marchandin *et al*. (2010). The respective proposals are summarized in Table S6. Summing up, protein-based phylogenetic trees fully support the 16S rRNA-based trees in Ludwig *et al*. (2009) and Tocheva *et al*. (2011) and the well-supported branches of the trees in Rainey (2009) and Marchandin *et al*. (2010), as well as the taxonomic assignments for the *Veillonellaceae* in Bergey's and in the SILVA and Greengenes databases. Of the changes proposed by Marchandin and colleagues (2010), creation of the class *Negativicutes* does not seem to be justified, at least from the phylogenetic point of view. The name "*Negativicutes*" could be retained as a mnemonic synonym to the *Selenomonas-Megasphaera-Sporomusa* group (i.e. the order *Selenomonadales*) within the class *Clostridia*. The family *Acidaminococcaceae* is only warranted if there are two other new families, *Sporomusaceae* and *Selenomonadaceae*; creation of the latter family is also needed to justify the order *Selenomonadales*. That said, a formal adoption of this classification should probably await completion of the genomes of *Sporomusa ovata*, *Anaeroarcus burkinensis*, and *Anaeromusa acidaminophila* (currently in progress), which would also help in resolving the weak affiliation of the latter two organisms with the *Sporomusa* group.

Phylogeny of the bacteria mis-assigned to the genus *Clostridium*

While members of *Veillonellaceae* stain Gram-negative owing to the structure of their cell envelopes, many *Clostridium* spp. have been reported to stain Gram-negative despite having a typical Gram-positive cell wall (e.g. Freier *et al*., 1988). Just 10 years ago, Sydney Finegold described the state of clostridial classification this way: “The genus *Clostridium* defies all the simple rules we learnt years ago when it was thought to consist of Gram-positive, spore-forming, anaerobic rods. Now the genus includes Gram-negatives, non-sporeformers, cocci, and non-anaerobes” (Finegold *et al*., 2002). Since that time, taxonomy of the genus *Clostridium sensu stricto* (cluster I in the classification of Collins *et al*., 1994) has been mostly resolved through concerted efforts of several different groups, assisted by the rapidly growing amount of genome sequence data (Stackebrandt *et al*., 1999, Gupta and Gao, 2009, Rainey *et al*., 2009). An extensive update of clostridial classification has been performed in the latest edition of Bergey’s (Ludwig *et al*., 2009). In the course of that update, more than 50 bacteria previously placed in the genus *Clostridium* (Garrity *et al*., 2007) have been reassigned to other taxonomic groups, based on their 16S rRNA sequences and some other features. However, despite this reassignment, many organisms still retained the *Clostridium* name (Ludwig *et al*., 2009; Rainey *et al*., 2009), causing a major confusion in the clostridial taxonomy (see, e.g., McDonald *et al*., 2012). Thus, these bacterial species remain listed as *Clostridium* spp. in the LPSN and in GenBank\ENA\DDBJ, which creates a false impression that they are legitimate members of the *Clostridium* genus in the family *Clostridiaceae*. As a way to resolve this conundrum, the NCBI Taxonomy database and SILVA database currently display some of these organisms as [*Clostridium*], but this name has its own problems and can hardly be considered a permanent solution. We have compared the positions of several questionable *Clostridium* spp. on the ribosomal proteins-based tree (Fig. 1B) with the taxonomic assignments in the latest edition of Bergey’s and in SILVA, RDP, Greengenes, and GenBank databases. We hope that this analysis and the suggestions listed below (Table 2, see Tables S7 and S8 for details) would help in removing these stumbling blocks, or will at least stimulate a discussion on how to properly do that.

***Clostridium difficile*.** In their classical paper 20 years ago, Collins and colleagues (1994) already noted that *C. difficile* and its close relatives *C. paradoxum* and *C. sticklandii*, along with *Peptostreptococcus anaerobius*, belong to a distinct family-level group (cluster XI or Family 13). Accordingly, the recent edition of Bergey’s reclassified these 13 species into the family *Peptostreptococcaceae* (Ludwig *et al*., 2009). Unfortunately, the names of these organisms have not been changed. As a result, many biologists remain unaware that *Clostridium difficile* is substantially distinct from *Clostridium butyricum* (the type species of the genus) and its close relatives, such as *C. botulinum*, *C. perfringens*, and *C. tetani*. This distance is manifested, for example in the sporulation gene patterns: most *C. difficile* genomes lack such genes as *spoIVFB*, *bofA*, *cotS*, *cotM*, *ydhD*, *gerA*, and *gerC*, which are widespread among *Clostridium sensu stricto* (Xiao *et al*., 2011, Galperin *et al*., 2012), but encode certain proteins that are not found in other clostridia (Lawley *et al*., 2009). The current versions of the NCBI Taxonomy database and SILVA database display *C. difficile* and its relatives as [*Clostridium*] spp., which is somewhat better but hardly resolves the confusion. We suggest renaming these organisms *Peptoclostridium* spp. Complete genomes of numerous isolates of *C. difficile* (*Peptoclostridium difficile)* and of *C. sticklandii* (*Peptoclostridium sticklandii*) strain DSM 519 are already available, and sequencing of other representatives of this group is currently under way. Analysis of their genome sequences may suggest that these organisms represent as many as four genera as has been suggested by Collins and colleagues (1994).

Clostridium acidurici. Protein trees agree with the 16S rRNA-based phylogeny (Hartwich *et al*., 2012), placing *C. acidurici* (cluster XII) as a sister group of *Anaerococcus prevotii* and *Finegoldia magna* (cluster XIII), which are currently assigned to *Clostridiales* Family XI Incertae Sedis. The complete genome sequences of these three organisms, as well as draft genomes of several other members of the Family XI Incertae Sedis, are already available, which should allow a better characterization of this family. Meanwhile, we propose assigning *C. acidurici* and its close relative *C. purinilyticum* to the new genus *Gottschalkia* as *Gottschalkia acidurici* and *Gottschalkia purinilytica*, respectively.

Clostridium cellulolyticum, *C. thermocellum* (both cluster III), and *C. leptum* (cluster IV), along with 13 other *Clostridium* spp., have been reassigned by Ludwig *et al*. (2009) to the family *Ruminococcaceae*. In protein-based trees, these three bacteria confidently clustered with *Ruminococcus albus*, confirming this placement. This group includes three other *Clostridium* spp. with completely sequenced genomes, *C. clariflavum*, *C. stercorarium* and *Clostridium* sp. BNL1100. These organisms are being intensively studied owing to their ability to metabolize cellulose, a common trait of all cluster III organisms described so far. Obviously, keeping these bacteria under the name *Clostridium* is counterproductive, as they are often being confused with non-cellulolytic clostridia. As a tentative solution, based on the relatively high rRNA similarity levels (Izquierdo *et al*., 2012), we suggest assigning cluster III members to the new genus *Ruminiclostridium*. In the future, some of these organisms might need to be moved to the genus *Acetivibrio* whose type species *A. cellulolyticus* is closely related to *C. clariflavum*.

Clostridium phytofermentans. In the 2nd edition of Bergey's (Ludwig *et al*., 2009), *Clostridium symbiosum* and 31 other *Clostridium* species that fall within clusters XIVa and XIVb of Collins *et al*. (1994) have been transferred to the family *Lachnospiraceae*, in full agreement with the assignments of SILVA and RDP databases. One of these species, *Clostridium lentocellum*, has been renamed *Cellulosilyticum lentocellum* (Cai and Dong, 2010), whereas, as far as we could see, the rest still retain the *Clostridium* name. Several well-known species from clusters XIVa and XIVb, such as *C. sphenoides* or *C. piliforme*, have not been mentioned by Ludwig *et al*. (2009) or Rainey *et al*. (2009), creating further confusion as to which *Clostridium* spp. belong to the family *Lachnospiraceae* and which should stay in *Clostridiaceae*. Thus, the web site http://www.broadinstitute.org/annotation/genome/clostridium_group/ of the Broad Institute, which has obtained draft genomic sequences of several organisms from this group (*C. aldenense*, *C. bolteae*, *C. citroniae*, *C. clostridioforme*, *C. hathewayi*, and *C. symbiosum*), lists them all as *Clostridium* spp. Again, protein trees supported 16S rRNA-based assignments, placing *C. phytofermentans* and *C.*

symbiosum in a tight cluster with *Butyrivibrio proteoclasticus*, *Roseburia hominis*, and other members of *Lachnospiraceae*. We propose tentatively assigning all cluster XIVa organisms that are still listed as *Clostridium* spp. to the new genus *Lachnoclostridium*. For the cluster XIVb organisms, which include *C. piliforme*, the causative agent of Tyzzer's disease, we propose the new genus *Tyzzerella* (see below).

Clostridium ramosum and *C. spiroforme*, members of the Collins *et al*. (1994) cluster XVIII, along with two other *Clostridium* species, have been transferred by Ludwig *et al*. (2009) to the family *Erysipelotrichaceae* in the class *Erysipelotrichi*. Our data confirm their close relationship to *Erysipelothrix rhusiopathiae*, as well as clustering with mollicutes (Marchandin *et al*., 2010; Ogawa *et al*., 2011), which are currently assigned to the separate phylum, the *Tenericutes*. We propose assigning *C. ramosum*, *C. spiroforme*, and three related species, to the new genus *Erysipelatoclostridium*.

The above suggestions are based primarily on the results from ribosomal protein-based phylogenetic trees (Fig. 1) and therefore miss those *Clostridium* spp. for which sequence information has been unavailable or insufficient. However, the excellent agreement of our protein trees with 16S rRNA-based classification presented in the SILVA and RDP databases indicates that, at least in the case of *Clostridia*, the assignments of these databases could be used to build a fairly reliable phylogeny-based taxonomy. Hence, based on the assignments in Bergey's, RDP and SILVA, as well as results of 16S rRNA similarity searches, we propose the following six new genera (Table 2, see Table S8 for details).

Description of *Peptoclostridium* gen. nov

Peptoclostridium [Pep.to.clos.tri'di.um. Gr. v. *peptô*, digest; N.L. neut. dim. n. *Clostridium*, a bacterial genus name (from Gr. n. *klôstêr*, a spindle); N.L. neut. dim. n. *Peptoclostridium*, the digesting clostridium].

Gram-staining-positive, motile, spore-forming rods 0.3–1.5 μm×1.5–20 μm. Obligate anaerobes, no microaerophilic or aerobic growth. Strains are mesophilic or thermophilic (temperature range from 20°C to 63°C) and grow in neutral to alkaline pH (some strains up to pH 11). Chemoorganotrophs. Oxidase and catalase negative. Peptone may serve as nitrogen source. Yeast extract can be used as the sole carbon and energy source. Several members require 1.5% NaCl for growth. Some mono- and disaccharides can be fermented, acetate is produced as a major end product. Sulfate is not reduced. The G+C content of the genomic DNA ranges from 25 to 32 mol%. The type species is *Peptoclostridium difficile* (formerly *Clostridium difficile*), the type strain is ATCC 9689 = DSM 1296.

The newly proposed genus *Peptoclostridium* is equivalent to genus *Clostridium* XI in the RDP and the *Peptostreptococcaceae* genus Incertae Sedis in SILVA, see the 16S rRNA trees in Song *et al*. (2004) and Pikuta *et al*. (2009). It includes 11 validly described species that have been transferred to the family *Peptostreptococcaceae* in the recent edition of Bergey's, as well as *C. mayombei* and *C. thermoalcaliphilum* (Table 2). In addition, we propose that the genus include *Eubacterium tenue*, *Eubacterium yurii* and the following species that have not been validly described but whose 16S rRNA sequences are available in GenBank: *C. maritimum* (GenBank accession number EU089965), *C. metallolevans*

(DQ133569), *C. ruminantium (*EU089964), *C. venationis* (EU089966), and the misnamed *C. hungatei* strain mc (JX073559; other *C. hungatei* strains go to *Ruminiclostridium*, see below). Two more members of the family *Peptostreptococcaceae*, *Clostridium sticklandii* and *C. litorale*, have been tentatively assigned to the genus *Peptoclostridium* to resolve the naming conundrum but might deserve to be put into a separate genus (or genera), see Fig. 1B and Pikuta *et al*. (2009). *Sporacetigenium mesophilum* falls within the diversity of the new genus but is left as is because of its unusual metabolic properties (Chen *et al*., 2006).

Description of *Gottschalkia* gen. nov

Gottschalkia (Gott.shal'ki.a. N.L. fem. n., named after Gerhard Gottschalk, in recognition of his important contributions to the studies of various anaerobic bacteria, including clostridia).

Obligately anaerobic purinolytic spore-forming rods that, in the presence of 0.1% yeast extract, are capable of utilizing uric acid as sole carbon and energy source. Gram-staining is variable, motility is by peritrichous flagella. Optimal growth at 19–37°C and pH 7.3–8.1. No utilization of carbohydrates, no reduction of nitrate, no production of H_2. The DNA G+C content is 28–29 mol%.

The proposed genus has been first suggested by Collins *et al*. (1994); it is equivalent to *Clostridiales* Family XI Incertae Sedis genus Incertae Sedis in SILVA and includes two validly described organisms: *Clostridium acidurici* and *C. purinilyticum*, see Hartwich *et al*. (2012) and references therein. Based on the similar 16S rRNA sequence and metabolic properties, *Eubacterium angustum* could be assigned to the same genus, despite its inability to form spores and higher G+C content (Beuscher and Andreesen, 1984). The type species is *Gottschalkia acidurici* (formerly *Clostridium acidurici*), the type strain is ATCC 7906 = DSM 604.

Description of *Ruminiclostridium* gen. nov

Ruminiclostridium [Ru.mi.ni.clos.tri'di.um. L. n. *rumen -inis*, the rumen; N.L. neut. dim. n *Clostridium*, a bacterial genus name (from Gr. n. *klôstêr*, a spindle); N.L. neut. dim. n. *Ruminiclostridium*, clostridia-like bacteria in the family *Ruminococcaceae*].

Obligately anaerobic, mesophilic or moderately thermophilic, spore-forming, straight or slightly curved rods 0.5–1.5 μm×1.5–8 μm. The cells have a typical Gram-positive cell wall, although often stain Gram-negative. Produce spherical or oblong terminal spores, which results in swollen cells. Most species are motile and have polar, subpolar, or peritrichous flagella. The temperature range for various species is from 203°C to 70°C with T_{opt} between 33 and 65°C. Optimal pH values are between 7 and 9 (some members can grow at pH as low as 5.9 or as high as 10.2). Oxidase and catalase are not produced. Yeast extract or vitamins are usually required for anabolic purposes. All known members can use cellulose, xylan, and/or cellobiose as substrates, fermenting them primarily to acetate, ethanol, H_2, and CO_2, as well as lactate, propionate, butyrate, or other end products. The ability to ferment other carbohydrates varies between species. Several species are capable of fixing N_2. Sulfate is not reduced. The G+C content of the genomic DNA is typically 39–41.5%, but ranges from 27 to 51 mol% [while two species, *C. alkalicellulosi* and *C. papyrosolvens*, have been initially reported to have the G+C content of 29.9–30.0%, the genomic sequence of *C. papyrosolvens* DSM 2782 showed G+C content of 36.9% (Hemme *et al*., 2010)]. The type species is *Ruminiclostridium thermocellum* (formerly *Clostridium thermocellum*), the type strain is ATCC 27405 = DSM 1237.

The proposed genus *Ruminiclostridium* includes organisms from clostridial cluster III of Collins and colleagues (1994) and is equivalent to the genus *Clostridium* III in the RDP and the *Ruminococcaceae* genus Incertae Sedis in SILVA, see the 16S rRNA trees in Shiratori *et al*. (2009) and Izquierdo *et al*. (2012). It includes 15 validly described species, 12 of which have been transferred to the family *Ruminococcaceae* in the recent edition of Bergey's, as well as *C. caenicola*, *C. clariflavum*, and *C. sufflavum* (Table 2). The *Clostridium* strain Rt51.B1 (GenBank: L09175), misnamed as *C. sporogenes*, and *Clostridium* sp. BNL1100 also belong to this genus.

Several *Clostridium* spp. that fall within the family *Ruminococcaceae* have been tentatively assigned to the genus *Ruminiclostridium* but will have to be reclassified and renamed after their phylogenetic status is better resolved. These include five members of the Collins *et al*. (1994) cluster IV (and genus *Clostridium* IV in the RDP): *Clostridium leptum*, *C. cellulosi*, *C. methylpentosum*, *C. sporosphaeroides*, and *C. viride*. One more member of *Ruminococcaceae*, *Clostridium orbiscindens*, has been recently reclassified as *Flavonifractor plautii* (Carlier *et al*., 2010). In addition to former *Clostridium* spp., *Bacteroides cellulosolvens*, *Eubacterium desmolans*, and *Eubacterium siraeum* fall within the proposed new genus. However, in future some of its members might have to be reassigned to *Acetanaerobacterium*, *Acetivibrio*, *Flavonifractor*, *Oscillibacter*, *Ruminococcus*, and/or new genera of *Ruminococcaceae*.

Description of *Lachnoclostridium* gen. nov

Lachnoclostridium [Lach.no.clos.tri'di.um. Gr. n. *lachnos*, wool; N.L. neut. dim. n. *Clostridium*, a bacterial genus name (from Gr. n. *klôstêr*, a spindle); N.L. neut. dim. n. *Lachnoclostridium*, the clostridia within the family *Lachnospiraceae*].

Gram-positive, motile, obligately anaerobic spore-forming rods 0.3–1.5 μm×1.5–20 μm. Strains are mesophilic or thermophilic (temperature range from 203°C to 633°C) and grow in neutral to alkaline pH (some up to pH 11). Chemoorganotrophs. Oxidase and catalase are not produced. Some mono- and disaccharides can be fermented, acetate is produced as a major end product. Sulfate is not reduced. The G+C content of the genomic DNA ranges from 25.6 to 52 mol%. The type species is *Lachnoclostridium phytofermentans* (formerly *Clostridium phytofermentans*), the type strain is ATCC 700394 = DSM 18823.

The proposed genus *Lachnoclostridium* includes organisms from clostridial cluster XIVa of Collins *et al*. (Collins *et al*., 1994), the genus *Clostridium* XIVa in the RDP, and the *Lachnospiraceae* genus Incertae Sedis in SILVA, see the 16S rRNA trees in Warren *et al*. (2006) and Domingo *et al*. (2009). It includes 30 validly described species, most of which have been assigned to the family *Lachnospiraceae* in the recent edition of Bergey's (Table 2). It also includes the following species that have not been validly described but whose 16S rRNA sequences are available in GenBank: *C. boliviensis* (AY943862), *C. fusiformis* (AB702934), C. *sulfatireducens* (AY943861), and a misnamed strain of *C. leptum* (AF262239; *C. leptum* type strain DSM 753 goes to *Ruminiclostridium*, see Fig. 1B). *Desulfotomaculum guttoideum*, *Eubacterium contortum*, *Eubacterium fissicatena*, and *Ruminococcus torques* also belong to this genus.

Description of *Tyzzerella* gen. nov

Tyzzerella [Ti.ze.rel'.la] N.L. fem. n. *Tyzzerella*, named after Ernest Tyzzer, an American pathologist who isolated and described "*Bacillus piliformis*", the causative agent of Tyzzer's disease.

A closely related cluster of organisms in the family *Lachnospiraceae* includes six *Clostridium* spp. that warrant assignment to a separate genus. The description of the new genus is essentially the same as that of *Lachnoclostridium* (see above), although some of its members are non-motile and non-spore-forming and have higher G+C contents, from 40% in *C. nexile* to 46.8% in *C. colinum*. The genus is named after Ernest Edward Tyzzer (1875–1965), who in 1917 characterized "*Bacillus piliformis*", the causative agent of an infectious diarrhea of laboratory mice, which was later found in a variety of animals and became known as "Tyzzer's disease". Unfortunately, no *Tyzzerella piliformis* (formerly *Clostridium piliforme*) strains have been deposited in public culture collections so far (deposition is currently in progress). Accordingly, *Tyzzerella nexilis* [formerly *Clostridium nexile* (Holdeman and Moore, 1974)] is selected as the type species and ATCC 27757 = DSM 1787 as the type strain.

Description of *Erysipelatoclostridium* gen. nov

Erysipelatoclostridium [E.ry.si.pe.la.to.clos.tri'di.um. Gr. n. *erusipelas -pelatos*, erysipelas; N.L. masc. n. *Clostridium*, a bacterial genus name (from Gr. n. *klôstêr*, a spindle); N.L. neut. dim. n. *Erysipelatoclostridium*, Clostridium-like members of the order *Erysipelotrichales*].

Gram-positively staining, nonmotile, obligately anaerobic straight or helically curved rods 0.3–1.0 μm×2–4 μm. Spore formation is rare or absent. The G+C content of the genomic DNA is 27–33 mol%. Ferment glucose, fructose and sucrose, see Kaneuchi *et al*. (1979) for a detailed comparison. The type species is *Erysipelatoclostridium ramosum* (formerly *Clostridium ramosum*); the type strain is ATCC 25582 = DSM 1402.

The newly proposed genus *Erysipelatoclostridium* is equivalent to the *Clostridium* XVIII genus in RDP and *Erysipelotrichaceae* genus Incertae Sedis in SILVA, see the 16S rRNA trees in Clavel *et al*. (2007) and Ogawa *et al*. (2011). It includes four validly described species: *Clostridium cocleatum*, *C. ramosum*, *C. saccharogumia*, and *C. spiroforme*. In addition, *Clostridium innocuum*, which is more distantly related to the rest of the group and has G+C content of 43–44%, is tentatively assigned to this species but might have to be reclassified in the near future. The ability of *C cocleatum*, *C. ramosum*, and *C. spiroforme* to form spores contradicts the current description of the family *Erysipelotrichaceae*, which is why the proposed genus *Erysipelatoclostridium* should either be placed in the order

Erysipelotrichales outside the family *Erysipelotrichaceae* or the description of the family be emended.

Protein-based and rRNA-based phylogeny *versus* cell wall structure

This study, in agreement with many earlier ones, demonstrated a high degree of coherence between 16S rRNA-based and protein-based trees for various members of the *Firmicutes*. While the congruity between 16S rRNA and ribosomal proteins S2–S20 that bind to this rRNA is hardly surprising, it must be noted that the concatenated alignment of 50 ribosomal proteins used in this work included 6164 unambiguously aligned positions, of which only 2367 (or 38%) were provided by 20 small subunit proteins, while the rest came from the large subunit of the ribosome (see the Supplementary Materials). Sequences of two other proteins, RpoB and GyrB (1154 and 631 positions, respectively) provided an additional, independent measure of the evolutionary proximity of the studied organisms.

In general, the results of this work reaffirm that protein sequences deduced from selected groups of informational genes, as defined by (Rivera *et al*., 1998), provide a valid tool for phylogenetic analysis of distant bacterial species, see, e.g. (Wolf *et al*., 2001, Ciccarelli *et al*., 2006, Gupta and Gao, 2009, Yutin *et al*., 2012) and could successfully complement 16S rRNA-based trees in building the ultimate genome-based classification of *Bacteria* and *Archaea* (Klenk and Göker, 2010). This conclusion becomes particularly important when the results of phylogenetic analyses contradict taxonomic assignments that are based on phenotypic traits, such as the structure (or even presence) of the bacterial cell wall, Gram-staining pattern, motility, or metabolic properties. Thus, despite the Gram-negative structure of their cellular envelopes, members of the family *Veillonellaceae* clearly belong within the *Clostridia*, and do not deserve placement into a separate class of *Firmicutes*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Boris Belitsky, Elizaveta Bonch-Osmolovskaya, Ilya Borovok, Jean Paul Euzéby, William B. Whitman, and Juergen Wiegel for critically reading the manuscript and many helpful comments and Drs. Yuri Wolf and Eugene Koonin for advice on the phylogenetic trees. This work was supported by the NIH Intramural Research Program at the National Library of Medicine.

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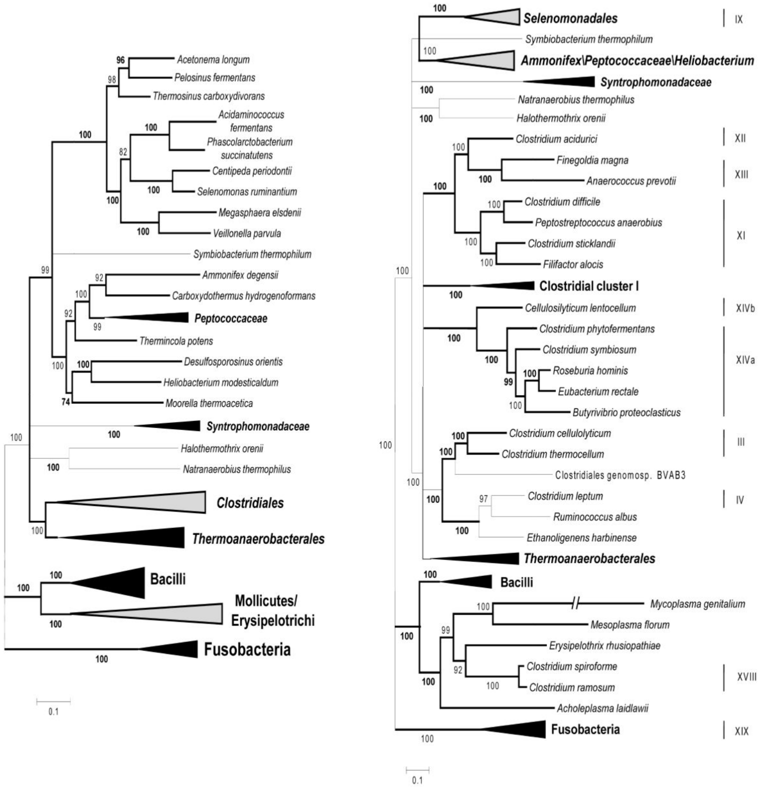

Figure 1.

A ribosomal proteins-based phylogenetic tree of the Firmicutes that shows the positions of (A) Gram-negative members of the *Firmicutes* and (B) mis-assigned *Clostridium* spp. The maximum-likelihood tree was built from a concatenated alignment of 50 ribosomal proteins from 70 organisms with a total of 6,164 unambiguously aligned positions, essentially as described in Yutin *et al*. (2012). The tree was rooted using two members of the *Fusobacteria*, *Fusobacterium nucleatum* and *Leptotrichia buccalis*, as an outgroup. The numbers on the branches show TreeFinder confidence values. Those branches shared with the RpoB tree are indicated with thick lines, bootstrap values of those branches that are shared with the GyrB tree are shown in bold. The Roman numerals on the right correspond to the clusters of Collins *et al*. (1994). Grey triangles indicate clusters that are shown in more details on the other panel (see Supplementary Materials for details, original trees, and Table S9 for the full list of organisms).

Table 1

Genome sequences of the Gram-negative members of the Firmicutes[a]

Organism name	Spore- former	Complete genomes[b]	Genome size, Mb[c]	Proteins[c]	GenBank accession number, reference[d]	Proposed family assignment
Acetonema longum	Yes	0 (1)	4.32	4,284	AFGF00000000	*Sporomusaceae*
Acidaminococcus fermentans	No	1	2.33	2,026	CP001859[d]	*Acidaminococcaceae*
Centipeda periodontii	No	0 (1)	2.72	2,631	AFHQ00000000	*Selenomonadaceae*
Megasphaera elsdenii	No	0 (1)	2.47	2,219	HE576794[d]	*Veillonellaceae*
Pelosinus fermentans						*Sporomusaceae*
strain A11	Yes	0 (1)	5.06	4,754	AKVM00000000[d]	
strain A12	Yes	0 (1)	4.85	5,138	AKVL00000000	
strain B3	Yes	0 (1)	4.88	5,140	AKVK00000000	
strain B4	Yes	0 (1)	5.04	4,691	AKVJ00000000[d]	
strain DSM 17108	Yes	0 (1)	4.93	4,593	AKVN00000000[d]	
strain JBW45	Yes	0 (1)	5.28	4,762	AKVO00000000[d]	
Phascolarctobacterium succinatutens	No	0 (1)	2.12	2,150	AEVN00000000	*Acidaminococcaceae*
Selenomonas ruminantium	No	1	3.63	3,512	AP012292	*Selenomonadaceae*
Thermosinus carboxydivorans	No	0 (1)	2.89	2,750	AAWL00000000	*Sporomusaceae*
Veillonella parvula	No	1	2.13	1,844	CP001820[d]	*Veillonellaceae*

[a] As of April 1st, 2013. Only those genomes used in the phylogenetic trees (Fig. 1, S1–S3) are listed here. The complete genome list is available as Table S1 and proposed family assignments as Table S6 in Supplementary Materials.

[b] The number in parentheses shows the number of unfinished genome projects that are registered with the NCBI's RefSeq database (Pruitt *et al*., 2012).

[c] Exact or rounded numbers for complete genomes, approximate numbers for draft genomes

[d] Some of these genomes have been described (Bowen De León *et al*., 2012; Brown *et al*., 2012; Chang *et al*., 2010; Gronow *et al*., 2010; Marx *et al*., 2011).

Table 2

Proposed genus assignments for former *Clostridium* spp.

Organism name[a]	Proposed family and genus name
Clostridium ramosum, *C. cocleatum*, *C. innocuum*, *C. saccharogumia*, *C. spiroforme*	*Erysipelotrichaceae*, *Erysipelatoclostridium*
Clostridium acidurici, *C. purinilyticum*[b], *Eubacterium angustum*[b]	Unassigned, *Gottschalkia*
Clostridium phytofermentans, *C. aerotolerans*, *C. aldenense*, *C. algidixylanolyticum*, *C. aminophilum*, *C. aminovalericum*, *C. amygdalinum*, *C. asparagiforme*, *C. bolteae*, *C. celerecrescens*, *C. citroniae*, *C. clostridioforme*, *C. fimetarium*, *C. glycyrrhizinilyticum*, *C. hathewayi*, *C. herbivorans*, *C. hylemonae*, *C. indolis*, *C. jejuense*, *C. lavalense*, *C. methoxybenzovorans*, *C. oroticum*, *C. polysaccharolyticum*, *C. populeti*, *C. saccharolyticum*, *C. scindens*, *C. sphenoides*, *C. symbiosum*, *C. xylanolyticum*, *C. xylanovorans*, *Desulfotomaculum guttoideum*, *Eubacterium contortum*, *Eubacterium fissicatena*	*Lachnospiraceae*, *Lachnoclostridium*
Clostridium difficile, *C. bartlettii*, *C. bifermentas*, *C. ghonii*, *C. glycolicum*, *C. hiranonis*, *C. irregulare*, *C. litorale*, *C. lituseburense*, *C. mangenotii*, *C. mayombei*, *C. paradoxum*, *C. sordelii*, *C. sticklandii*, *C. thermoalcaliphilum*, *Eubacterium tenue*, *Eubacterium yurii*	*Peptostreptococcaceae*, *Peptoclostridium*
Clostridium thermocellum, *C. aldrichii*, *C. alkalicellulosi*, *C. caenicola*, *C. cellobioparum*, *C. cellulolyticum*, *C. cellulosi*, *C. clariflavum*, *C. hungatei*, *C. josui*, *C. leptum*, *C. methylpentosum*, *C. papyrosolvens*, *C. sporosphaeroides*, *C. stercorarium*, *C. straminisolvens*, *C. sufflavum*, *C. termitidis*, *C. thermosuccinogenes*, *C. viride*, *Bacteroides cellulosolvens*, *Eubacterium siraeum*	*Ruminococcaceae*, *Ruminiclostridium*
Clostridium nexile [b], *C. colinum* [b], *C. lactatifermentans*, *C. neopropionicum* [b], *C. piliforme*[b], *C. propionicum* [b]	*Lachnospiraceae*, *Tyzzerella*

[a] The list includes only those *Clostridium* spp. that (i) have been validly described and listed in the List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.net/, Euzéby, 1997), (ii) do not belong to the family *Clostridiaceae*, and (iii) fall within the suggested new genera. Proposed type species are listed first and indicated in bold typeface.

[b] These species designations have been changed: *Gottschalkia purinilytica, Gottschalkia angusta,* ***Tyzzerella nexilis***, *Tyzzerella colina*, *Tyzzerella neopropionica, Tyzzerella piliformis, Tyzzerella propionica.*