INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, Oct. 1994, p. 812–826 Vol. 44, No. 4
0020-7713/94/$04.00+0

The Phylogeny of the Genus *Clostridium*: Proposal of Five New Genera and Eleven New Species Combinations

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The 16S rRNA gene sequences of 34 named and unnamed clostridial strains were determined by PCR direct sequencing and were compared with more than 80 previously determined clostridial sequences and the previously published sequences of representative species of other low- G+C-content gram-positive genera, thereby providing an almost complete picture of the genealogical interrelationships of the clostridia. The results of our phylogenetic analysis corroborate and extend previous findings in showing that the genus *Clostridium* is extremely heterogeneous, with many species phylogenetically intermixed with other spore-forming and non-spore-forming genera. The genus *Clostridium* is clearly in need of major revision, and the rRNA structures defined in this and previous studies may provide a sound basis for future taxonomic restructuring. The problems and different possibilities for restructuring are discussed in light of the phenotypic and phylogenetic data, and a possible hierarchical structure for the clostridia and their close relatives is presented. On the basis of phenotypic criteria and the results of phylogenetic analyses the following five new genera and 11 new combinations are proposed: *Caloramator* gen. nov., with *Caloramator fervidus* comb. nov.; *Filifactor* gen. nov., with *Filifactor villosus* comb. nov.; *Moorella* gen. nov., with *Moorella thermoacetica* comb. nov. and *Moorella thermoautotrophica* comb. nov.; *Oxobacter* gen. nov., with *Oxobacter pfennigii* comb. nov.; *Oxalophagus* gen. nov., with *Oxalophagus oxalicus* comb. nov.; *Eubacterium barkeri* comb. nov.; *Paenibacillus durum* comb. nov.; *Thermoanaerobacter kivui* comb. nov.; *Thermoanaerobacter thermocopriae* comb. nov.; and *Thermoanerobacterium thermosaccharolyticum* comb. nov.

Great advances have been made over the past decade in unravelling the phylogenetic complexities within the gram-positive endospore-forming bacteria. For example, 16S rRNA oligonucleotide cataloging and, more recently, almost complete rRNA (or gene) sequencing have shown that the aerobic endospore-forming bacilli are phylogenetically very heterogeneous, consisting of at least six highly divergent lines (1, 11, 51). As a result of these studies, taxonomic reorganization of the genus *Bacillus* was initiated with the introduction of the genus *Alicyclobacillus* for some acidophilic species (51) and the genus *Paenibacillus* (2) for *Bacillus polymyxa* and its close relatives (rRNA group 3 [1]). Although the remainder of the genus is still in need of taxonomic revision, the phylogenetic groups established by rRNA analysis are already forming the foundation for a new molecular data-based taxonomy for this group of organisms. Knowledge of the natural interrelationships within the anaerobic genus *Clostridium* is more fragmented than knowledge of the interrelationships among the aerobic bacilli. The earliest, and until recently the most comprehensive, phylogenetic study of the genus *Clostridium* was published by Johnson and Francis (22), who demonstrated that there is considerable diversity within the genus by using DNA-rRNA pairing methods but unfortunately did not include nonclostridial gram-positive reference organisms. It was only with the advent of oligonucleotide cataloging that the very considerable phylogenetic incoherence of the genus *Clostridium* was realized (15, 32, 46, 47). During the 1980s the oligonucleotide catalogs of more than 30 clostridia were determined (see reference 43 for a review), and members of the genus were shown to belong to several deeply branching lineages, some of which also include nonclostridial species.

Although 16S rRNA oligonucleotide cataloging has without question provided valuable insights into the phylogenetic interrelationships of the clostridia and their relatives, it is now recognized that full rRNA sequences provide far greater precision for constructing phylogenetic trees (52). If phylogenetic data are to be used as the basis for future taxonomic restructuring of the genus *Clostridium*, it is imperative that as many species as possible be sequenced and that the branching patterns of trees be resolved with confidence. In recent years there has been considerable progress toward this end, with complete (or nearly complete) 16S rRNA sequences available for more than 80 clostridial species (5, 19, 20, 27, 29, 30, 34, 35, 37, 38, 50). In this paper we describe the 16S rRNA gene sequences of an additional 34 clostridial strains and the results of a comparative sequence analysis and thereby provide an almost complete picture of the genealogical interrelationships in the genus. The results of our phylogenetic analysis are discussed in the context of a possible future taxonomic rearrangement of the genus.

MATERIALS AND METHODS

Cultures. With the exception of the *Clostridium tetani* strain (National Collection of Type Cultures strain NCTC 279) and the *Clostridium rectum* strain (National Collection of Industrial and Marine Bacteria strain NCIMB 10651^T [T = type strain]), all clostridial strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. Details concerning the strains which we examined are shown in Table 1, and the strains were cultivated as recommended in the culture collection catalogs.

16S rRNA gene sequence determination. Genomic DNA was

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TABLE 1. *Clostridium* strains examined and their 16S rRNA accession numbers

Species	Strain[a]	EMBL accession no.
C. absonum	DSM 599^T	X77842
C. aerotolerans	DSM 5434^T	X76163
"*C. aminobutyricum*"	DSM 2634	X76161
"*C. caliptrosporum*"	DSM 5905	X77843
C. celatum	DSM 1785^T	X77844
C. colinum	DSM 6011^T	X76748
"*C. corinoforum*"	DSM 5906	X76742
C. durum	DSM 1735^T	X77846
"*C. favososporum*"	DSM 5907	X76749
C. felsineum	DSM 794^T	X77851
"*C. filamentosum*"	DSM 6645	X77847
C. formicoaceticum	DSM 92^T	X77836
C. glycolicum	DSM 1288^T	X76750
C. halophilum	DSM 5387^T	X77837
C. hastiforme	DSM 5675^T	X77848
C. homopropionicum	DSM 5847^T	X76744
C. intestinalis	DSM 6191^T	X76740
"*C. kainantoi*"	DSM 523	X77834
C. lentocellum	DSM 5427^T	X76162
C. litorale	DSM 5388^T	X77845
"*C. longisporum*"	DSM 8431	X76164
C. magnum	DSM 2767^T	X77835
"*C. neopropionicum*"	DSM 3847^T	X76746
C. oxalicum	DSM 5503^T	X77840
C. pfennigii	DSM 3222^T	X77838
C. polysaccharolyticum	DSM 1801^T	X77839
C. propionicum	DSM 1682^T	X77841
"*C. quinii*"	DSM 6736^T	X76745
C. rectum	NCIMB 10651^T	X77850
Clostridium sp.	DSM 6877	X76747
Clostridium sp.	BN11	X75909
C. tetani	NCTC 279^T	X74770
"*C. thermoamylolyticum*"	DSM 2335	X76743
C. xylanolyticum	DSM 6555^T	X76739

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; NCIMB, National Collection of Industrial and Marine Bacteria; NCTC, National Collection of Type Cultures.

extracted from cells in the mid-logarithmic growth phase and was purified by the method of Lawson et al. (26). 16S rRNA gene fragments were generated by PCR as previously described (19). Amplified products were purified by using a Magic DNA Clean-up System (Promega) and were sequenced directly by using α-^{35}S-labeled dATP and a Sequenase version 2.0 sequencing kit (United States Biochemicals) (19).

Analysis of sequence data. The 16S rRNA gene sequences which we determined and the sequences of other clostridia and reference strains (obtained from the EMBL Data Library and the Ribosomal Database Project) were aligned by using programs in the Wisconsin Molecular Biology Package (7) and were analyzed by the distance matrix method of Fitch and Margoliash (13) and the neighbor-joining method of Saitou and Nei (39), using a VAX computer. The stability of relationships was assessed by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE of the PHYLIP package (12). A minimum of 1,000 bootstrap trees were generated for each data set.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences which we determined have been deposited in the EMBL Data Library under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

The almost complete 16S rRNA sequences of 34 strains of clostridia were determined by direct sequencing of PCR-amplified rRNA gene products. These new sequences were aligned and compared with homologous sequences of more than 200 other clostridia and selected reference strains belonging to low-G+C-content gram-positive genera available from EMBL and the Ribosomal Database Project. Because of incomplete sequence data for some reference strains, and also to eliminate possible errors due to the extremely variable V1 region, approximately 100 nucleotides at the 5′ end of the rRNA were omitted from the alignments. Levels of sequence similarity for an approximately 1,330-nucleotide region (ranging from position 101 to position 1410 in the *Escherichia coli* numbering system) were calculated, and derived distances were used to determine the phylogenetic interrelationships of clostridia and other low-G+C-content taxa. The significance of the branching order of trees was estimated by bootstrapping. A complete matrix of the levels of sequence similarity which we determined is available upon request from P. A. Lawson.

The results of the treeing programs confirmed and extended the results of previous comparative rRNA studies and demonstrated the marked phylogenetic incoherence of the genus *Clostridium*. From our analyses it was evident that many clostridia formed very deep, but nevertheless distinct, clusters (Fig. 1 and 2). Almost one-half of the clostridial species, including *Clostridium butyricum*, the type species of the genus, belonged to a phylogenetically well-defined cluster (designated cluster I). The detailed phylogenetic interrelationships within this cluster are shown in Fig. 1. The remaining clostridial species exhibited very considerable degrees of phylogenetic diversity and formed numerous clusters and individual lines of descent. Two species, *Clostridium durum* and *Clostridium oxalicum*, were found to be phylogenetically remotely related to other clostridial species and were recovered within the confines of a large supercluster which encompassed members of the genus *Bacillus* and their non-spore-forming relatives (e.g., the genera *Staphylococcus* and *Listeria*). Figure 2 is a tree which shows the full genealogical complexities of the clostridia and their close relatives. Brief descriptions of the taxonomic compositions of the various clusters and sublines are given below.

Cluster I. Cluster I is the largest of the clostridial groups and is equivalent to rRNA group I of Johnson and Francis (22). Of the 34 new strains sequenced, 13 were found to be members of this cluster. Several of these strains exhibited specific affinities with other species or species groups within cluster I. For example, *Clostridium absonum* was closely related to *Clostridium baratii*, while the invalid vesicular cap-forming species "*Clostridium caliptrosporum*" (strain DSM 5905), which was isolated from soil (24), was highly related to *Clostridium acetobutylicum* and *Clostridium beijerinckii* (Fig. 1). Two other vesicular cap-forming species, "*Clostridium corinoforum*" (strain DSM 5906) and "*Clostridium favososporum*" (strain DSM 5907), which were also isolated from soil (24), were found to be almost identical to each other (level of similarity, 99.9%) and were highly related to the pectinolytic amylolytic species *Clostridium puniceum* (level of similarity, 99.5%). Clearly, chromosomal DNA-DNA hybridization data will be necessary to determine whether the vesicular cap-forming organisms and their close relatives (*C. acetobutylicum*, *C. beijerinckii*, and *C. puniceum*) warrant separate species status. A similar problem of species identification was encountered with the bison rumen organism "*Clostridium longisporum*"

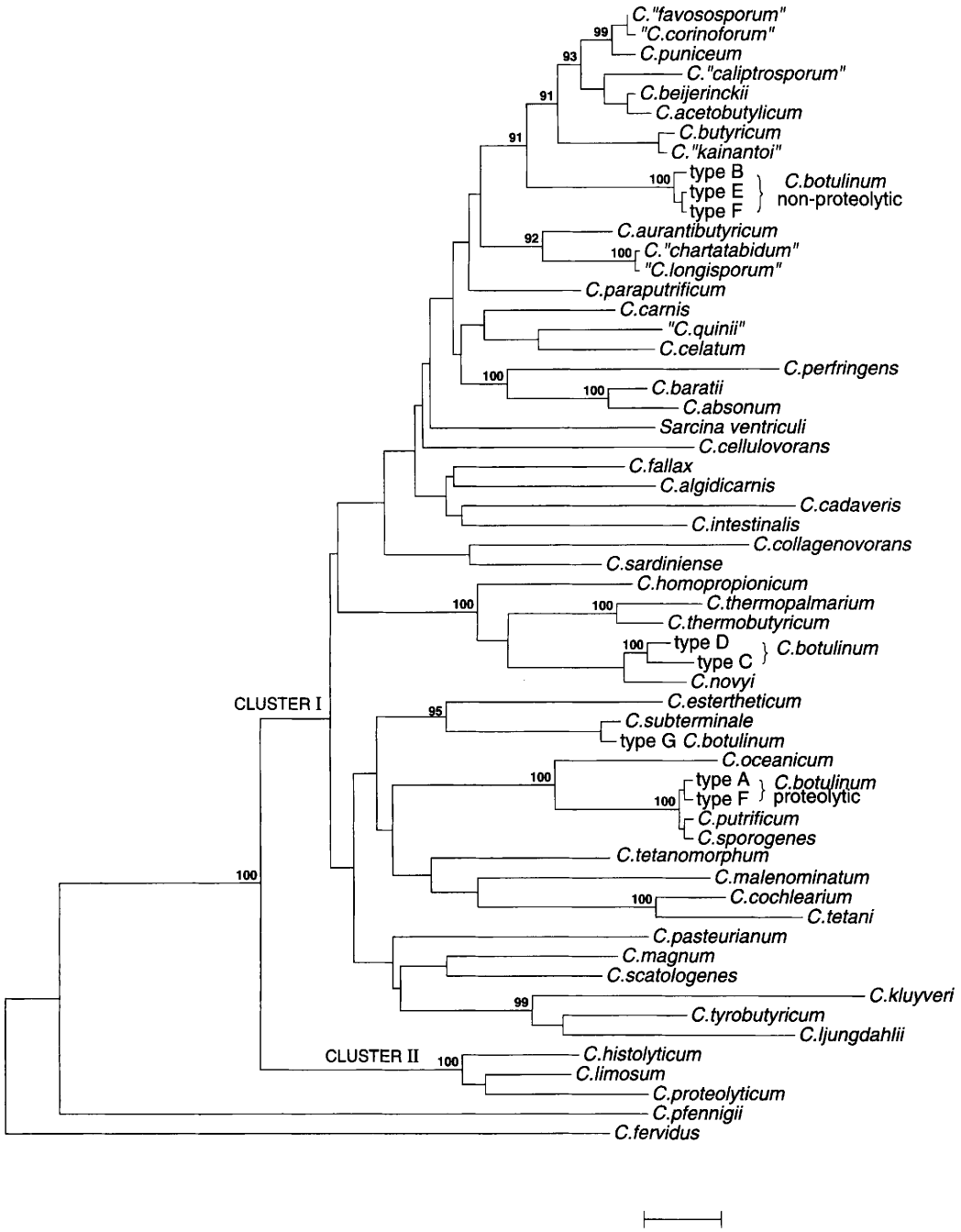

FIG. 1. Dendogram showing the interrelationships within cluster I (*Clostridium* group I) and closely related species. The tree was constructed by using the neighbor-joining method and bootstrap values calculated from 1,000 trees. Bar = 1% sequence divergence.

DSM 8431, which exhibited 99.9% 16S rRNA sequence similarity with the ovine rumen species "*Clostridium chartatabidum*" (23). *C. tetani* NCTC 279 was significantly related to *Clostridium cochlearium* (level of similarity, 97.5%). The recovery of *C. tetani* in cluster I was not expected as this species was placed in a quite separate group (rRNA homology group II; equivalent to cluster XI in this study) by Johnson and Francis (22). A second strain of *C. tetani* (NCTC 5404), which originally was isolated from a case of human tetanus, was also examined (unpublished data) and was found to be identical to the type strain, thereby confirming its placement in cluster I. *Clostridium homopropionicum* surprisingly exhibited high levels of sequence relatedness (>95%) with *Clostridium novyi*, *Clostridium botulinum* types C and D, and the thermophilic species *Clostridium thermobutyricum* and *Clostridium thermopalmarium*. The results of bootstrap calculations (bootstrap value, 100) reinforced the significance of this subgroup (Fig. 1). In a previous study Rainey et al. (38) demonstrated that *C. thermobutyricum* and *C. thermopalmarium* were members of *Clostridium* group I, although a close association with *C. botulinum* types C and D and their relatives was not reported by these authors. *Clostridium celatum*, *Clostridium magnum*,

FIG. 2. Dendrogram showing the phylogenetic interrelationships of non-group I *Clostridium* species. The tree was constructed by using the neighbor-joining method and bootstrap values calculated from 1,000 trees. Bar = 1% sequence divergence.

and *Clostridium intestinalis* also grouped in cluster I but exhibited only loose associations with other species (Fig. 1).

As shown in previous studies, *Clostridium* group I of Johnson and Francis (22) is a phylogenetically distinct group (3, 27, 37, 50). Members of this group exhibit relatively high levels of intracluster similarity (generally >90%) despite having markedly different phenotypes (for example, the group includes saccharolytic and proteolytic species, as well as psychrophiles, mesophiles, and thermophiles). In this study this group was found to be well separated from other clostridial clusters or lines irrespective of the treeing programs employed. Since group I contains the type species, *C. butyricum*, it is now generally accepted that the genus *Clostridium* should be retained for organisms belonging to this group.

Cluster II. As shown previously (27), cluster II is composed of three species (*Clostridium histolyticum*, *Clostridium limosum*, and *Clostridium proteolyticum*) and represents a distinct line at the periphery of cluster I. Species belonging to this group exhibit levels of sequence similarity of >96% with each other but are significantly less closely related (levels of similarity, generally <92%) to cluster I species. Phenotypically, *C. histolyticum*, *C. limosum*, and *C. proteolyticum* resemble each other in producing acetate as the major end product of metabolism and in being highly proteolytic. These species probably constitute a natural group worthy of generic status, although clear phenotypic separation from a redefined genus *Clostridium* (cluster I) may prove to be problematical.

Cluster III. Cluster III consists of eight cellulolytic species and corresponds to group E of Rainey et al. (38). The levels of intracluster similarity ranged from 87 to 99%. Although cluster III contains both mesophiles and thermophiles and the members of this cluster differ considerably in their chromosomal DNA base compositions (range, approximately 28 to 42 mol% G+C), this group was identified consistently irrespective of the type of analysis performed and was confirmed (37, 38) to be statistically significant (bootstrap value, 100).

Cluster IV. Consistent with the results of previous investigations (27), *Clostridium leptum* and *Clostridium sporosphaeroides* were found to be closely related (level of similarity, approximately 94%; bootstrap value, 100), with *Clostridium cellulosi* exhibiting a more peripheral association (level of similarity, approximately 89%; bootstrap value, 100). The members of cluster IV are phenotypically heterogeneous (e.g., this cluster includes both mesophiles and thermophiles) and exhibit a broad chromosomal DNA G+C content range (approximately 27 to 52 mol%) (18). However, the results of bootstrap calculations indicate that the relationship among these species is significant, and, in contrast to the results of a previous study (38), the thermophile *C. cellulosi* did not exhibit any rearrangement when transversion analysis was used (data not shown). In terms of sequence divergence cluster IV probably represents a suprageneric or family group.

Cluster V. Rainey et al. (38) showed that *Thermoanaerobacter* species, *Acetogenium kivui*, and *Clostridium thermocopriae* constitute a phylogenetically coherent group (designated group A). The results of our analysis confirmed these findings. Despite some phenotypic differences, high sequence similarity values (approximately 93 to 99%) and bootstrap calculations (bootstrap values, 100) clearly indicated that *A. kivui* and *C. thermocopriae* should be reclassified in the genus *Thermoanaerobacter* (38).

Cluster VI. Cluster VI consists of *Clostridium thermoaceticum* and *Clostridium thermoautotrophicum* and corresponds to group B of Rainey et al. (38). These homoacetogenic spore-forming species differ from other clostridia in their high DNA base compositions (approximately 53 to 55 mol% G+C) and in the presence of LL-diaminopimelic acid in their cell wall peptidoglycans and, as shown previously, form a distinct line worthy of genus status (38). *Desulfotomaculum australicum* was peripherally associated with this cluster.

Cluster VII. Cluster VII is equivalent to group C of Rainey et al. (38) and corresponds to the genus *Thermoanaerobacterium*. As shown by Rainey et al. (38), *Clostridium thermosaccharolyticum* is also a member of this group and is clearly misclassified. The invalid species "*Clostridium thermoamylolyticum*" (strain DSM 2335) exhibited approximately 98 to 99% sequence relatedness with *Thermoanaerobacterium* species. In view of these very high levels of sequence similarity, chromosomal DNA-DNA hybridization data will be necessary to determine whether this starch-degrading organism, which is able to grow at pH 3.5, represents a distinct species within the genus *Thermoanaerobacterium*.

Cluster VIII. The fatty acid β-oxidizing species *Syntrophomonas wolfei* and *Syntrophospora bryantii* constitute cluster VIII. The genera *Syntrophomonas* and *Syntrophospora* have recently been assigned to a new family, the *Syntrophomonadaceae* (53). However, the level of evolutionary divergence (approximately 6%) between the two species seems to be more consistent with classification as members of a genus than with classification as separate genera in a family.

Cluster IX. Cluster IX corresponds to the *Sporomusa* branch of the *Clostridium* phylum and, as shown previously (8, 27), includes a heterogeneous collection of spore-forming and non-spore-forming organisms, many of which are gram negative. Although this cluster exhibits considerable internal structure and overall depth (range of intracluster similarity values, approximately 80 to 99%), bootstrap calculations (bootstrap value, 99) indicate that it is a phylogenetically significant group at the suprageneric level. Within this cluster, the taxa *Sporomusa*, *Acidaminococcus*, *Pectinatus*, *Selenomonas*, *Megasphaera*, *Quinella*, and *Phascolarctobacterium* are distinct genera. In contrast to the results of a previous study and despite a significantly lower reported chromosomal DNA base composition (41), *Zymophilus paucivorans* was found to be phylogenetically intermixed with *Selenomonas* species (Fig. 2). The phylogenetic separateness of the genera *Selenomonas* and *Zymophilus* clearly merits further investigation. Consistent with previous studies, *Clostridium quercicolum* exhibited a specific association with the genus *Sporomusa* (level of similarity, approximately 89 to 90%). In terms of evolutionary divergence, *C. quercicolum* is as distant from the genus *Sporomusa* as, for example, the genera *Phascolarctobacterium* and *Acidaminococcus* are from each other (8) and should therefore probably be placed in a new genus.

Cluster X. Cluster X corresponds to group D of Rainey et al. (38) and consists of several highly saccharolytic spore-forming thermophiles ("*Anaerocellum thermophilum*," "*Caldocellum saccharolyticum*," "*Thermoanerobacter cellulolyticus*"), none of which have been validly described. High intracluster similarity values (>95%) and bootstrap calculations (bootstrap value, 100) support the view that this group represents a distinct genus.

Cluster XI. Cluster XI, which includes *Clostridium lituseburense* and its relatives, is equivalent to rRNA homology group II-A of Johnson and Francis (22). Five new sequences were recovered within the confines of this group. *Clostridium glycolicum* was found to be genetically highly related to *Clostridium mayombei* (level of similarity, >99%), whereas *Clostridium felsineum* was found to be closely related to *Clostridium formicoaceticum* (level of similarity, approximately 97%). The remaining two species, "*Clostridium aminobutyricum*" and *Clostridium halophilum*, formed relatively distinct lines. Cluster

XI is taxonomically heterogeneous and, as noted previously (27), includes the non-spore-forming species *Peptostreptococcus anaerobius* and *Eubacterium tenue*. Despite the marked range of phenotypes exhibited by members of cluster XI, the group is phylogenetically well defined (bootstrap significance, 100). The relatively broad range of intracluster similarity values (approximately 85 to 99%) indicates that cluster XI represents a suprageneric, possibly family, group. *C. litusebu-rense* and several other species (*Clostridium bifermentans*, *Clostridium difficile*, *C. glycolicum*, *Clostridium ghonii*, *Clostridium irregularis*, *C. mayombei*, *Clostridium mangenotii*, *Clostridium sordellii*, *Eubacterium tenue*) form a distinct subgroup (levels of similarity, >93%; bootstrap value, 100) within cluster XI, as do *C. felsineum* and *C. formicoaceticum* and the recently described thermotolerant alkaliphiles *Clostridium paradoxum* and *Clostridium thermoalcaliphilum* (29, 30). These three subclusters clearly represent new genera. All of the remaining cluster XI species form relatively distinct sublines exhibiting no specific associations.

Cluster XII. Cluster XII is phenotypically incoherent and is a loose association of four clostridial species and an unnamed non-spore-forming bacterium (strain BN11) that was isolated from a wastewater pond of a sugar refinery and is used as a source of a potent creatinine deiminase (16). Within this cluster, *Clostridium acidiurici* and *Clostridium purinolyticum* were specifically related (level of similarity, approximately 94%; bootstrap value, 100) and probably belong to a single genus. The recovery of *Clostridium hastiforme* in cluster XII was completely unexpected. Although *C. hastiforme* has been shown to be distinct from *C. botulinum* type G and *Clostridium subterminale* on the basis of DNA-DNA pairing results (45), because of the phenotypic resemblance of these organisms it has generally been assumed that the relationship among them may be similar to the relationship which exists between *C. botulinum* group I and *Clostridium sporogenes*. However, *C. botulinum* type G and *C. subterminale* form a distinct subline within cluster I and are phylogenetically remote from *C. hastiforme*. Even more remarkable was the highly significant association (level of similarity, approximately 95%) between *C. hastiforme* and the unidentified *Clostridium* sp. (strain BN11). The latter organism contains a cell wall based on ornithine (type A4 β) and does not produce spores! The remaining species in cluster XII, "*Clostridium filamentosum*," exhibited only a loose association with other organisms and clearly constitutes a distinct subline.

Cluster XIII. As shown by Ezaki and colleagues (10) *Peptostreptococcus asaccharolyticus*, "*Peptostreptococcus hydrogenalis*," "*Peptostreptococcus lacrimalis*," *Peptostreptococcus micros*, *Peptostreptococcus prevotii*, and *Peptostreptococcus tetradius* form a natural group. The results of our study indicate that *Helcococcus kunzii* and possibly *Clostridium hydroxybenzoicum* are peripherally associated with this group. "*Peptostreptococcus hydrogenalis*," *Peptostreptococcus prevotii*, and *Peptostreptococcus tetradius* are specifically related to each other (levels of similarity, >92%), whereas the remaining peptostreptococcal species and *H. kunzii* form relatively long individual lines (levels of similarity, generally <87%) and probably constitute separate genera. It seems likely that this natural grouping of peptostreptococci and *H. kunzii* is at a suprageneric, possibly family, level.

Cluster XIV. Cluster XIV comprised more than 20 clostridial species, "*Acetitomaculum ruminis*," "*Epulopiscium*" sp., *Ruminococcus torques*, *Coprococcus eutactus*, *Eubacterium cellulolyticum*, *Roseburia cecicola*, and *Streptococcus hansenii* and exhibited very considerable phylogenetic depth (range of intracluster similarity values, approximately 80 to 99%). With the exception of *Clostridium aminovalericum* (33 mol% G+C) and *Clostridium populeti* (28 mol% G+C) cluster XIV species are generally characterized by high G+C contents (approximately 38 to 52 mol%).

Considerable internal structure was present within cluster XIV, and two major subgroups (designated subclusters XIVa and XIVb) were readily discernable. Subcluster XIVa consisted of a phenotypically heterogeneous collection of organisms, including several non-spore-forming cocci (e.g., coprococci and ruminococci). Within subcluster XIVa, a biochemically unusual *N*-methylhydantoin-degrading mesophilic spore former (strain FS41 [= DSM 6877]) isolated from sewage sludge (17) was found to be specifically related to *Clostridium clostridiiforme*. This unnamed strain (17) phenotypically resembles *C. clostridiiforme*, but the observed level of sequence divergence (approximately 2.5%) indicates that this organism represents a new species. Overall, the diverse phenotypes exhibited by members of subcluster XIVa, together with the wide range of observed evolutionary distances, indicate that this cluster is probably a suprageneric cluster. In this context, we believe that the recent assignment of *Peptostreptococcus productus* and *Streptococcus hansenii* to the genus *Ruminococcus* by Ezaki et al. (10) may prove to be premature. Several clostridial species (e.g., *Clostridium oroticum*) are more closely related to the ruminococcal species examined by Ezaki et al. (10) than *Peptostreptococcus productus* and *Streptococcus hansenii* are. The phylogenetic placement of *Ruminococcus flavefaciens*, the type species of the genus *Ruminococcus*, clearly needs to be resolved before taxonomic proposals for species of this subgroup can be made with confidence.

Subcluster XIVb consisted of a loose association of six species. Within this subcluster *Clostridium propionicum* was highly related to "*Clostridium neopropionicum*" (level of similarity, approximately 98%), whereas the cellulolytic species *Clostridium lentocellum* exhibited a specific relationship with the "giant" nonculturable bacterium *Epulopiscium* sp. (level of similarity, >90%). Similarly, *Clostridium colinum*, which is associated with ulcerative enteritis of chickens, exhibited a highly significant affinity (level of similarity, approximately 95%; bootstrap value, 100) with *Clostridium piliforme*, the noncultured causative agent of Tyzzer's disease (9). It is probable that these three sublines represent distinct genera.

Cluster XV. In agreement with previous studies (27, 51), *Clostridium barkeri* and *Eubacterium limosum* were found to be highly related on the basis of 16S rRNA analysis data (level of similarity, approximately 95%). Oligonucleotide cataloging data also indicated that the acetogen *Acetobacterium woodii* is a member of this group (47). Members of cluster XV possess an unusual type B cell wall peptidoglycan (47), have relatively high chromosomal DNA base compositions (approximately 38 to 45 mol% G+C), and clearly should be classified in a single genus.

Cluster XVI. Cluster XVI is a loose, albeit phylogenetically significant, association of three species (*Clostridium innocuum*, *Eubacterium biforme*, and *Streptococcus pleomorphus*) within the mycoplasma supercluster. Members of this cluster are incoherent with respect to cellular morphology, spore formation, and DNA base composition (32 to 44 mol% G+C). The relatively wide range of intracluster similarity values (approximately 88 to 93%) indicates that the rank of this group is at a suprageneric, possibly family, level. *C. innocuum* is the most peripheral member of the group, and its level of sequence divergence (approximately 11 to 12%) compared with the other two species indicates that it is a member of a separate genus. In contrast, the species *Eubacterium biforme* and *Streptococcus pleomorphus* are more highly related to each other

(level of divergence, approximately 7%), although whether these taxa represent a single genus or two closely related genera is currently unclear.

Cluster XVII. *Lactobacillus catenaformis* and *Lactobacillus vitulinus* clustered together, in accordance with the data of Weisburg et al. (48), and are phylogenetically far removed from the *Lactobacillales*. Although these organisms are undoubtedly related, the level of sequence divergence (approximately 10%) is probably more consistent with classification as members of two closely related genera than classification as members of a single genus. *L. catenaformis* and *L. vitulinus* exhibited a loose, albeit significant, association with the species *Clostridium spiroforme* and *Clostridium ramosum*.

Cluster XVIII. As reported previously (27) *C. spiroforme* and *C. ramosum* belong to a natural group within a supercluster which includes mycoplasmas and their relatives. The very high level of sequence relatedness of *C. spiroforme* and *C. ramosum* (level of divergence, <4%), together with their phenotypic resemblance and similar DNA base compositions (26 to 27 mol% G+C), indicates that these species should be reclassified in a single genus.

Cluster XIX. *Propionigenium modestum* and *C. rectum* form a distinct cluster with members of the genus *Fusobacterium*. The genus *Propionigenium* was described by Schink and Pfenning (40) to accomodate a novel gram-negative, non-spore-forming, propionate-producing, strictly anaerobic bacterium. Although this genus was originally assigned to the family *Bacteroidaceae* (40), the phylogenetic analysis clearly placed this taxon proximal to the periphery of the genus *Fusobacterium* in the *Clostridium* subphylum of the gram-positive bacteria. Evolutionary distance data indicate that the relationship between *Propionigenium modestum* and fusobacteria is at a family level rather than a genus level. The association of *C. rectum* with the genus *Fusobacterium* was unexpected, as *C. rectum* produces endospores. Despite this major phenotypic difference, *C. rectum* clusters well within the boundaries of the genus and exhibits a particularly close affinity with *Fusobacterium necrogenes* (level of sequence similarity, approximately 99%). On the basis of phylogenetic findings *C. rectum* should be reclassified in the genus *Fusobacterium*, and the definition of the latter taxon should be emended to take into account the possibility of spore formation.

Individual lines. **(i) *Clostridium pfennigii*.** Bootstrap data indicate that *C. pfennigii* exhibits no significant affinity with any other species or group and belongs to a relatively long and isolated line. *C. pfennigii* exhibits some metabolic similarities to *Acetobacterium woodii* (25), but *Acetobacterium woodii* does not produce endospores and has a type B cell wall murein, and oligonucleotide cataloging (46, 47) has shown that it is phylogenetically closely related to *C. barkeri* and *Eubacterium limosum* (Fig. 2, cluster XV). In view of its isolated phylogenetic position and unique metabolism (it catabolyzes methoxybenzenoids to corresponding hydroxybenzenoids and butyrate, pyruvate to acetate and CO_2, and CO to acetate and butyrate), *C. pfennigii* clearly merits a new genus.

(ii) *Clostridium fervidus*. As previously reported by Rainey et al. (38), *C. fervidus* exhibits a loose association with some other thermophilic species groups when normal evolutionary distances are used (clusters V to VII), while transversion analysis data (Fig. 2) indicate a peripheral association with cluster I and its relatives. However, despite some uncertainty with respect to these higher associations, *C. fervidus* clearly forms a distinct line at the genus level.

(iii) *Clostridium durum*. *C. durum* differs from most other clostridia in having a relatively high chromosomal DNA base composition (50 mol% G+C) (42). Although *C. durum* is aerotolerant, this species was assigned to the genus *Clostridium* because it sporulates under anaerobic conditions (sporulation under aerobic conditions is a characteristic of the genus *Bacillus*) (42). It is evident from our data that *C. durum* is only remotely related to other clostridia, is phylogenetically a member of the genus *Paenibacillus* (rRNA group 3 bacilli [1, 2]), and is specifically related to *Paenibacillus azotofixans* (level of similarity, approximately 98%).

(iv) *Clostridium oxalicum*. Although *C. oxalicum* is strictly anaerobic, the treeing programs showed that this organism is only remotely related to clostridia and consistently placed it within the boundaries of the supercluster encompassing the genus *Bacillus* and its aerobic and facultatively anaerobic relatives (Fig. 2). It is evident from both distance calculations (level of sequence similarity, generally <87%) and tree branching patterns that *C. oxalicum* represents a previously undescribed lineage. Phenotypically, *C. oxalicum* differs from all other spore-forming taxa in utilizing oxalate as a sole energy source and in its inability to utilize other organic acids, sugars, or alcohols (6). The highly unusual metabolism of *C. oxalicum*, together with its isolated phylogenetic position, indicates that this organism merits a new genus.

Taxonomic considerations. The genus *Clostridium* includes organisms which are obligately anaerobic, form endospores, are not able to carry out dissimilatory sulfate reduction, and possess a gram-positive type of wall structure. As a result of this simple definition, the genus *Clostridium* has become one of the largest genera of bacteria and presently contains more than 100 species. Although it has long been recognized that the genus is phenotypically heterogeneous, it is only with the advent of oligonucleotide cataloging (see reference 3 for a review of the early literature) and, more recently, 16S rRNA sequencing (27, 37, 38) that the full phylogenetic diversity of the clostridia has become apparent. A remarkable result that has emerged from such studies is the phylogenetic intermixing of some clostridia with non-spore-forming organisms, challenging our perception of spore formation as an important criterion for determining relatedness. It is now clear that any future taxonomic rearrangement of the clostridia must include their non-spore-forming relatives and take into account phylogenetic relatedness.

Knowledge of the interrelationships of the genus *Clostridium* and its relatives is increasing so rapidly that a naturally based classification may be realized in the next few years. Considerable progress toward this goal would be made if the genus *Clostridium* were restricted to homology group I of Johnson and Francis (22). Despite considerable phenotypic diversity, numerous studies have shown that this group is phylogenetically distinct, and it is now generally recognized that homology group I should form the basis of a redefined genus *Clostridium* (50). However, the placement of *Sarcina ventriculi*, the type species of the genus *Sarcina*, in this group raises a serious nomenclatural problem, as the name *Sarcina* has priority (50). Even if the rules of nomenclatural priority were set aside, the inclusion of sporulating cocci in the genus *Clostridium* could present a major hurdle to deriving a phenotypically tight genus delineation. Restriction of the genus *Clostridium* to group I organisms would result in the remaining clostridial species losing their status as members of the genus, and they would require reclassification.

The results of previous studies and our analysis indicate that the non-group I clostridia represent a phylogenetically very broad range of organisms and form a plethora of lines and groups of various complexities and depths. Numerous phylogenetically significant associations can be discerned at higher (suprageneric) levels, indicating that a hierarchical system for

these organisms may now be achievable. Fox et al. (15) outlined a possible hierarchy for gram-positive bacteria which was subsequently updated by Cato and Stackebrandt (3). An amended hierarchical structure for the clostridia and their relatives in light of current knowledge is shown in Table 2.

It now seems clear that any comprehensive taxonomic rearrangement of clostridia will result in a major proliferation of new genera. It is important to emphasize that this will not be due to overzealous taxonomic splitting but will be a consequence of the immense genealogical diversity of these organisms, which has become apparent only since molecular sequence data have been available. Some clostridial species form phylogenetically distinct groups with sufficient phenotypic coherence that they can be readily equated with genera. However, many other species form individual lines or sublines and exhibit only loose associations with other organisms. The taxonomic rank of many of these organisms is far more problematic. Although it is not possible to derive precise boundary conditions based on percentage 16S rRNA sequence relatedness to delineate taxonomic rank, as an approximation most generic groupings exhibit a sequence divergence of <6%. When this guideline is used in conjunction with tree topology considerations (including confidence values), it seems likely that many of the lines and sublines will represent the nuclei of previously unrecognized genera. The considerable genealogical complexities in the suprageneric association designated cluster XI in this study are a case in point. This cluster contains a phylogenetically and phenotypically diverse assemblage of organisms. Within this cluster *C. lituseburense* and related species and two pairs of organisms, *C. felsineum* plus *C. formicoaceticum* and *C. paradoxum* plus *C. thermoalcaliphilum*, form three phylogenetically significant subgroups worthy of separate generic status. The evolutionary distances exhibited by most other members of cluster XI (e.g., *Peptostreptococcus anaerobius*, *C. villosum*, and *C. aminobutyricum*) are such that they too could be designated distinct genera with reasonable confidence. Not only is such a subdivision phylogenetically justifiable, but it would also reduce many phylogenetic and phenotypic inconsistencies. Subdivision into more manageable units would simplify the search for suitable characteristics to allow clear phenotypic circumscription and differentiation of new genera, which is recognized as a major obstacle (18, 27) in constructing a naturally based classification of the clostridia. Table 2 shows the lines and sublines which in our opinion represent new genera. This scheme will undoubtedly be modified and expanded as other clostridia and related organisms (e.g., the genus *Eubacterium*) are characterized. However, we are confident that this compilation of relationships established from the results of this and previous phylogenetic analyses will provide a firm basis for a comprehensive taxonomic restructuring of the clostridia and their low-G+C-content relatives in the near future.

It is not the purpose of this paper to formally propose a major taxonomic revision of the clostridia. However, some clostridial species are phylogenetically so distinct that they merit reclassification as separate genera, whereas others should be assigned to existing genera. Details of our proposals are given below.

Taxonomic proposals. (i) Description of *Oxobacter* gen. nov. *Oxobacter* (Ok.so.bac'ter. Gr. n. *oxos*, vinegar; Gr. hyp. masc. n. *bakter*, rod; M. L. Masc. n. *Oxobacter*, acetogenic rod). Cells are gram positive and rod shaped. Oval, subterminal to terminal spores are produced. Obligately anaerobic. Pyruvate is catabolized to acetate and CO_2, CO is catabolized to acetate and butyrate, and methoxybenzenoids are catabolized to butyrate and hydroxybenzenoids. Other substrates, such as sugars, amino acids, organic acids, and alcohols, are not utilized as energy sources. The G+C content of the DNA is 38 mol%. The type species is *Oxobacter pfennigii.*

(ii) Description of *Oxobacter pfennigii* (Krumholz and Bryant) comb. nov. The description of *Oxobacter pfennigii* comb. nov. is identical to that proposed for *C. pfennigii* Krumholz and Bryant 1985, 455^{AL} (25). The type strain is DSM 3222.

(iii) Description of *Oxalophagus* gen. nov. *Oxalophagus* (Ok. sa.lo'pha.gus. Gr. n. *oxalis*, wood sorrel [from which the name of oxalic acid is derived]; Gr. masc. n. *phagos*, glutton; N. L. masc. n. *Oxalophagus*, oxalate eater). Cells are gram-positive, straight rods. Endospores are formed. The spores are oval and are located subterminally to centrally. Strictly anaerobic. Catalase negative; cytochromes are not produced. Oxalate and oxamate are decarboxylated to formate. Acetate is assimilated for cell carbon synthesis; no growth occurs with other organic acids, sugars, or alcohols. The G+C content of the DNA is 35.4 to 37.2 mol%. The type species is *Oxalophagus oxalicus.*

(iv) Description of *Oxalophagus oxalicus* (Dehning and Schink) comb. nov. The description of *Oxalophagus oxalicus* comb. nov. is identical to that proposed for *C. oxalicum* Dehning and Schink 1989, 83^{AL} (6). The type strain is DSM 5503.

(v) Description of *Caloramator* gen. nov. *Caloramator* (Ca.lo ra.ma'tor. L. n. *calor*, heat; L. masc. n. *amator*, lover; N. L. masc. n. *caloramator*, heat lover). Cells are rod shaped and stain gram negative but are gram positive in wall structure. Endospores are produced; the endospores are spherical and subterminal to terminal and do not swell the sporangia. Obligately anaerobic. Glucose and some other sugars are fermented. The fermentation products from glucose are acetate, isobutyrate, isovalerate, valerate, lactate, and ethanol. Xylan is degraded. Cellulose is not degraded. Thermophilic (temperature range, approximately 37 to 80°C). Unable to grow autotrophically or to produce sulfur from thiosulfate. The G+C content of the DNA is 37 mol%. The type species is *Caloramator fervidus.*

(vi) Description of *Caloramator fervidus* (Patel, Monk, Littleworth, Morgan, and Daniel) comb. nov. The description of *Caloramator fervidus* comb. nov. is identical to that proposed for *C. fervidus* Patel, Monk, Littleworth, Morgan, and Daniel 1987, 125^{AL} (36). The type strain is ATCC 43204.

(vii) Description of *Filifactor* gen. nov. *Filifactor* (Fi.li.fac'tor. L. n. *filum*, thread; L. masc. n. *factor*, maker; N. L. masc. n. *Filifactor*, thread maker). Cells are long rods. Filaments may be formed. Cells in young cultures are gram positive; the Gram reaction is variable in old cultures. Spores are oval and subterminal. The products of fermentation are acetate, butyrate, isobutyrate, formate, and isovalerate. Pyruvate is converted to butyrate. The cell walls contain an ornithine-D-asparagine-type murein. The type species is *Filifactor villosus.*

(viii) Description of *Filifactor villosus* (Love, Jones, and Bailey) comb. nov. The description of *Filifactor villosus* comb. nov. is identical to that proposed for *C. villosum* Love, Jones, and Bailey 1979, 241^{AL} (31). The type strain is NCTC 11220.

(ix) Description of *Moorella* gen. nov. *Moorella* (Moo. rel'la. N. L. fem. n. *Moorella*, in honor of W. E. C. Moore, an American bacteriologist). Cells are rod shaped and occur singly, in pairs, or in chains. Cells are generally gram positive, although older cultures may stain gram negative. Spores are round to slightly oval and terminal or subterminal and distend the cells. Thermophilic. The optimum temperature for growth is 56 to 60°C, and the maximum temperature for growth is 65 to 68°C. Homoacetogenic, growing chemolithotrophically with H_2 and CO_2 or CO, as well as chemoorganotrophically with some carbohydrates or methanol, producing acetate. Produces

TABLE 2. Possible hierarchical structure for clostridia and their close relatives

Family 1: *Clostridiaceae*
- Genus 1: Redefined genus *Clostridium* based on *C. butyricum* and its relatives (equivalent to group I of Johnson and Francis), including the genus *Sarcina*
- Genus 2: *C. histolyticum*, *C. limosum* and *C. proteolyticum*
- Genus 3: *C. pfennigii*
- Genus 4: *C. fervidus*[a]

Family 2: *Thermoanaerobacteriaceae*
- Genus 1: *Thermoanaerobacter*
- Genus 2: *Thermoanaerobacterium*
- Genus 3: *C. thermoaceticum* and *C. thermoautotrophicum*
- Genus 4: *Desulfotomaculum australicum*

Family 3
- Genus 1: *C. leptum* and *C. sporosphaeroides*[b]
- Genus 2: *C. cellulosi*

Family 4
- Genus 1: *C. stercorarium* and *C. thermolacticum*
- Genus 2: *C. cellobioparum*, *C. cellulolyticum*, *C. papyrosolvens*, *C. termitidis*, *C. aldrichii*[c], and *C. thermocellum*[c]

Family 5: *Syntrophomonadaceae*
- Genus 1: *Syntrophomonas*
- Genus 2: *Syntrophospora*[d]

Family 6: *Selenomonadaceae*
- Genus 1: *Selenomonas*
- Genus 2: *Acidaminococcus*
- Genus 3: *Megasphaera*
- Genus 4: *Pectinatus*
- Genus 5: *Phascolarctobacterium*
- Genus 6: *Quinella*
- Genus 7: *Sporomusa*
- Genus 8: *C. quercicolum*
- Genus 9: *Zymophilus*[e]

Family 7
- Genus 1: "*Anaerocellum thermophilum*," "*Caldocellum saccharolyticum*," and "*Thermoanaerobacter cellulolyticus*"

Family 8: *Ruminococcaceae*
- Genus 1: *Ruminococcus*
- Genus 2: *Coprococcus*
- Genus 3: *Roseburia*
- Genus 4: "*Acetitomaculum*"
- Genus 5: *C. coccoides*, *Peptostreptococcus productus*, and *Streptococcus hansenii*
- Genus 6: *Lachnospira*
- Genus 7: *C. aminophilum*
- Uncertain status: *C. aerotolerans*,[f] *C. aminovalericum*, *C. celerecrescens*, *C. populeti*, *C. polysaccharolyticum*, *C. sphenoides*, *C. xylanolyticum*,[f] *C. clostridiiforme*, *C. nexile*, *C. symbiosum*, *Clostridium* sp. strain DSM 6877, and *Eubacterium cellulosolvens*

Family 9: *Fusobacteriaceae*
- Genus 1: *Fusobacterium* (including *C. rectum*)[h]
- Genus 2: *Propionigenium*
- Genus 3: *Leptotrichia*
- Genus 4: *Sebaldella*

Family 10
- Genus 1: "*Epulopiscium*" spp.
- Genus 2: *C. lentocellum*
- Genus 3: *C. propionicum* and "*C. neopropionicum*"
- Genus 4: *C. colinum* and *C. piliforme*

Family 11: *Eubacteriaceae*
- Genus 1: *Eubacterium* (*Eubacterium limosum*) and *C. barkeri*

Family 12
- Genus 1: *C. acidiurici* and *C. purinolyticum*
- Genus 2: *C. hastiforme* and *Clostridium*. sp. strain BN11
- Genus 3: "*C. filamentosum*"

Family 13
- Genus 1: *Peptostreptococcus* (*Peptostreptococcus anaerobius*)
- Genus 2: *C. bifermentans*, *C. difficile*, *C. irregularis*, *C. ghonii*, *C. glycolicum*, *C. lituseburense*, *C. mayombei*, *C. mangenotii*, *C. sordellii*, and *Eubacterium tenue*
- Genus 3: *C. paradoxum* and *C. thermoalcaliphilum*
- Genus 4: *C. sticklandii*
- Genus 5: *C. villosum*
- Genus 6: "*C. aminobutyricum*"
- Genus 7: *C. formicoaceticum* and *C. felsineum*
- Genus 8: *C. halophilum*
- Genus 9: *C. litorale*

Continued on following page

TABLE 2—*Continued*

Family 14: *Helcococcaceae*
Genus 1: *Helcococcus*
Genus 2: *Peptostreptococcus micros*
Genus 3: *Peptostreptococcus tetradius*, *Peptostreptococcus prevotii*, *Peptostreptococcus vaginalis*, *Peptostreptococcus lactolyticus*, and "*Peptostreptococcus hydrogenalis*"
Uncertain status: *Peptostreptococcus asaccharolyticus*, "*Peptostreptococcus lacrimalis*", *Peptostreptococcus magnus*, and *C. hydroxybenzoicum*
Distinct genera of uncertain higher affiliation
Genus 1: *Desulfotomaculum* (*Desulfotomaculum nigrificans* and *Desulfotomaculum ruminis*)
Genus 2: *Heliobacterium*
Genus 3: *Peptococcus* (*Peptococcus niger*)
Clostridia and relatives which are associated with members of the *Mycoplasmatales*
Genus 1: *C. spiroforme* and *C. ramosum*
Genus 2: *L. catenaformis*[g] and *L. vitulinus*[g]
Genus 3: *Eubacterium biforme* and *Streptococcus pleomorphus*
Genus 4: *C. innocuum*
Genus 5: *Erysipelothrix*

[a] The position of *C. fervidus* is uncertain. When normal distances are used, this species is associated with some thermophilic species, while transversion analysis data indicate that it is peripherally associated with the group I clostridia and their relatives.
[b] It is not known whether these species are members of two closely related genera or a single genus.
[c] The significance of the association of these organisms with other species is unclear.
[d] The small evolutionary distance between the genera *Syntrophomonas* and *Syntrophospora* indicates that these taxa may represent a single genus.
[e] Whether this taxon can be separated from the genus *Selenomonas* is unclear.
[f] These organisms have almost identical sequences and may be members of the same species.
[g] These organisms are loosely associated with each other and may belong to separate genera.
[h] Although *C. rectum* forms endospores, it is clearly a member of the genus *Fusobacterium*.

a variety of electron carriers, including cytochrome *b* and menaquinone MK-7. The cell walls contain LL-diaminopimelic acid and glycine. The G+C content of the DNA is 53 to 55 mol%. The type species is *Moorella thermoacetica*.

(x) Description of *Moorella thermoacetica* (Fontaine, Peterson, McCoy, and Johnson) comb. nov. The description of *Moorella thermoacetica* comb. nov. is identical to that proposed for *C. thermoaceticum* Fontaine, Peterson, McCoy, and Johnson 1942, 707[AL] (14). The type strain is DSM 521.

(xi) Description of *Moorella thermoautotrophica* (Wiegel, Braun, and Gottschalk) comb. nov. The description of *Moorella thermoautotrophica* comb. nov. is identical to that proposed for *C. thermoautotrophicum* Wiegel, Braun, and Gottschalk 1982, 384[VP] (49). The type strain is DSM 1974.

(xii) Description of *Paenibacillus durum* (Smith and Cato) comb. nov. The description of *Paenibacillus durum* comb. nov. is identical to that proposed for *C. durum* Smith and Cato 1974, 1394[AL] (42). The type strain is ATCC 27763.

(xiii) Description of *Eubacterium barkeri* (Stadtman, Stadtman, Pastan, and Smith) comb. nov. The description of *Eubacterium barkeri* comb. nov. is identical to that proposed for *C. barkeri* Stadtman, Stadtman, Pastan, and Smith 1972, 760[AL] (45) and given in detail in *Bergey's Manual of Systematic Bacteriology* (4). The type strain is ATCC 25849.

(xiv) Description of *Thermoanaerobacter kivui* (Leigh and Wolfe) comb. nov. The description of *Thermoanaerobacter kivui* comb. nov. is identical to that proposed for *Acetogenium kivui* Leigh and Wolfe 1983, 886[AL] (28). The type strain is ATCC 33488.

(xv) Description of *Thermoanaerobacter thermocopriae* (Jin, Yamasato, and Toda) comb. nov. The description of *Thermoanaerobacter thermocopriae* comb. nov. is identical to that proposed for *C. thermocopriae* Jin, Yamasato, and Toda 1988, 280[AL] (21). The type strain is IAM 13577.

(xvi) Description of *Thermoanaerobacterium thermosaccharolyticum* (McClung) comb. nov. The description of *Thermoanaerobacterium thermosaccharolyticum* comb. nov. is identical to that proposed for *C. thermosaccharolyticum* McClung 1939, 200[AL] (33). The type strain is ATCC 7956.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Agriculture, Fisheries and Food and by grants BIOT-CT91-0294 and BIOT-CT93-0119) from the European Community.

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