Phylogenetic Relationships of the Genera *Acetobacterium* and *Eubacterium* Sensu Stricto and Reclassification of *Eubacterium alactolyticum* as *Pseudoramibacter alactolyticus* gen. nov., comb. nov.

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16S rRNA gene sequences of the type strains of the seven previously described *Acetobacterium* species were determined. The *Acetobacterium* species were found to form a tight phylogenetic cluster within the *Clostridium* subphylum of the gram-positive bacteria. Within this subphylum these organisms belong to cluster XV as defined by Collins et al. (M. D. Collins, P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow, Int. J. Syst. Bacteriol. 44:812–826, 1994) together with *Eubacterium alactolyticum*, *Eubacterium barkeri*, *Eubacterium callanderi*, and *Eubacterium limosum*. Our data indicate that *Clostridium* cluster XV consists of at least the following three genera: the genus *Acetobacterium*, the genus *Eubacterium* sensu stricto (comprising *E. limosum*, *E. barkeri*, and *E. callanderi*), and the genus *Pseudoramibacter* gen. nov., which is created for *E. alactolyticum*, which we reclassify as *Pseudoramibacter alactolyticus* comb. nov.

The genus *Eubacterium* (Prévot) as currently defined contains anaerobic, non-spore-forming, gram-positive rods which are distinguished from other genera mainly on the basis of negative metabolic characteristics (13). Because of its broad definition, this genus has over the years acted as a depository for a large number of phenotypically diverse species (1). In addition to this marked phenotypic heterogeneity, it is recognized that the eubacteria are not phylogenetically homogeneous, with species dispersed among many of the different groups of the *Clostridium* subphylum (5, 16, 27). In a recent phylogenetic study in which the nearly complete 16S rRNA gene sequences of more than 200 species of clostridia and related organisms were used, *Eubacterium limosum* (the type species of the genus *Eubacterium*) and the spore-forming species *Clostridium barkeri*, together with *Eubacterium alactolyticum*, formed a distinct subgroup designated cluster XV (5), which corroborated the findings of Weizenegger et al. (26). Earlier oligonucleotide cataloging studies had revealed that *Acetobacterium woodii*, the type species of the genus *Acetobacterium*, also exhibits a close phylogenetic affinity with *Eubacterium limosum* and *C. barkeri* (22, 23). No phylogenetic analysis based on complete 16S rRNA sequences has been performed with *Acetobacterium woodii*, and thus the precise relationships between this species and other members of cluster XV remain unknown. In addition to *Acetobacterium woodii*, the genus *Acetobacterium* contains the following six species for which no phylogenetic data are available: *Acetobacterium wieringae* (3), *Acetobacterium carbinolicum* (8), *Acetobacterium malicum* (20), and the psychrophilic species *Acetobacterium bakii*, *Acetobacterium paludosum*, and *Acetobacterium fimetarium* (10). To investigate the phylogenetic relationships within the genus *Acetobacterium* and to obtain a clearer picture of the taxonomic affinities and separateness of this genus within cluster XV, we determined the 16S rRNA gene sequences of the type

strains of the seven *Acetobacterium* species. We also determined the 16S rRNA sequence of *Eubacterium callanderi*, a species that has been described as genotypically similar but phenotypically distinct from *Eubacterium limosum* (14). In this paper we present the results of a phylogenetic analysis of these data and discuss the taxonomic implications of our findings.

MATERIALS AND METHODS

Bacterial strains studied. *Acetobacterium bakii* DSM 8239^T (T = type strain), *Acetobacterium carbinolicum* DSM 2925^T, *Acetobacterium fimetarium* DSM 8238^T, *Acetobacterium malicum* DSM 4132^T, *Acetobacterium paludosum* DSM 8237^T, *Acetobacterium wieringae* DSM 1911^T, *Acetobacterium woodii* DSM 1030^T, and *Eubacterium callanderi* DSM 3662^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

16S rRNA gene amplification and sequencing. Lyophilized cells obtained from a culture collection ampoule were suspended in 500 µl of TES buffer (0.05 M Tris-HCl, 0.005 M EDTA, 0.05 M NaCl; pH 8.0), and the DNA was extracted by using the procedure of Lawson et al. (11). The almost complete 16S rRNA gene (*Escherichia coli* positions 31 to 1420) was then amplified by PCR by using primers ARI (5'-GAGAGTTTGATCCTGGCTCAGGA-3') and pH (5'-AAG GAGGTGATCCAGCCGCA-3'). The PCR products were purified by using a Prep-A-Gene kit (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions and were sequenced directly by using an ABI PRISM DNA sequencing kit (Perkin Elmer, Foster City, Calif.) and a model 373A automatic DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.).

Phylogenetic analysis. Analyses were performed by using the sequence analysis programs in the Genetics Computer Group package (6) and the phylogeny inference package PHYLIP (9). The program FASTA (6) was used to determine which sequences in the EMBL data library were most similar to the new *Acetobacterium* sequences. These data were then retrieved from the EMBL data library and included in a phylogenetic analysis together with our new data and a selection of sequence data for other taxa obtained from the EMBL data library. A multiple-sequence alignment was prepared with the program LOCALPILEUP (6) and was corrected manually. Approximately the first 100 bases were omitted from further analyses because of alignment ambiguities in the hypervariable region. In all, 1,309 positions were used for distance calculations. A distance matrix in which the Kimura-2 parameter was used was prepared with the programs PRETTY (6) and DNADIST (9), and a phylogenetic tree was constructed by using the neighbor-joining method and the program NEIGHBOR (9). To assess the statistical significance of the groups, a bootstrap analysis (500 replications) was performed by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE (9). The sequence similarity values given below and in Table 1 were calculated by using the program GAP (6).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of the type strains of the seven *Acetobacterium* species and *Eubacterium callanderi* have been deposited in the EMBL data library under accession numbers X96954 to X96961.

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TABLE 1. Levels of 16S rRNA gene sequence similarity within *Clostridium* cluster XV

Species	% Similarity									
	<i>Acetobacterium bakii</i>	<i>Acetobacterium carbinolicum</i>	<i>Acetobacterium fimetarium</i>	<i>Acetobacterium malicum</i>	<i>Acetobacterium paludosum</i>	<i>Acetobacterium wieringae</i>	<i>Acetobacterium woodii</i>	<i>Eubacterium barkeri</i>	<i>Eubacterium callanderi</i>	<i>Eubacterium limosum</i>
<i>Acetobacterium carbinolicum</i>	97.3									
<i>Acetobacterium fimetarium</i>	97.1	98.2								
<i>Acetobacterium malicum</i>	96.5	98.4	98.5							
<i>Acetobacterium paludosum</i>	97.3	97.0	96.8	96.7						
<i>Acetobacterium wieringae</i>	96.2	97.7	97.7	99.2	96.5					
<i>Acetobacterium woodii</i>	96.9	98.4	97.3	97.8	96.2	98.1				
<i>Eubacterium barkeri</i>	91.5	92.0	91.5	92.3	91.7	92.6	92.1			
<i>Eubacterium callanderi</i>	93.1	93.3	93.1	93.4	93.2	93.7	93.4	94.5		
<i>Eubacterium limosum</i>	92.9	93.4	93.0	93.5	93.0	93.5	93.3	94.6	99.6	
<i>Pseudoramibacter alactolyticus</i>	90.1	89.6	89.6	90.1	90.7	90.6	89.6	91.7	90.6	91.0

RESULTS AND DISCUSSION

The nearly complete sequences of the 16S rRNA genes of the seven *Acetobacterium* species were determined. These sequences consisted of 1,459 to 1,475 nucleotides; the differences in length were due to deletions in the V1 region of the gene. The type strains of *Acetobacterium carbinolicum*, *Acetobacterium fimetarium*, and *Acetobacterium malicum* had a deletion of 6 bases in the V1 region compared with the type strains of *Acetobacterium wieringae* and *Acetobacterium woodii*, and the type strains of *Acetobacterium bakii* and *Acetobacterium paludosum* had a deletion of 16 bases in the V1 region compared with the type strains of *Acetobacterium wieringae* and *Acetobacterium woodii*. The levels of sequence similarity between *Acetobacterium* species ranged from approximately 96 to 99% (Table 1), demonstrating that these organisms form a tight phylogenetic group. This finding was confirmed by the neighbor-joining tree topology; the *Acetobacterium* group was supported by a bootstrap value of 100% (Fig. 1). Kotsyurbenko et al. (10) performed chromosomal DNA-DNA hybridization experiments with *Acetobacterium* strains belonging to different species and obtained levels of hybridization of less than 37% for all species combinations except one. The one exception was *Acetobacterium woodii* and *Acetobacterium carbinolicum*, for which a level of DNA-DNA hybridization of 69% was reported; this value is close to the boundary value of 70% used for species delineation and indicated that these organisms were closely related, possibly at the species level. In our 16S rRNA gene analyses, *Acetobacterium woodii* and *Acetobacterium carbinolicum* exhibited 98.4% sequence relatedness (Table 1), which supported their classification as separate species. In addition, the 16S rRNA genes of *Acetobacterium malicum* and *Acetobacterium wieringae* exhibited less than 1% divergence (Table 1), whereas a level of genomic DNA-DNA homology of only 30% was obtained (10). The three psychrophilic species, *Acetobacterium bakii*, *Acetobacterium fimetarium*, and *Acetobacterium paludosum*, did not exhibit closer phylogenetic affinity with each other than with nonpsychrophilic *Acetobacterium* species (Fig. 1).

When the genus *Acetobacterium* was originally described by Balch et al. in 1977 (2), homoacetogenic fermentation was considered a rare phenomenon, with *Clostridium acetium*,

Clostridium thermoaceticum, and *Clostridium formicoaceticum* the only other taxa that had been reported to have this phenotype (2, 18). Today, anaerobic acetogenesis is thought to be a more widespread microbial process, which occurs in soils, sediments, sewage, and the gastrointestinal tracts of many animals, including humans (7, 17). Acetogenesis has now been observed in many more species that have a diverse range of phenotypes (e.g., several gram-positive spore-forming rod-shaped members of the genus *Clostridium*, including some thermophilic species; non-spore-forming organisms, such as the coccoid bacterium *Ruminococcus* [*Peptostreptococcus*] *productus*; the rod-shaped organism *Eubacterium limosum*; and the gram-negative *Sporomusa* species). It is worth noting that since acetogenesis is not routinely determined as a physiological trait, many other taxa (including currently recognized species) may possess this attribute. All of the homoacetogenic species that have been described so far belong to the domain *Bacteria* (17), and most of them belong phylogenetically to the *Clostridium* subphylum (24). The only exceptions are *Acetohalobium arabaticum*, an extremely halophilic homoacetogenic bacterium that was recently shown to belong to a separate phylum of halophilic anaerobes within the *Bacteria* (25), and *Holophaga foetida*, a bacterium that is capable of degrading methoxylated aromatic compounds and is peripherally related to the δ subclass of the *Proteobacteria* (12). To obtain a clearer picture of the position of the genus *Acetobacterium* within the *Clostridium* subphylum, we performed a phylogenetic analysis in which we included representative members of the different clostridial clusters. The results of our analysis (Fig. 1) confirmed that homoacetogenic species (Fig. 1) are found in several different clusters and thereby reinforced the known phylogenetic diversity of this group of organisms (18, 22, 24).

Consistent with 16S rRNA oligonucleotide cataloging evidence, the closest phylogenetic relatives of the genus *Acetobacterium* were found to be *C. barkeri* (levels of 16S rRNA gene sequence similarity, 91.5 to 92.3% [Table 1]), *Eubacterium limosum* (92.9 to 93.5%), and *Eubacterium alactolyticum* (89.6 to 90.7%). These three species make up cluster XV of the *Clostridium* subphylum as defined by Collins et al. (5), and our phylogenetic analysis revealed that *Acetobacterium woodii* and the other *Acetobacterium* species are members of this cluster,

FIG. 1. Dendrogram showing the phylogenetic position of the genus *Acetobacterium* within cluster XV of the *Clostridium* subphylum. Known homoacetogenic species are indicated by boldface type. The tree was constructed by the neighbor-joining method. Bootstrap values (which are expressed as percentages of 500 replications) of 90% or more are indicated at the branch points. The cluster numbers are the numbers used by Collins et al. (5).

in which they form a distinct subgroup (Fig. 1). High bootstrap values confirmed the significance of these groups. Collins et al. (5) were of the opinion that cluster XV probably represented a single genus. In addition, *C. barkeri*, which exhibited a close phylogenetic affinity with *Eubacterium limosum* (level of sequence divergence, <5%), was reclassified as *Eubacterium barkeri* by these authors (5). However in view of our new sequence data, which place the genus *Acetobacterium* in cluster XV, the taxonomic structure of this cluster clearly needs to be reevaluated. It is now evident from the rather high levels of sequence divergence (up to 10% [Table 1]) and considerable internal structure of cluster XV that the members of this group do not belong to a single genus. Within cluster XV the *Acetobacterium* species form a tight phylogenetic group, and this fact, together with the distinct phenotype of these organisms, clearly justifies retention of the *Acetobacterium* species as members of a single genus. *Eubacterium limosum*, *Eubacterium callanderi*, and *Eubacterium barkeri* form a statistically significant phylogenetic group (bootstrap value, 90) that is close to, albeit distinct from, the genus *Acetobacterium*. Species belonging to this group exhibited intragroup levels of sequence divergence of up to 6% (Table 1). Higher levels of sequence divergence (approximately 7 to 8%) were obtained with *Acetobacterium* species. Within this group *Eubacterium callanderi*, a species which demethoxylates *o*-methylated aromatic acids to

fatty acids (14), was found to be genealogically very closely related to *Eubacterium limosum* (level of 16S rRNA gene sequence similarity, 99.6% [Table 1]). Although *Eubacterium callanderi* differs from *Eubacterium limosum* in being unable to utilize one-carbon substrates for growth (14), the exceptionally high level of 16S rRNA similarity suggests that these organisms may be related at the species level or at least may be genomically very closely related species. *Eubacterium barkeri* was the most peripheral species in this group, although its levels of 16S rRNA divergence (less than 6%), together with its highly unusual type B cell wall murein, are consistent with assignment to the genus *Eubacterium* (5). Although the genus *Eubacterium* is very heterogeneous, a comprehensive taxonomic revision along phylogenetic lines is not possible at this time (because, for example, it is difficult to obtain good phenotypic traits which distinguish the newly delineated taxa). However, it is evident from our data and previous data (5) that *Eubacterium limosum*, *Eubacterium barkeri*, and *Eubacterium callanderi* could form the nucleus of a redefined genus *Eubacterium*. On the basis of the characteristics of this group, a preliminary working definition of *Eubacterium sensu stricto* could be as follows: gram-positive rod-shaped organisms that are nonmotile and obligately anaerobic, may form endospores, and are saccharoclastic. The main end products from glucose fermentation are butyrate, acetate, lactate, and H_2 . Formate or CO_2

TABLE 2. Characteristics that differentiate the genera *Pseudoramibacter*, *Acetobacterium*, and *Eubacterium*

Genus	Autotrophic growth with H ₂ -CO ₂	Peptidoglycan type ^a	Fermentation end product(s) from glucose	G+C content (mol%)
<i>Acetobacterium</i> ^b	+	B	Acetate	39–46
<i>Eubacterium sensu stricto</i> ^c	√ ^d	B	Acetate, butyrate, lactate, formate, ^e H ₂	45–50
<i>Pseudoramibacter</i> ^f	–	A1γ	Formate, acetate, butyrate, caproate, H ₂	61

^a Nomenclature of Schleifer and Kandler (19).^b Data from references 10, 18, and 21.^c The genus *Eubacterium sensu stricto* comprises *Eubacterium limosum*, *Eubacterium barkeri*, and *Eubacterium callanderi*. Data from references 1, 4, 13, 14, and 23.^d √, variable. *Eubacterium limosum* is positive, and *Eubacterium barkeri* and *Eubacterium callanderi* are negative.^e Formate is produced only by *Eubacterium callanderi*.^f Data from references 1, 13, and 15.

may also be produced by some strains. The cell walls contain a type B peptidoglycan. The G+C content of the DNA is 45 to 47 mol%. Although the remaining cluster XV species, *Eubacterium alactolyticum*, exhibits a statistically significant affinity (bootstrap value, 100), it is only loosely associated with the genus *Acetobacterium* and *Eubacterium limosum* and its close relatives. The levels of 16S rRNA sequence divergence (approximately 10%) (Table 1), together with its deep branching position, show that *Eubacterium alactolyticum* represents a third genus within cluster XV that is separate from the genus *Acetobacterium* and the genus *Eubacterium sensu stricto*. Support for the phylogenetic separateness of *Eubacterium alactolyticum* within cluster XV comes from the quite distinct phenotype of this organism (Table 2). A particularly noteworthy chemotaxonomic difference is the presence of a type A peptidoglycan in the cell walls of *Eubacterium alactolyticum* (1). The cell walls of members of the genus *Acetobacterium* and *Eubacterium limosum* and its relatives contain the rarer compound type B peptidoglycan (1, 18, 23). Thus, because of compelling phylogenetic evidence supported by the phenotypic distinctiveness of *Eubacterium alactolyticum*, we believe that this species should be placed in a separate genus, for which we propose the name *Pseudoramibacter* gen. nov.

Description of *Pseudoramibacter* gen. nov. *Pseudoramibacter* (Pseu.do.ra.mi.bac'ter. Gr. adj. *pseudes*, false; L. masc. n. *ramus*, a branch; M. L. masc. n. *bakter*, the equivalent of Gr. neut. n. *bakterion*, rod, staff; M. L. masc. n. *Pseudoramibacter*, false branching rod) cells are rod shaped and occur in pairs resembling flying birds, clumps, or Chinese characters. Non-motile. No endospores are formed. Gram positive. Strictly anaerobic. Growth is stimulated by fermentable carbohydrates. The fermentation end products are formate, acetate, butyrate, caproate, and H₂. The cell wall type is type A, and the cell walls contain *meso*-diaminopimelic acid as the dibasic acid. The type species is *Pseudoramibacter alactolyticus* comb. nov.

Description of *Pseudoramibacter alactolyticus* (Prévot and Taffanel 1942) comb. nov. The description below is based on the description of *Eubacterium alactolyticum* (Prévot and Taffanel 1942) Holdeman and Moore 1970 (13). Cells grown in peptone-yeast extract-glucose (PYG) broth are 0.3 to 0.6 by 1.6 to 7.5 µm. They occur in pairs resembling flying birds, clumps, or Chinese characters. After 2 to 3 days colonies on horse blood agar plates are punctate to 0.5 mm in diameter, circular, entire, convex to pulvinate, smooth, and shiny. Smaller colonies can be translucent, and larger colonies are often opaque. Glucose broth cultures are usually turbid with sediment formation; occasionally a sediment is formed without turbidity in broth cultures. The terminal pH in glucose cultures is 5.0 to 5.6 or, occasionally 5.8 to 6.0. The optimal growth temperature is 35 to 37°C, but most strains grow at 30°C and some strains grow at 25 and 45°C. Growth is stimulated by fermentable

carbohydrates but not by 0.02% Tween 80 or 5% rumen fluid and is inhibited by 20% bile. Gas is produced in glucose agar deep cultures.

Neutral red is reduced, and resazurin is usually not reduced. Hippurate, esculin, and starch are not hydrolyzed. No H₂S production occurs in SIM medium. Indole and acetylmethylcarbinol are not produced. Milk reaction negative. Meat and gelatin are not digested. Nitrate is not reduced. In PYG broth cultures the following compounds are produced: formate (0.01 to 1.0 meq/100 ml of culture), acetate (0.01 to 0.5 meq/100 ml), butyrate (0.1 to 0.5 meq/100 ml), and caproate (0.2 to 2.0 meq/100 ml). Abundant H₂ is produced, and small amounts of caprylate may also be produced. Pyruvate is converted to acetate and formate. Lactate is not utilized. Threonine is not converted to propionate. In the presence of glucose and propionic acid, valeric and heptanoic acids are produced. Acid (pH < 5.5) is produced from fructose and glucose but not from amygdalin, arabinose, cellobiose, glycogen, lactose, maltose, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, starch, sucrose, trehalose, and xylose. Some strains produce acid from mannitol or sorbitol (pH < 5.5) or from esculin (weak reaction). Susceptible to (25 strains tested, including the type strain) chloramphenicol (12 µg/ml), clindamycin (1.6 µg/ml), erythromycin (3 µg/ml), penicillin G (2 U/ml), and tetracycline (6 µg/ml).

Isolated from dental calculus and the gingival crevices of patients with periodontal disease, from root canals, and from patients with various infections, including purulent pleurisy, jugal cellulitis, postoperative wounds, and abscesses of the brain, lung, intestinal tract, and mouth. The type strain is strain ATCC 23263. This strain does not ferment adonitol, dextrin, dulcitol, galactose, glycerol, inulin, or sorbose and does not produce ammonia from peptone, arginine, or threonine. The G+C content of the DNA of the type strain is 61 mol% (as determined by high-performance liquid chromatography [15]).

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