Phenotypic and Phylogenetic Characterization of Some *Eubacterium*-Like Isolates Containing a Novel Type B Wall Murein from Human Feces: Description of *Holdemanian filiformis* gen. nov., sp. nov.

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A group of *Eubacterium*-like strains (designated group S14), isolated from the feces of healthy people, was characterized by biochemical tests, fatty acid analysis, cell wall murein analysis, and 16S rDNA analysis. Our results indicate that group S14 is phylogenetically a member of the *Clostridium* subphylum of the gram-positive bacteria. Despite a phenotypic resemblance to the genus *Eubacterium*, group S14 was shown to be phylogenetically distantly related to the type species of the genus, *Eubacterium limosum*. Group S14 showed a specific phylogenetic association with *Erysipelothrix rhusiopathiae*. Group S14 resembled *Erysipelothrix* in possessing the uncommon type B cell wall murein. Structural analyses, however, revealed the presence of a previously unknown B1δ (L-Ala)–D-Glu–Gly–L-Lys murein type. Based on a 16S rRNA sequence divergence of greater than 10% with *E. rhusiopathiae* and the presence of a unique murein type, a new genus, *Holdemanian*, is proposed for group S14, with one species, *Holdemanian filiformis*. Type strain of *H. filiformis* is ATCC 51649.

The genus *Eubacterium*, as currently defined (10), contains all gram-positive, anaerobic, non-spore-forming, rod-shaped bacteria which do not produce the following acids from fermentation: (i) propionic acid as a major end product (as do members of the genera *Propionibacterium* and *Arachnia*), (ii) lactic acid alone (in contrast to members of the genus *Lactobacillus*), (iii) acetic acid in larger amounts than lactic acid (in contrast to members of the genus *Bifidobacterium*), with or without formic acid, as sole end products, and (iv) succinic acid (in the presence of CO₂) and lactic acid with small amounts of acetic and formic acids (in contrast to members of the genus *Actinomyces*). This definition, by elimination from other anaerobic genera on the basis of negative fermentation characteristics, provided a convenient, pragmatic classification at a time when little or no phylogenetic data were available and allowed effective scientific communication (10). However, from the nature of this definition, it was inevitable that the resulting genus would become rather large and it now contains many named and unnamed species and species groups which are phylogenetically unrelated (10, 17). As more *Eubacterium* species and strains are studied by genotypic and chemotaxonomic methods, they should become genotypically and phenotypically distinguishable, which will permit the gradual taxonomic dissection of the genus *Eubacterium*.

Eubacteria represent an important group of organisms of the human and animal fecal microflora. In recent years, many fecal isolates and groups of isolates have been described which qualify as members of the genus *Eubacterium* but could not be assigned to any of the named species (9, 11–13, 16, 18, 19). In this paper, we report the phenotypic and phylogenetic characterization of a group of human fecal isolates provisionally

designated *Eubacterium* group S14. On the basis of the results presented, we propose that these organisms be classified as *Holdemanian filiformis* gen. nov., sp. nov.

MATERIALS AND METHODS

Bacterial strains. Strains J1-31B-1, J1-37, and S4B-1 from the Moore collection (Virginia Polytechnic Institute and State University, Blacksburg) were isolated from the feces of healthy humans.

Biochemical characterization. Fermentation and enzymatic reactions determined in prereduced media and analyses of fermentation end products were performed as described previously (5).

Fatty acid analysis. The fatty acid methyl ester contents of whole-cell sediments obtained from prereduced, anaerobically sterilized peptone-yeast extract-Tween (PYT)-glucose broth cultures were determined as described by Johnson et al. (6).

Cell wall murein analysis. Preparation of cell walls and determination of murein structure were carried out by the methods described by Schleifer and Kandler (14) with the modification that thin-layer chromatography on cellulose sheets (Merck, Darmstadt, Germany) was used instead of paper chromatography. Briefly, 1 mg of freeze-dried cell walls was hydrolyzed in 0.2 ml of 4 N HCl at 100°C for 16 h (total hydrolysate) or 45 min (partial hydrolysate). Diamino acids were determined from the total hydrolysate by one-dimensional chromatography in the solvent system methanol-pyridine-water–10 N HCl (320:40:70:10 [vol/vol]). Amino acids and peptides from the total and partial hydrolysates were identified after two-dimensional chromatography in the systems described by Schleifer and Kandler (14) by their mobilities and staining characteristics with ninhydrin spray. The resulting "fingerprints" could be compared with those of known peptidoglycan structures. The configurations of aspartic acid and lysine were determined by using L-aspartase (Sigma A 8147) and L-lysine decarboxylase (Sigma L 0882), respectively.

16S rRNA gene sequencing. The nearly complete 16S rRNA gene sequence of strain J1-31B-1 and the partial 16S rRNA gene sequence of strain S4B-1 were determined. Lyophilized cells were resuspended in 500 µl of TES buffer (0.05 M Tris-HCl, 0.005 M EDTA, 0.05 M NaCl; pH 8.0), and DNA was extracted by the method of Lawson et al. (8). A large fragment of the 16S rRNA gene (corresponding to positions 30 to 1491 of the *Escherichia coli* 16S rRNA gene) was amplified by PCR with primers close to the 3' and 5' ends of the gene. The PCR products were purified by using a Prep-A-Gene kit (Bio-Rad, Hercules, Calif.) in accordance with the manufacturer's instructions and directly sequenced by using a Taq Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and a 373A automatic DNA sequencer (Applied Biosystems).

Phylogenetic analysis. The closest known relatives of the new isolates were determined by performing a sequence database search by using the FASTA program of the Genetics Computer Group package (3). The sequences of closely related strains were retrieved from the EMBL or Ribosomal Database Project data library and aligned with the newly determined sequence data by using the

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FIG. 1. Scheme of a thin-layer chromatogram of a partial acid hydrolysate of cell walls of strain J1-31B-1. 1, lysine; 2, aspartic acid; 3, glutamic acid; 4, serine; 5, alanine; 6, glucosamine; 7, muramic acid; 8, α -(aminosuccinyl)lysine; 9, L-Ser-D-Glu; 10, γ -D-Glu-L-Ala; 11, N^6 -D-Ala-L-Lys; 12, α -(aminosuccinyl)lysine-D-Ala; 13, L-Ala-D-Ala. I, 2-Propanol-acetic acid-water; II, α -picoline-ammonia-water.

PILEUP program (3). The resulting multiple sequence alignment was corrected manually, and approximately 100 bases at the 3' end of the gene were omitted from further analyses because of alignment ambiguities. In total, a continuous stretch of 1,376 bases was used for distance matrix analysis. A distance matrix was calculated by using the programs PRETTY (3) and DNADIST (using the Kimura-2 correction parameter) (4). A phylogenetic tree was constructed by the neighbor-joining method with the NEIGHBOR program (4). The stability of the grouping was estimated by bootstrap analysis (500 replications) by using the DNABOOT, DNADIST, NEIGHBOR, and CONSENSE programs (4).

Nucleotide sequence accession number. The EMBL accession number for the 16S rDNA sequence of strain J-31B-1 is Y11466.

RESULTS AND DISCUSSION

Three strains of eubacterium-like organisms isolated from the feces of healthy people were characterized. The strains stained gram positive and consisted of non-spore-forming, rod-shaped organisms which formed pairs or short chains. The isolates were obligately anaerobic and catalase negative, producing moderate amounts of acetic and lactic acids and small amounts of succinic acid from PYT broth. The strains were nonproteolytic (as determined by failure to digest gelatin and meat) and did not reduce nitrate nor deaminate arginine. They hydrolyzed esculin. Based on the above phenotypic characteristics, the strains were consistent with the genus *Eubacterium* and were therefore preliminarily designated *Eubacterium* group S14.

The structure of the cell wall murein of strain J1-31B-1 was determined. The murein contained, besides muramic acid and glucosamine, the amino acids lysine, glutamic acid, aspartic acid, serine, and alanine in a molar ratio of 1:1:1:1:2, respectively. Both aspartic acid and lysine occur in the L configuration. Up to now, only D-aspartic acid has been found in bacterial cell walls. In the hydrolysate of dinitrophenylated cell walls, only ϵ -2,4-dinitrophenol-lysine is present. From the chromatogram of a partial acid hydrolysate, as depicted schematically in Fig. 1, the primary structure of the peptidoglycan, as shown in Fig. 2, was deduced. This structural type—B1 δ (L-Ala)—D-Glu—L-Asp—L-Lys—represents a hitherto unknown group B murein type and most closely resembles that of *Erysipelothrix rhusiopathiae*, B1 δ (L-Ala)—D-Glu—Gly—L-Lys (14), which contains a glycine residue instead of an aspartic acid in the interpeptide bridge. It is pertinent, however, that group B

FIG. 2. Fragment of the primary structure of the cell wall murein of strain J1-31B-1. The interpeptide bridge is boxed.

mureins are relatively uncommon but are also found in *Acetobacterium* species, *Clostridium barkeri*, and *Eubacterium limosum*.

The fatty acid composition of the cells was analyzed and found to be of the straight-chain, nonhydroxylated type, predominantly monounsaturated or saturated. Methylated cellular constituents detected at greater than 4% of total acids (mean values for four analyses of two isolates) were as follows: C_{18:1} *cis*9 fatty methyl ester (50% of total acid + 11.48% standard deviation [SD]), C_{18:1} *cis*9 dimethyl acetal (12% + 4.51% SD), C_{16:1} *cis*9 fatty acid methyl ester (6% + 3.43% SD), C_{10:1} fatty acid methyl ester (5% + 4.03% SD), C_{16:0} fatty acid methyl ester (4% + 3.24% SD), and C_{16:0} dimethyl acetal (4% + 0.65% SD). No hydroxylated or branched-chain fatty acids were detected. By contrast, *Erysipelothrix* does contain 2- and 3-hydroxylated long-chain fatty acids and small amounts of *iso*- and *anteiso*-methyl branched fatty acids (7).

To establish the precise phylogenetic affiliation of *Eubacterium* group S14, we determined the 16S rRNA gene sequence of strain J1-31B-1. In addition, we determined the partial sequence of a second strain (S4B-1) of this group. The primary sequence of strain J1-31B-1 obtained consisted of 1,391 bases (corresponding to positions 99 to 1491 of the *E. coli* 16S rRNA). The sequence of strain S4B-1 obtained consisted of approximately 800 bases (corresponding to *E. coli* positions 99 to 900) and displayed two base differences from the corresponding sequence of strain J1-31B-1, thereby demonstrating the genotypic homogeneity of group S14. A database search revealed that strain J1-31B-1 belongs to the *Clostridium* subphylum of the gram-positive bacteria and its 16S rRNA sequence is most similar to that of members of *Clostridium* cluster XVI (see reference 2). A phylogenetic analysis (Fig. 3) was performed including all taxa from cluster XVI and representatives of other clostridial clusters defined by Collins et al. (2). Our analysis shows that *Eubacterium* group S14 belongs to a larger group consisting of *Clostridium* clusters XVI, XVII, and XVIII, although it does not belong to any of these clusters (2).

FIG. 3. Dendrogram showing the phylogenetic position of *H. filiformis* J1-31B-1 (ATCC 51649) and some close relatives. The dendrogram was constructed by using the neighbor-joining method. Bootstrap values were calculated from 500 replications.

It is noteworthy that many of these taxa, like those in group S14, have been isolated from human feces. Within this group, strain J1-31B-1 formed a distinct line exhibiting a specific phylogenetic association (approximately 89% 16S rRNA sequence relatedness) with *E. rhusiopathiae*, the causative agent of erysipelas in swine. Bootstrap resampling (value, 94%) showed this association to be statistically significant. A second species, *Erysipelothrix tonsillarum*, is biochemically and phylogenetically highly similar to the type species (1, 15). No close phylogenetic affinity with any of the other reference species examined was shown (sequence divergence values, >13%; data not shown). In particular, *E. limosum*, the type species of the genus *Eubacterium*, was found to be phylogenetically only remotely related to the unknown fecal bacterium (approximately 80% sequence similarity).

The unknown isolates from human feces clearly belong to a hitherto unrecognized, gram-positive, catalase-negative species within the *Clostridium* subphylum (2). From murein structural analysis and 16S rRNA gene sequence comparisons, it is evident that the bacterium has a close phylogenetic relationship with *E. rhusiopathiae*. Although the association between these taxa is statistically significant, a sequence divergence of >10% suggests that this relationship is that of two phylogenetically closely related, but nevertheless different, genera. In contrast to members of the genus *Erysipelothrix*, our isolates do not contain hydroxylated or branched-chain fatty acids. Based on 16S rRNA sequence considerations and the unique type B murein of the unknown rod, we believe that this bacterium merits classification as a new genus, for which the name *Holdemanifiliformis* gen. nov., sp. nov. is proposed.

Description of *Holdemanifiliformis* gen. nov. *Holdemanifiliformis* (Holdeman'ia. *Holdemanifilium* L. n., named to honor Lillian V. Holdeman Moore, a contemporary American microbiologist, for her outstanding contribution to anaerobic bacteriology) cells are rod shaped and occur in pairs and short chains. No endospores are formed. Gram positive. Strictly anaerobic. Catalase negative. Growth in broth media is enhanced by addition of Tween 80. Main fermentation end products from glucose are acetic and lactic acids; succinic acid is produced in smaller amounts. The cell walls contain a B1δ-type peptidoglycan with an (L-Ala)-D-Glu-L-Asp-L-Lys interpeptide bridge. Long-chain fatty acids are of the nonhydroxylated, monounsaturated or saturated type. The DNA base composition is 38 mol% G+C. The type species is *H. filiformis*. The genus *Holdemanifiliformis* is a member of the *Clostridium* subphylum of the gram-positive bacteria and exhibits a close phylogenetic association with the genus *Erysipelothrix*.

Description of *Holdemanifiliformis* sp. nov. *Holdemanifiliformis* (fi.li.for'mis. L. n. *filum*, thread; L. n. *forma*, shape; M. L. adj. *filiformis*, filiform). This description is based on the study of three strains isolated from the feces of healthy humans. Cells are short-to-long, gram-positive bacilli that decolorize easily and occur in pairs and short chains. The longer cells may have central to terminal swellings, but neither spores nor heat resistance has been demonstrated. Colonies on brain heart infusion agar streak tubes incubated in an anaerobic atmosphere are 1.0 mm in diameter, circular, entire, low convex, and translucent, with a granular appearance. There is no growth on the surface of solid medium incubated in air enriched to 10% with CO₂. Broth cultures in PYT-glucose medium are turbid with a smooth, white sediment. In broth media, growth is enhanced by 0.2% (vol/vol) Tween 80. There is good growth in PYT broth medium containing esculin, fructose, glucose, salicin, or sucrose, even though the pH is only 5.8 to 6.2 in this medium. Growth in PYT broth without carbohydrate and PYT with amygdalin, arabinose, cellobiose, erythri-

tol, glycogen, inositol, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, sorbitol, starch, trehalose, or xylose is marginal; the pH of cultures in this medium is 6.2 to 6.5. Moderate growth sometimes is observed in peptone-yeast extract broth with lactose or maltose. Esculin is hydrolyzed. Nitrate is not reduced; indole and catalase are not produced. Gelatin and meat are not digested, and milk is unchanged. Arginine is not deaminated. Acids detected in PYT-glucose broth cultures acidified to pH 2.0 (in milliequivalents per milliliter of culture) are acetic acid (0.8), lactic acid (0.9), and succinic acid (0.2); formic acid is sometimes detected. Methylated cellular lipid constituents are C_{18:1} *cis*9 fatty methyl ester (50% of total acids + 11.48% SD), C_{18:1} *cis*9 dimethyl acetal (12% + 4.51% SD), C_{16:1} *cis*9 fatty acid methyl ester (6% + 3.43% SD), C_{10:0} fatty acid methyl ester (5% + 4.03% SD), C_{16:0} fatty acid methyl ester (4% + 3.24% SD), and C_{16:0} dimethyl acetal (4% + 0.65% SD). The DNA base composition is 38 mol% G+C. The type strain of *H. filiformis* is strain J1-31B-1 (= ATCC 51649). Isolated from human feces.

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