Evidence for the Placement of the Gram-Negative *Catonella morbi* (Moore and Moore) and *Johnsonella ignava* (Moore and Moore) within the *Clostridium* Subphylum of the Gram-Positive Bacteria on the Basis of 16S rRNA Sequences

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Comparative 16S rRNA analysis was used to determine the phylogenetic positions of *Catonella morbi* and *Johnsonella ignava*, which are members of two monospecific genera of gram-negative anaerobic bacilli isolated from human gingival crevices. Both of these genera were found to belong to cluster XIVa (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) of the *Clostridium* subphylum of gram-positive bacteria. Within this cluster, which contains several *Clostridium*, *Coprococcus*, *Eubacterium*, and *Ruminococcus* species, *C. morbi* and *J. ignava* formed two distinct lines that were separate from all other taxa. Our findings support the separate generic status of the genera *Catonella* and *Johnsonella* and show that these genera do not belong to the family *Bacteroidaceae* but instead belong to the gram-positive *Clostridium* subphylum.

Recently, Moore and Moore (9) described five new genera (the genera *Catonella*, *Dialister*, *Hallella*, *Johnsonella*, and *Oribaculum*) within the family *Bacteroidaceae* for groups of anaerobic, gram-negative bacilli that occur in human gingival crevices and periodontal pockets. In previous studies 16S rRNA gene sequence analysis was used to determine the phylogenetic positions of three of these new genera. *Dialister pneumosintes* (formerly *Bacteroides pneumosintes*) was found to belong to the *Sporomusa* subbranch of the *Clostridium* subphylum of gram-positive bacteria (10). In contrast, *Hallella seregens* and *Oribaculum catoniae* grouped within the *Bacteroidaceae* but formed separate lineages within the genera *Prevotella* and *Porphyromonas*, respectively (11, 12). In this paper, we describe the phylogenetic positions of members of the remaining two genera, *Catonella morbi* and *Johnsonella ignava*.

Catonella morbi, formerly known as “*Bacteroides* D42,” was described for a group of gram-negative saccharolytic bacilli associated with periodontitis. *Catonella morbi* strains have a G+C content of 34 mol% (9) and can be distinguished from members of other genera of gram-negative anaerobes with similar G+C contents by the fact that in peptone-yeast extract-glucose (PYG) broth they produce predominantly acetic acid and moderate amounts of formic and lactic acids (9). *J. ignava* was described for a group of gram-negative, nonfermentative, nonproteolytic bacilli that were previously referred to as “*Bacteroides* D19” (9) and are associated with gingivitis and periodontitis. *J. ignava* has a G+C content of 32 mol% and produces moderate amounts of acetate and isovalerate and trace amounts of lactate, succinate, and isobutyrate in PYG broth (9). In this study we determined the 16S rRNA gene sequences of the type strains of *Catonella morbi* and *J. ignava* in order to investigate the phylogenetic distinctiveness and higher affiliations of these taxa.

Lyophilized cells of *Catonella morbi* ATCC 51271^T (T = type strain) and *J. ignava* ATCC 51276^T were obtained from

the American Type Culture Collection, Rockville, Md. DNA was extracted directly from freeze-dried cells, resuspended in 500 µl of TES buffer (0.05 M Tris-HCl, 0.005 M EDTA, 0.05 M NaCl; pH 8.0), as described by Lawson et al. (8). A PCR fragment containing the almost-complete 16S rRNA gene (positions 30 to 1521; *Escherichia coli* numbering) was generated by using primers for highly conserved sequences at the 3' and 5' ends of the gene. PCR products were cleaned by using a Prep-A-Gene kit (Bio-Rad, Hercules, Calif.) as recommended by the manufacturer and were sequenced directly by using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and an Applied Biosystems model 373A automatic DNA sequencer.

A search of the EMBL and GenBank data libraries was performed by using the FASTA program of the Genetics Computer Group sequence analysis package (3) to determine the sequences most similar to the newly determined sequences. On the basis of the information obtained a set of sequences of reference organisms was retrieved from the EMBL, GenBank, Ribosomal Database and Project databases for further analysis. The sequences were aligned by using the PILEUP and PRETTY programs (3), and the multiple alignment was then corrected manually. Approximately 100 nucleotides at the 5' end of the molecule were omitted from the analysis because of alignment ambiguities; this resulted in a continuous stretch of 1,306 nucleotides being used for each strain in the analysis. Using the PHYLIP package (5), we constructed a distance matrix with the program DNADIST (by using the Kimura-2 parameter), and a phylogenetic tree was produced by using the neighbor-joining method with the program NEIGHBOR. The stability of the groups obtained was verified by the bootstrapping method (500 bootstrap replications) by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE of the PHYLIP package. For comparison, a pairwise similarity matrix was constructed by repeatedly using the GAP program of the Genetics Computer Group package; the similarity values were converted to nucleotide substitution values (K_{nuc} values [7]), and a phylogenetic tree was drawn by using the NEIGHBOR program. A parsimony analysis was per-

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formed with the multiple sequence alignment by using the DNAPARS program in the PHYLIP package.

The 16S rRNA sequences which we determined for *Catonella morbi* ATCC 51271^T and *J. ignava* ATCC 51276^T consisted of 1,467 and 1,478 nucleotides, respectively. Preliminary database searches revealed that both of these species were only remotely related to members of the gram-negative family *Bacteroidaceae* (levels of sequence similarity, approximately 71 to 74%) and were more closely affiliated with the *Clostridium* subphylum of gram-positive bacteria. To determine the positions of *Catonella morbi* and *J. ignava* within this subphylum, we first performed distance matrix-based phylogenetic analyses (by using both multiple and pairwise sequence alignments) with 75 strains representing the 19 clostridial clusters described by Collins et al. (2), as well as the *Bacteroidaceae*, the *Proteobacteria*, the *Lactobacillaceae*, the *Bacillaceae*, the *Mycoplasmatales*, and the *Actinomycetales*. These analyses (data not shown) revealed that both *Catonella morbi* and *J. ignava* belong to cluster XIVa of the *Clostridium* subphylum as defined by Collins et al. (2). Cluster XIVa contains several *Clostridium*, *Coprococcus*, *Eubacterium*, and *Ruminococcus* species, as well as *Acetitomaculum ruminis*. To assess the relationships of *Catonella morbi* and *J. ignava* within cluster XIVa, we performed additional phylogenetic analyses in which we included all known species belonging to this cluster, as well as cluster XIVb, and *Clostridium butyricum* (cluster I), the type species of the genus *Clostridium*, as an outgroup. *Catonella morbi* and *J. ignava* exhibited levels of sequence similarity of approximately 84 to 88 and 84 to 87%, respectively, with other taxa belonging to cluster XIVa, and a level of sequence similarity of 84.6% with each other. The three approaches which we used for sequence analysis (distance matrix analysis based on both multiple and pairwise sequence alignments and parsimony analysis) resulted in very similar phylogenetic trees and clearly showed that within cluster XIVa *Catonella morbi* and *J. ignava* belong to distinct sublineages. For example, the phylogenetic tree obtained from a neighbor-joining analysis of a distance matrix calculated from the multiple sequence alignment is shown in Fig. 1. The low bootstrap values obtained for *Catonella morbi* and *J. ignava* indicate that there is considerable uncertainty concerning the precise positions of these organisms within cluster XIVa, although the lengths of the branches clearly support recognition of these taxa as members of separate genera (9). All groups with bootstrap values of 80% or more (Fig. 1) were also recovered in phylogenetic trees (data not shown) obtained by performing a distance matrix analysis after pairwise sequence alignment and by performing a parsimony analysis (three most parsimonious trees were obtained [data not shown]). *Eubacterium saburreum* was consistently recovered as the closest neighbor of *J. ignava* in all of the analyses performed. However, the observed level of sequence divergence (13%) and the low bootstrap value (Fig. 1) indicate that this relationship is probably not particularly significant.

The finding that gram-negative genera group together with gram-positive genera is quite surprising, but not exceptional. So far, most gram-negative organisms that are phylogenetically related to the clostridia belong to the *Sporomusa* subbranch. To our knowledge, the only other example of a gram-negative species that belongs to one of the other clostridial clusters is *Tissierella praeacuta*, which belongs to cluster XII and is closely related genealogically to *Clostridium hastiforme* (4). In this context it is worth noting that although some organisms (e.g., *Butyrivibrio* species) are considered gram-negative when traditional staining methods are used, they have been shown by ultrastructural electron microscopy studies to possess unusually thin but gram-positive cell walls (1, 6).

FIG. 1. Unrooted phylogenetic tree obtained by a neighbor-joining analysis of a distance matrix calculated from a multiple sequence alignment, showing the positions of *Catonella morbi* and *J. ignava* within the *Clostridium* subphylum of gram-positive bacteria. Bootstrap values (expressed as percentages of 500 replications) are indicated at the branch points; values of 90% or more were considered significant.

The 16S rRNA gene sequences of *Catonella morbi* ATCC 51271^T and *J. ignava* ATCC 51276^T have been deposited in the EMBL data library under accession numbers X87151 and X87152, respectively.

We gratefully acknowledge the support of Commission of the European Communities grant Bio-2-CT93-0119.

REFERENCES

- Cheng, K.-J., and J. W. Costerton. 1977. Ultrastructure of *Butyrivibrio fibrisolvens*: a gram-positive bacterium? *J. Bacteriol.* **129**:1506-1512.
- Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int. J. Syst. Bacteriol.* **44**:812-826.
- Devereux, J., P. Haerberli, and D. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
- Farrow, J. A. E., P. A. Lawson, H. Hippe, U. Gauglitz, and M. D. Collins. 1995. Phylogenetic evidence that the gram-negative nonsporulating bacterium *Tissierella (Bacteroides) praeacuta* is a member of the *Clostridium* subphylum of the gram-positive bacteria and description of *Tissierella creatinini* sp. nov. *Int. J. Syst. Bacteriol.* **45**:436-440.
- Felsenstein, J. 1989. PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5**:164-166.
- Hespell, R. B., K. Kato, and J. W. Costerton. 1993. Characterization of the cell wall of *Butyrivibrio* species. *Can. J. Microbiol.* **39**:912-921.
- Hori, H., and S. Osawa. 1979. Evolutionary change in 5S rRNA secondary structure and phylogenetic tree of 54 5S rRNA species. *Proc. Natl. Acad. Sci. USA* **76**:381-385.
- Lawson, P. A., S. E. Gharbia, H. N. Shah, and D. R. Clark. 1989. Recognition of *Fusobacterium nucleatum* subgroups Fn-1, Fn-2 and Fn-3 by ribosomal RNA gene restriction patterns. *FEMS Microbiol. Lett.* **65**:41-46.
- Moore, L. V. H., and W. E. C. Moore. 1994. *Oribaculum catoniae* gen. nov., sp. nov.; *Catonella morbi* gen. nov., sp. nov.; *Hallella seregens* gen. nov., sp. nov.; *Johnsonella ignava* gen. nov., sp. nov.; and *Dialister pneumosintes* comb. nov., nom. rev., anaerobic gram-negative bacilli from the human gingival crevice. *Int. J. Syst. Bacteriol.* **44**:187-192.
- Willems, A., and M. D. Collins. 1995. Phylogenetic placement of *Dialister*

- pneumosintes* (formerly *Bacteroides pneumosintes*) within the *Sporomusa* sub-branch of the *Clostridium* subphylum of the gram-positive bacteria. Int. J. Syst. Bacteriol. **45**:403–405.
11. **Willems, A., and M. D. Collins.** 1995. Reclassification of *Oribaculum catoniae* (Moore and Moore 1994) as *Porphyromonas catoniae* comb. nov. and emendation of the genus *Porphyromonas*. Int. J. Syst. Bacteriol. **45**:578–581.
12. **Willems, A., and M. D. Collins.** 1995. 16S rRNA gene similarities indicate that *Hallella seregens* (Moore and Moore) and *Mitsuokella dentalis* (Haapasalo et al.) are genealogically highly related and are members of the genus *Prevotella* (Shah and Collins): emended description of the genus *Prevotella* (Shah and Collins) and description of *Prevotella dentalis* comb. nov. Int. J. Syst. Bacteriol. **45**:832–836.