Whipple's disease is a rare chronic illness associated with an unculturable bacterium that is constantly present in affected tissues. This bacterium was previously characterized at the molecular level by PCR and sequencing of the 16S rRNA gene. On the basis of 1,321 nucleotides of the sequence of its gene coding for 16S rRNA (16S rDNA), a phylogenetic relationship to the actinomycetes was established. In this study, we determined an almost complete 16S rDNA sequence (1,495 nucleotides), the 16S-23S ribosomal intergenic spacer sequence, and 200 nucleotides of the 23S rRNA gene. The 16S rDNA sequence was compared with the large number of actinomycete sequences that have been added to the database since the original study. Phylogenetic analysis revealed a branching position as the deepest branch of the cluster comprising the actinomycetes with group B peptidoglycan between this group and the family Cellulomadaceae. This provides additional information on the phylogenetic position of this bacterium and some clues as to its characteristics. The spacer region between the 16S and 23S rRNA genes is 294 nucleotides long and does not contain tRNA genes. As has been shown in other instances, the increased variability of the ribosomal intergenic spacer compared with the 16S rRNA gene makes it a potential target for use in the differentiation of strains of the bacterium associated with Whipple's disease.
ple's disease bacterium and a universal bacterial primer binding in the 23S rDNA, and the sequence was determined.

MATERIALS AND METHODS

Preparation of DNA from biopsy material. DNA from the Whipple's disease bacterium was extracted from the duodenal biopsy of a patient whose case was previously reported (27). Prior to PCR, the biopsy was deparaffinized by shaking it twice in 1 ml of n-xylene and twice in 500 μl of ethanol, each step lasting for 30 min. The samples were then washed in 0.5 ml water and air-dried for 5 min. DNA was extracted by these steps. The biopsy was then dried under vacuum, subsequently digested for 2 h at 56°C in 40 μl of lysis buffer (50 mM KCl, 10 mM Tris, 1.5 mM MgCl2, 1% Triton X-100, 200 μg of proteinase K per ml), and boiled for 10 min. The DNA was precipitated with ethanol, washed, and then dissolved in 50 μl of sterile water.

PCR amplification. The composition of the PCR mix was the same as described previously (26), and the cycling profile consisted of initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30s, annealing at 58°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 2 min. To amplify a 1,249-bp fragment from the 16S rDNA gene, the universal bacterial primer p3FPL (5’-AGTTTGATCCTGGCTCAG-3’) and the Whipple’s disease bacterium-specific primer pW2RB (5’-ATTTCCATTGCAAGGCAATA-3’) of Relman et al. (36) were used in a modified version, both without restriction enzyme recognition sites. To obtain the 5' end of the 16S rDNA gene, the 5' end of the 23S rRNA gene and the intergenic spacer, the Whipple's disease bacterium-specific primer pW3FE (5’-AGAGATACGCCCCCCGCAA-3’) and primer pW3RE (5’-ATGACGAGGTTGCAATA-3’) of Kostman et al. (20) were used. PCR products were checked on 5% polyacrylamide gels with previously described electrophoresis conditions (26), subsequently transferred to nylon membranes, and hybridized at 60°C with the 32P-labeled oligonucleotide “whip3” (5’-GTGATCAAGGTTGCAATA-3’), which is located on the 16S rDNA of the Whipple’s disease bacterium between the primers p3FPL and pW2RB of Relman et al. (36). To obtain pure DNA for sequencing, electrophoresis was performed on 1% agarose gels. DNA fragments were cut out from the agarose gels and purified with the Jet sequencing kit (Genomed, Research Triangle Park, N.C.). Sequencing was performed with the Applied Biosystems 373A DNA sequencer. Sequences were manually aligned by the combination of primers described above, two PCR products were obtained; both were slightly larger than 1 kbp on polyacrylamide gels and both hybridized with the oligonucleotide whip3. The sequences of both PCR products contained an identical overlapping fragment of 230 bases which is located between the Whipple’s disease bacterium-specific primers pW3FE and pW2RB of Relman et al. (36).

The whole sequence is 1,989 nucleotides long and ranges from position 28 in the 16S rRNA to position 188 in the 23S rRNA of the corresponding genes of E. coli (accession no. J01695). The sequence contains 1,495 nucleotides of the 16S rRNA gene and 200 nucleotides of the 23S rRNA gene of the Whipple’s disease bacterium. The first 1,321 nucleotides are identical to the sequence of the Whipple’s disease bacterium that was reported earlier by Relman et al. (36).

RESULTS AND DISCUSSION

By using the combination of primers described above, two PCR products were obtained; both were slightly larger than 1 kbp on polyacrylamide gels and both hybridized with the oligonucleotide whip3. The sequences of both PCR products contained an identical overlapping fragment of 230 bases which is located between the Whipple’s disease bacterium-specific primers pW3FE and pW2RB of Relman et al. (36). The whole sequence is 1,989 nucleotides long and ranges from position 28 in the 16S rRNA to position 188 in the 23S rRNA of the corresponding genes of E. coli (accession no. J01695). The sequence contains 1,495 nucleotides of the 16S rRNA gene and 200 nucleotides of the 23S rRNA gene of the Whipple’s disease bacterium. The first 1,321 nucleotides are identical to the sequence of the Whipple’s disease bacterium that was reported earlier by Relman et al. (36).

All methods of phylogenetic analysis used in this study gave identical branching patterns with respect to the phylogenetic position of the Whipple’s disease bacterium. Differences in the positions and relationships of the deep branching groups were observed between the different analyses. In all phylogenetic analyses, the branching position of the Whipple’s disease bacterium was as the deepest branch of the cluster composed of the actinomycetes with group B peptidoglycan, between this group and the members of the family Cellulomonadaceae. A phylogenetic tree was calculated with the 16S rDNA sequences of representatives of the major phylogenetic groups within the order Actinomycetiales (Fig. 1). Pairwise evolutionary distances were computed with the correction of Jukes and Cantor (13). The phylogenetic dendrogram shown in Fig. 1 was reconstructed from the distance matrices by the neighbor-joining method (38). Although the position of the Whipple’s disease bacterium between the actinomycetes with group B peptidoglycan and the cellulosanoms was recovered in all phylogenetic analyses, the bootstrap analyses do not indicate a very high level of confidence at 83% associated with this position. The bootstrap values indicated in Fig. 1 show that the cellulosanom group was also recovered in 83% of the resamplings while the cluster of actinomycetes with group B peptidoglycan was recovered in only 81% of the analyses. When the 16S rDNA sequence similarities of the Whipple’s disease bacterium, the representatives of the actinomycetes with group B peptidoglycan, and the cellulosanoms shown in Fig. 1 were calculated, the 16S rDNA similarities of the Whipple’s disease bacterium and the cellulosanoms with group B peptidoglycan are in the range of 90.0 to 91.6% compared with a range of 89.9 to 91.6% for the cellulosanoms.

Nucleotide sequence accession numbers. The accession numbers of the sequences of the reference strains (strain designations given when available) used in the phylogenetic analyses are as follows: Actinoplanes phthophilus DSM 43019T (X93187), Agrocybea jenensis DSM 95883T (X92492), Agromyces ramosus DSM 43045T (X77445), Arthrobacler globiformis DSM 20124T (M24311), Atopobium minutum ATCC 32326T (M59059), Aerobacteriium liquefaciens DSM 20638T (X74444), “Breitbereitrium hevelorum” DSM 20419 (X74440), Breitbereitrium linens DSM 20425T (X74552), Cellulomonas biazea DSM 20112T (X83802), Cellulomonas cellulosas DSM 20118T (X83804), Cellulomonas cellulans DSM 43897T (X83809), Cellulomonas fermentans DSM 3133T (X83805), Cellulomonas flavigena DSM 20109T (X73799), Cellulomonas gelida DSM 20111T (X83800), Cellulomonas hominis C14a (X82598), Clostridium michiganense subsp. michiganense DSM 46364T (X77445), Clostridium yilsi subsp. cynodontis (M69035), “Corynebacterium aquaticum” DSM 20146 (X77450), Curobacterium cirueum DSM 20578T (X74374), Deferribacteres nociocruensis DSM 20448T (X27575), Desulfovibrio desulfuricans DSM 20456T (X5382), Kocuria rosea DSM 20447T (X77576), Lentaia albidocapillata DSM 44073T (X84321), Microbacterium lactum DSM 20427T (X77441), Micrococcus luteus (M38242), Nesterenkonia halobia DSM 20641T (X80747), Nocardioides asteroides DSM 4375T (X80606), Promicromonospora aurantia DSM 43525T (X13870), Ralstoniabacter cathaysiensis DSM 7485T (X73949), Rothia denticola DSM 17391T (M50055), Sporichthys polymorpha DSM 46113T (X72377), Streptomyces griseus (M76388), and Streptomyces orientalis DSM 43021T (X89947).

The sequence determined for the Whipple’s disease bacterium in this study and the 16S rRNA gene sequence isolated from clinical samples are deposited in the EMBL database under accession no. X99636.

The phylogenetic position determined in this study for the Whipple’s disease bacterium based on comparison of almost
complete 16S rDNA sequences of more than 25 reference organisms from a group that Relman et al. (36) designated "actinobacteria" is different from the position previously shown (36). The Whipple's disease bacterium is not the deepest branching organism of the actinobacteria group but branches within this group. The phylogenetic analysis presented here allows us to eliminate the possibility that the Whipple's disease bacterium is highly related to any of the actinomycete taxa for which sequences have become available since the analysis of Relman (36) with the then-available limited database. The determination of the phylogenetic position of the Whipple's disease bacterium 16S rDNA sequence between the actinomycetes with group B peptidoglycan and the cellulomonads may provide some clues as to its characteristics. Future studies should now be aimed at the determination of the peptidoglycan type of the Whipple's disease bacterium in order to determine its affiliation to either neighboring taxon at the chemotaxonomic level. Such data would complement the phylogenetic data available and indicate the significance of the intermediate branching point of the Whipple's disease bacterium demonstrated in this study.

The spacer region between the 16S and 23S rRNA genes determined in this study is 294 nucleotides long and does not contain tRNA genes. Searches for sequence similarity to the spacers of other actinomycetes (Frankia spp., accession numbers M55343 and M88466; Streptomyces sp., M27245; Clavibacter spp., L43095 and U09379) revealed an overall low homology (approximately 40%) to the respective genes of these species, indicating the lack of comparability of spacer regions between phylogenetically distinct taxa. A region of higher sim-
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