APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1997, p. 1505–1514 Vol. 63, No. 4
0099-2240/97/$04.00+0

Bacterial Diversity of a Carolina Bay as Determined by 16S rRNA Gene Analysis: Confirmation of Novel Taxa

MARK G. WISE,[1] J VAUN MCARTHUR,[2] AND LAWRENCE J. SHIMKETS[1]*

Department of Microbiology, University of Georgia, Athens, Georgia 30602-2605,[1] and Savannah River Ecology Laboratory, Aiken, South Carolina 29802[2]

Received 22 July 1996/Accepted 31 January 1997

Carolina bays are naturally occurring shallow elliptical depressions largely fed by rain and shallow ground water. To identify members of the domain *Bacteria* which inhabit such an environment, we used PCR to construct a library of 16S rRNA genes (16S rDNAs) cloned from DNA extracted from the sediments of Rainbow Bay, located on the Savannah River Site, near Aiken, S.C. Oligonucleotides complementary to conserved regions of 16S rDNA were used as primers for PCR, and gel-purified PCR products were cloned into vector pGEM-T. Partial sequencing of the cloned 16S rDNAs revealed an extensive amount of phylogenetic diversity within this system. Of the 35 clones sequenced, 32 were affiliated with five bacterial groups: 11 clustered with the *Proteobacteria* division (including members of the alpha, beta, and delta subdivisions), 8 clustered with the *Acidobacterium* subdivision of the *Fibrobacter* division (as categorized by the Ribosomal Database Project's taxonomic scheme, version 5.0), 7 clustered with the *Verrucomicrobium* subdivision of the *Planctomyces* division, 3 clustered with the gram-positive bacteria (*Clostridium* and relatives subdivision), and 3 clustered with the green nonsulfur bacteria. One sequence branched very deeply from the *Bacteria* and was found not to be associated with any of the major divisions when phylogenetic trees were constructed. Two clones did not consistently cluster with specific groups and may be chimeric sequences. None of the clones exhibited an exact match to any of the 16S rDNA sequences deposited in the databases, suggesting that most of the bacteria in Rainbow Bay are novel species. In particular, the clones related to the *Acidobacterium* subdivision and the *Verrucomicrobium* subdivision confirm the presence of novel taxa discovered previously in other molecular surveys of this type.

Over the past decade, the use of 16S rRNAs or genes coding for 16S rRNA (16S rDNAs) as molecular markers has become a routine technique for microbial ecologists. This type of analysis can circumvent the limitations of traditional culturing techniques in the assessment of the biodiversity of microbial communities. In one such approach, nucleic acids extracted directly from environmental samples are purified and 16S rDNAs are amplified by the PCR and then cloned and sequenced. Phylogenetic analysis can then be used to determine relationships with 16S rRNA sequences from cultured organisms. Most examinations of this type, carried out in a variety of habitats, have revealed that natural ecosystems include novel species that are unknown to microbiologists (for a review, see reference 2). Evidently, the vast majority of bacteria have never been described. Nevertheless, most investigators using this technique have discovered that the observed diversity is not equally spread throughout the prokaryotic world. Instead phylogenetic analysis has shown that sequences often tend to cluster in distinct groups. These clusters are sometimes called "phylotypes" (although technically, a phylotype can consist of just one sequence), and they are often recovered in independent examinations of the same or similar habitat (or "ecotype") (40). This suggests that certain groups of organisms, phylogenetically related, are adapted to particular habitat types. An excellent example of this phenomenon is the marine water column. Independent studies of this habitat have shown the same novel taxa occurring in samples as geographically separated as the Atlantic Ocean, the Pacific Ocean, and an Antarctic sea (5, 9, 10, 14–16, 38).

* Corresponding author. Phone: (706) 542-2681. Fax: (706) 542-2674. E-mail: shimkets@bscr.uga.edu.

Sediments and soils probably represent the most complex microbial habitat on earth. There may be several thousand species of bacteria in a single gram of soil (43). In order to gain some insight into the types of bacteria found in the sediments of a freshwater pond, we focused on a Carolina bay, a common natural lentic ecosystem located on the Carolina coastal plain. These freshwater ponds of unknown origin are characterized by their elliptical shape and constant orientation (northwest to southeast) (26). Water levels in Carolina bays fluctuate seasonally as a function of precipitation, usually filling in the winter and drying in the spring or summer. Rainbow Bay, the subject of this study, has acidic water and moderate dissolved organic carbon concentrations (36). Primary production by macrophytes, algae, and autotrophic bacteria, as well as input from riparian vegetation, provides the base of the food web in this system.

The eukaryotic biological diversity of Rainbow Bay has been extensively studied with regard to groups ranging from zooplankton to copepods to amphibians (31, 42, 49). To complement these studies, we investigated the microbial diversity of members of the domain *Bacteria*. We cloned and sequenced 16S rDNAs amplified from DNA extracted from the sediments of Rainbow Bay. Here we report the retrieval of clones related to taxa found previously in DNA extracted from Australian soil (27, 39) and more recently in other habitats.

MATERIALS AND METHODS

Sampling. A sediment sample submerged under approximately 18 cm of standing water was collected near the center of Rainbow Bay, located on the Savannah River Site, near Aiken, S.C., on 8 December 1994. The sediment sample extended to approximately 5 cm below the benthic surface.

DNA extraction. DNA was extracted following the method of Tsai and Olson (44), with modifications from Barns et al. (3). Approximately 40 g of sediment (wet weight) was divided into four 10-g samples. To each 10-g sample, 10 ml of

TABLE 1. Summary of taxonomical groups and GenBank accession numbers for Rainbow Bay 16S rDNA clones

Phylogenetic affiliation and clone (RDP designation)	Accession no.
Proteobacteria (2.13)	
Alpha subdivision (2.13.1)	
RB10	U62833
RB13	U62836
RB25	U62846
Beta subdivision (2.13.2)	
RB06	U62830
RB11	U62834
RB12	U62835
RB23	U62844
RB33	U62851
RB37	U62854
Delta subdivision (2.13.4)	
RB18	U62841
RB28	U62848
Fibrobacter and relatives (2.11)	
Acidobacterium subdivision (2.11.2)	
RB04	U62828
RB05	U62829
RB07	U62831
RB08	U62832
RB16	U62839
RB17	U62840
RB29	U62849
RB38	U62855
Planctomyces and relatives (2.9)	
Verrucomicrobium subdivision (2.9.3)	
RB01	U62825
RB02	U62826
RB14	U62837
RB22	U62843
RB24	U62845
RB31	U62850
RB35	U62853
Gram-positive bacteria (2.15)	
Clostridium and relatives subdivision (2.15.2)	
RB15	U62838
RB26	U62847
RB34	U62852
Green nonsulfur bacteria and relatives (2.4)	
Chloroflexus subdivision (2.4.1)	
RB03	U62827
RB21	U62842
RB41	U62857
Inconsistent (possible chimeric sequence)	
RB09	ND[a]
RB36	ND
Unaffiliated	
RB39	U62856

[a] ND, sequence not deposited in database.

120 mM sodium phosphate (pH 8.0) was added, and the slurry was shaken at 150 rpm for 15 min. The slurry was pelleted by centrifugation at 6,000 × *g* for 10 min, and the pellet was resuspended in 10 ml of lysis solution (0.15 M NaCl, 0.1 M Na_2–EDTA [pH 8.0]) containing 15 mg of lysozyme ml^{-1} (added freshly). The mixture was incubated at 37°C for 1 h 30 min with occasional agitation. Proteinase K (2 mg ml^{-1}) was then added, and the samples were incubated at the same temperature for an additional 30 min. Ten milliliters of lysis buffer (0.1 M NaCl, 0.5 M Tris-HCl [pH 8.0], 10% sodium dodecyl sulfate) was added, and three cycles of freezing (−70°C ethanol bath) and thawing (70°C water bath) were conducted. Ten milliliters of 0.1 M Tris-HCl (pH 8.0)-saturated phenol was added to each tube, and the tubes were vortexed to obtain an emulsion. They were then centrifuged at 6,000 × *g* for 10 min, and 15 ml from the top aqueous layer was retained. To this, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the mixture was centrifuged as described above. Then, 12.5 ml from the top phase was collected and mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged as described above. Ten milliliters of the aqueous phase was collected, and 5 g of acid-washed polyvinylpolypyrollidone (PVPP) was added to each tube. PVPP binds humic acids often present in environmental samples that comigrate with DNA in the aqueous phase of phenol extractions (50). The mixture was vortexed and allowed to incubate at 37°C for 30 min with occasional agitation. PVPP was sedimented by centrifugation, and the supernatant was filtered through a 0.45-μm-pore-size filter to remove any remaining PVPP. Two volumes of ice-cold isopropanol was added to precipitate the nucleic acids, and the tubes were stored overnight at −20°C. Nucleic acids were pelleted at 10,000 × *g* for 10 min, and the sample was resuspended in 500 μl of TE (10 mM Tris-HCl, 1 mM EDTA) buffer. Then, 0.1 g of anhydrous ammonium acetate was added and the samples were spun at room temperature for 30 min in a microcentrifuge. One and one-half volumes of isopropanol was added to the supernatant, and the nucleic acids were allowed to precipitate at −20°C for 2 h. After centrifugation (as described above), the samples were suspended in 300 μl of TE and this DNA was pooled and further purified by CsCl-ethidium bromide density-gradient ultracentrifugation (35). As a final purification step, ethanol-precipitated DNA was passed through gel filtration columns (Sephadex G-200) preequilibrated with TE (35). DNA present in the second, third, fourth, and fifth elutions was retained.

PCR amplification and cloning. rDNAs present in the purified samples were amplified by using *Taq* polymerase (Perkin Elmer). The reaction mixture contained the *Bacteria*-specific primer 68F (5′-TNANACATGCAAGTCGAKCG-3′) (5) and the universal primer 1392R (5′-ACGGGCGGTGTGTRC-3′) (2) (final concentration of each, 2 μM), the four deoxynucleoside triphosphates (final concentration of each, 0.2 mM), *Taq* buffer (final concentration, 1×), $MgCl_2$ (final concentration, 4 mM), purified Rainbow Bay environmental DNA (final concentration, 0.6 ng/μl) and 10 U of Amplitaq DNA polymerase. The PCR conditions were as follows: 1 min 30 s at 94°C, followed by 10 cycles of 94°C for 30 s, 53°C for 45 s, and 70°C for 1 min. Then, 10 cycles of 94°C for 30 s, 53°C for 1 min, and 70°C for 2 min 30 s were carried out and followed by 12 cycles of 94°C for 30 s, 53°C for 1 min 15 s, and 70°C for 3 min 30 s. The final extension step was at 70°C for 7 min 30 s. The PCR products were visualized on a 0.7% agarose gel, the size was confirmed to be correct, and the band was excised and purified by the Prep-a-Gene (Bio-Rad) gel purification protocol. The purified PCR products were then cloned into pGEM-T (Promega). The pGEM-T vector is constructed with a 3′-terminal thymidine on each end of a blunt-end digestion product, thus improving the efficiency of ligation of PCR products into the vector by taking advantage of the non-template-dependent addition of a single deoxyadenosine to the 3′ end of PCR products by *Taq* polymerase.

Sequencing and phylogenetic analysis. The first 35 (of a total of 99) clones with the full-length insert (approximately 1.34 kbp) were sequenced by using an automated sequencer (model 373A; Applied Biosystems) and the universal rRNA primers 907R and 1392R (24). Sequence data from the two reactions was merged by using the Fragment Assembly System suite of programs that are part of the University of Wisconsin Genetics Computer Group (GCG) Sequence Analysis Software Package. This resulted in a range of 891 to 987 usable bases for each clone. These sequences were checked for chimeric artifacts by the CHECK_CHIMERA program of the Ribosomal Database Project (RDP), release number 5.0 (30), and compared to similar rDNA sequences retrieved from the RDP, as well as the GenBank and EMBL databases, using the FASTA program of the GCG package. Sequences were aligned by using the PILEUP program of the GCG package, and phylogenetic trees were constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm (18) and the neighbor-joining method (34) by using the PHYLIP package (version 3.5c, from J. Felsenstein, University of Washington). Bootstrap analyses for 100 replicates were performed to provide confidence estimates for tree topologies (11).

Combined PCR-DNA sequencing error rate. Since only one strand of each clone was sequenced and the fidelity of *Taq* polymerase is less than 100%, we decided to empirically determine the error rate of our clone sequences. To do this, *Escherichia coli* JM83 cells were added to sterile sediment (10^{10} cells/g [wet weight]) and DNA was extracted and purified in the same manner as described above. The *E. coli* 16S rDNA was amplified by using the same primers listed above and cloned into pGEM-T. One clone was isolated and sequenced by using the same sequencing primers previously described. The two contiguous sequences from the sequencing reactions were merged into a 968-bp sequence, and this sequence was compared to the published sequence of an *E. coli* 16S rDNA (7). The cloned sequence aligned with the published *E. coli* gene sequence from positions 374 to 1344. Along the length of the alignment there were two mismatches, including one ambiguous N position, and five single-base gaps, yielding an overall identity of 99.3%.

Nucleotide sequence accession numbers. The nucleotide sequences of all 16S rDNA Rainbow Bay clones (except those suspected of being chimeras) have been deposited into GenBank and assigned accession numbers U62825 through U62857. The accession numbers are correlated with specific clones in Table 1.

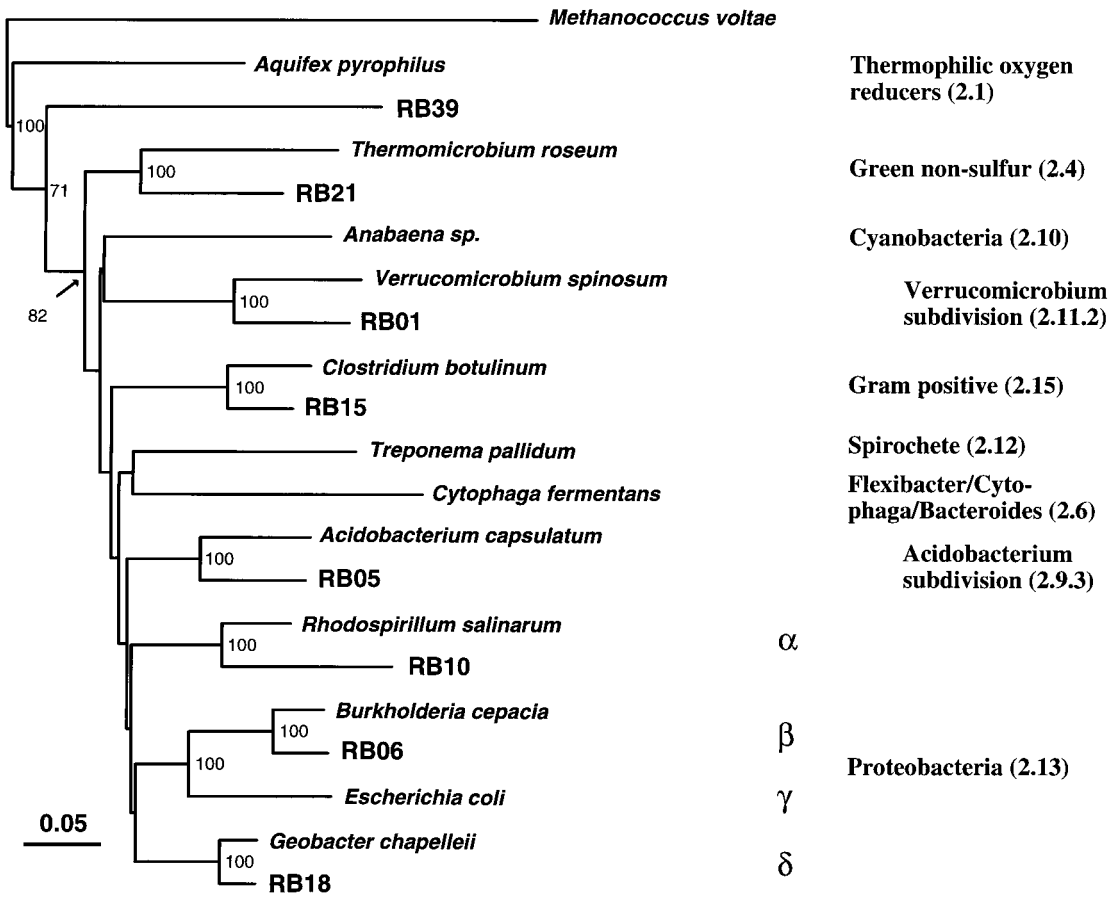

FIG. 1. Large unrooted phylogenetic tree showing the relationship of representative members (but not all members) of the main groups of Rainbow Bay 16S rDNA clones (listed in Table 1) to the major lines of radiation comprising the domain *Bacteria*. This tree was constructed from a matrix of pairwise genetic distances by the neighbor-joining method (34). A total of 917 aligned positions, corresponding to *E. coli* positions 476 to 1355, were used in this analysis. A member of the domain *Archaea* (*Methanococcus voltae*) was used as the outgroup. The scale bar represents 0.05 substitutions per base position. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 50 are not shown). The numbers in parentheses are the designations of the RDP's phylogenetic scheme.

RESULTS

Overall phylogenetic analysis. Large-scale phylogenetic trees (using either 600 or 900 aligned positions corresponding to *E. coli* positions 503 to 1069 and 491 to 1346, respectively) based on all the sequenced clones and representative members of the major lines of *Bacteria* descent were produced (data not shown). Trees constructed from a matrix of pairwise genetic distances by both the neighbor-joining method and the maximum-parsimony algorithm revealed that 32 of the 35 clones could be consistently associated with five major groups of the domain *Bacteria*: the *Proteobacteria* (including members of the alpha, beta, and delta subdivisions), the *Acidobacterium* subdivision of the *Fibrobacter* division, the *Verrucomicrobium* subdivision of the *Planctomyces* division, the gram-positive bacteria, and the green nonsulfur bacteria (Table 1). One clone (RB39) was not associated with any major group and branched deeply within the *Bacteria*. Two clones (RB09 and RB36) were not consistently affiliated with specific groups. These sequences tended to cluster with different taxa depending upon the tree-building algorithm employed. A large-scale phylogenetic tree showing most of the major radiations of the *Bacteria* (with an archaeon as an outgroup) is shown in Fig. 1. This tree was constructed on the basis of 917 aligned positions corresponding to *E. coli* positions 476 to 1355, and it includes representative members, but not all members, of the Rainbow Bay clusters corresponding to the major groups (and RB39) listed in Table 1. A more detailed examination of the relationship among all clones and some members of specific bacterial divisions is given in subsequent figures (see Fig. 2 to 6).

Proteobacteria. Figure 2 shows the results of the phylogenetic analysis of all Rainbow Bay clones related to members of the *Proteobacteria* (RDP designation, 2.13). This tree was constructed from a matrix of pairwise genetic distances by the neighbor-joining method (34) using 905 aligned positions corresponding to *E. coli* positions 500 to 1371. Six clones clustered within the beta subdivision (RDP designation, 2.13.2), three clustered within the alpha subdivision (RDP designation, 2.13.1) and two clustered within the delta subdivision (RDP designation, 2.13.4). Within the beta subdivision, four clones (RB06, RB12, RB33, and RB37) group near species in the *Nitrosomonas* group (RDP designation, 2.13.2.6). GenBank and EMBL database searches using the FASTA program of GCG revealed that two clones, RB33 and RB37, were most identical to *Nitrosospira* sp. isolate AF (46), at 89.2% identity in 931 bp and 90.4% in 934 bp, respectively. *Nitrosospira* is one of the genera comprising the nitrifying bacteria; these are obligate chemoautotrophs that use CO_2 as the sole source of carbon and obtain energy from the oxidation of ammonia to nitrite (20). RB06 shows 96.2% nucleotide identity to uniden-

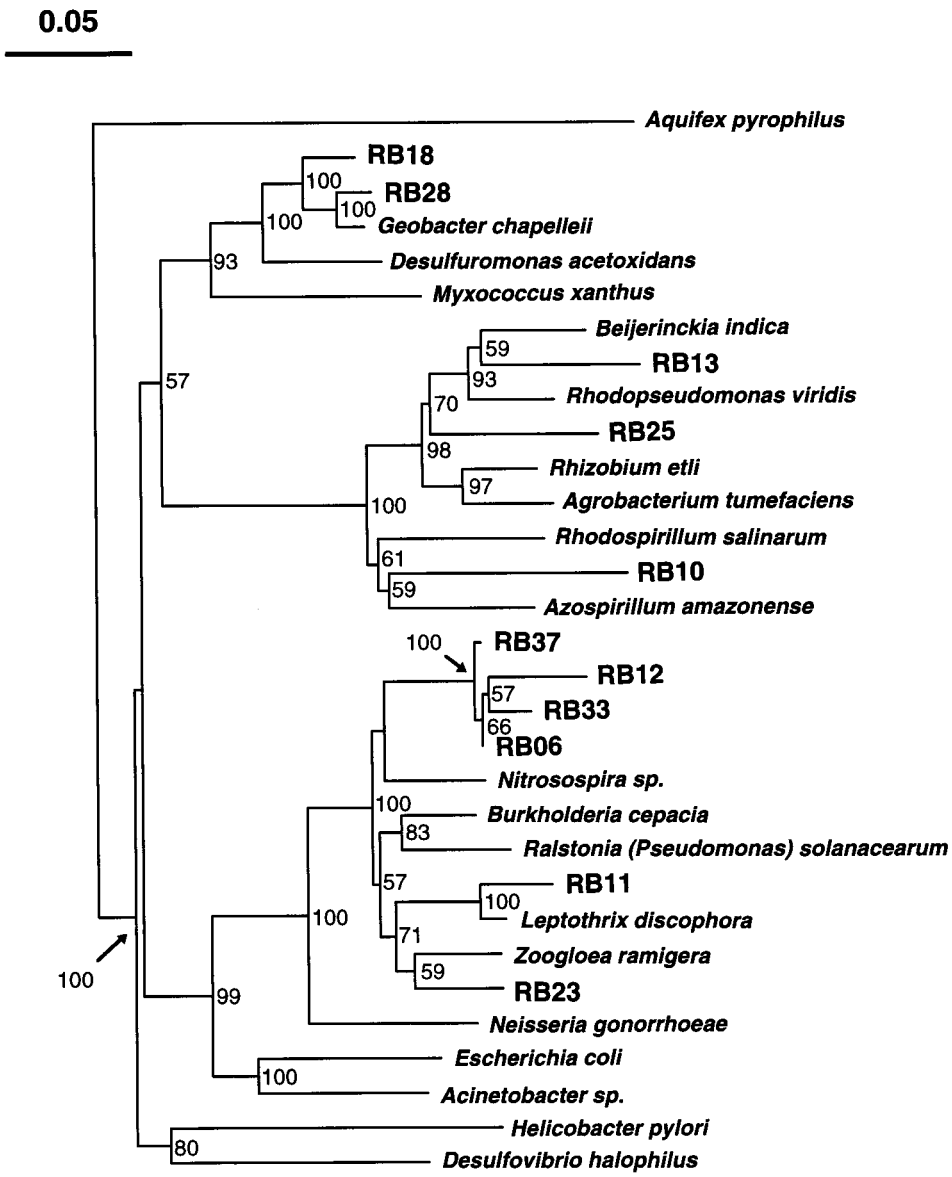

FIG. 2. Unrooted phylogenetic tree showing the relationship of selected Rainbow Bay 16S rDNA clones to representatives of the *Proteobacteria*. This tree was constructed from a matrix of pairwise genetic distances by the neighbor-joining method (34). A total of 905 aligned positions, corresponding to *E. coli* positions 500 to 1371, were used in this analysis. *Aquifex pyrophilus* was used as the outgroup. The scale bar represents 0.05 substitutions per base position. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 50 are not shown).

tified beta proteobacterium clone 10.40 (GenBank accession no. U34035), and RB12 is closely related to unclassified beta proteobacterium isolate B0265 (GenBank accession no. U12216), showing 94.8% identity in 842 bp. One clone (RB23) clusters with *Zoogloea ramigera* (90.8% identity in 975 bp). FASTA searches revealed, however, that this sequence shows slightly higher identity to *Burkholderia* sp. isolate N2P5, at 91.0% identity in 976 bp (GenBank accession no. U37342). RB11 is related to *Leptothrix discophora*, a member of the *Rubrivivax* subgroup (RDP designation, 2.13.2.2.6) as evidenced by the bootstrap value of 100 at that node. This sequence shows 94.9% identity in 891 bp to *L. discophora.*

Among the clones related to the alpha subdivision, two (RB13 and RB25) fall in the *Agrobacterium-Rhizobium* group (RDP designation, 2.13.1.8) and one (RB10) clusters within the *Rhodospirillum rubrum* assemblage (RDP designation, 2.13.1.1). RB13 shows the highest identity to *Rhodopseudomonas acidophila* (89.8% identity in 886 bp), and RB25 is most identical to *Rhizobium etli* (89.5% identity in 940 bp). RB10 is 85% identical to two species: *Rhodospirillum salinarum* and *Azospirillum brasilense* over 906 and 907 bp, respectively.

Both clones that cluster in the *Desulfuromonas* group of the delta subdivision (RDP designation, 2.13.4.3), RB18 and RB28, are closely related to the genus *Geobacter*, a collection of anaerobic dissimilatory metal reducers that has recently been described (29). Both clones show their highest identity to *Geobacter chapelleii*: RB18 is 93.3% identical in 949 bp, while RB28 is 94.9% identical in 945 bp, the latter being one of the highest percentage identities to a known, cultured organism seen in this study.

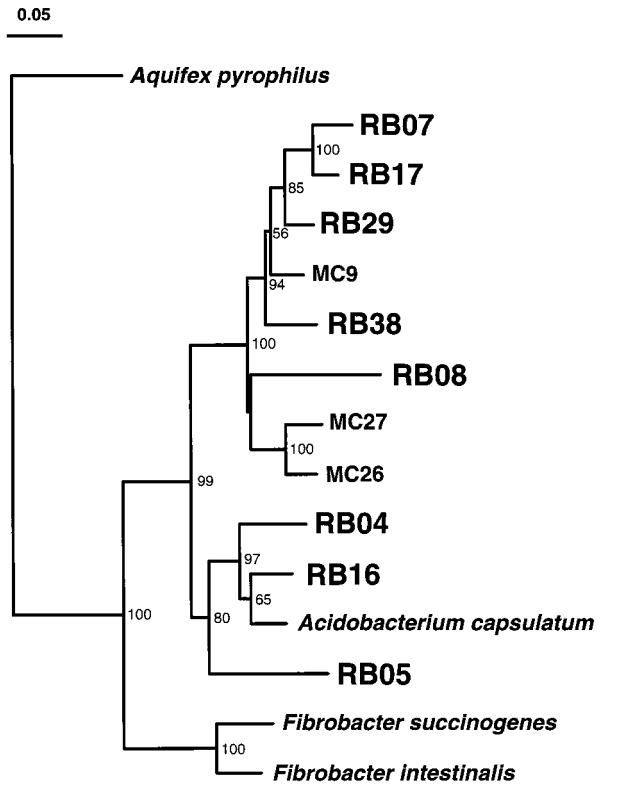

FIG. 3. Unrooted phylogenetic tree showing the relationship of selected Rainbow Bay 16S rDNA clones to representatives of the *Fibrobacter* division and environmental clones from Australian soil (soil cluster MC VI) (39). The tree was constructed from a matrix of pairwise genetic distances by the neighbor-joining method (34). A total of 631 aligned positions, corresponding to *E. coli* positions 482 to 1100, were used in this analysis. *Aquifex pyrophilus* was used as the outgroup. The scale bar represents 0.05 substitutions per base position. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 50 are not shown).

***Acidobacterium* subdivision.** Phylogenetic analysis suggested that eight Rainbow Bay clones belong to a unique group related to the *Acidobacterium* subdivision (RDP designation, 2.11.2), a group that contains the sequence of only one cultured organism, *Acidobacterium capsulatum*. Interestingly, the only other members of this group that we are aware of are environmental clones retrieved from forest soil in Brisbane, Queensland, Australia (39), and clover-grass pasture soil in Wisconsin (4). Some of the clones from Australia (designations with MC, for the Mount Coot-tha region from which they were obtained) have been included on the tree shown in Fig. 3. Since only partial 16S rDNA sequences were available from members of this group (termed soil cluster MC VI), the tree was constructed with only 631 overlapping aligned positions, corresponding to *E. coli* positions 482 to 1100. Nevertheless, the specific relationship among these Rainbow Bay clones, the MC VI clones, and *A. capsulatum* is evident. Since the *Acidobacterium* group is listed as a subdivision of the *Fibrobacter* division according to the most recent RDP phylogenetic scheme, we included two members of the *Fibrobacter* division (*F. succinogenes* and *F. intestinalis*) on this dendrogram. The phylogeny of the *Acidobacterium* subdivision, however, remains controversial: a recent study reported that *A. capsulatum* belongs to a unique lineage deeply branching from either the *Planctomyces* or the gram-positive line (19). Indeed, our analysis did not support the inclusion of *A. capsulatum* (or related clones) within the *Fibrobacter* division, as evidenced by the 100% bootstrap value separating the *Fibrobacter* division from the *Acidobacterium* subdivision. However, we did find evidence for a separate clade consisting of environmental clones related to, but apart from, the *Acidobacterium* subdivision. The three clones RB04, RB05, and RB16 are much more closely related to *A. capsulatum* (showing from 86.6 to 92.9% identity) than are the other five clones (RB07, RB08, RB17, RB29, and RB38) in this group. These five sequences branch off the main *Acidobacterium* cluster (with a 99% bootstrap value) and show a higher percentage identity to certain Mount Coot-tha clones than to *A. capsulatum*. For example RB29 is 94.6% identical to MC9 in 661 bp but only 83.0% identical to *A. capsulatum* in 936 bp.

***Verrucomicrobium* subdivision.** Figure 4 shows the phylogenetic relationship of several Rainbow Bay clones to members of the *Planctomyces* division. These clones are only peripherally associated with any true *Planctomyces* species but instead cluster near the *Verrucomicrobium* subdivision (RDP designation, 2.9.3), a subset of the *Planctomyces* division, according to the RDP. Until very recently, this subdivision contained only one known, cultured organism, *Verrucomicrobium spinosum*, and the sequences of several environmental clones. Included among these are four sequences from the Mount Coot-tha region of Brisbane, Australia (designated soil cluster MC III) (27), clones from the soil of a paddy field (PAD7, PAD18, and PAD50 [GenBank accession no. D26194, D26205, and D26237, respectively]), a clone from soil from a soybean field (FIE19) (45), a clone retrieved from DNA collected from 100 m below the surface of the Pacific Ocean (NH25-19) (15), and a clone obtained from surface soil in eastern Washington State (EA25) (25). Recently, the 16S rDNA of *Prosthecobacter fusiformis* was sequenced and phylogenetic analysis showed that it also fell within this group (17). The dendrogram presented in Fig. 4 shows the phylogenetic relationship of certain Rainbow Bay clones to the MC III soil clones, EA25, *V. spinosum*, and *P. fusiformis*. In order to perform this comparison, we had to reduce the number of positions examined to 609 (corresponding to *E. coli* positions 467 to 1065), the number of positions overlapping between some MC III clones and the Rainbow Bay sequences. Unfortunately, the other environmental clones reported to be related to this group consist of ca. 250 to 280 bases, too few to be useful for this analysis. One Rainbow Bay clone, RB35, groups specifically with *V. spinosum*, *P. fusiformis*, clone EA25, and the MC III soil cluster; this clone is 92.7% identical in 975 bp to EA25, 92.7% identical in 840 bp to MC18, and 86.0% identical in 910 bp to *V. spinosum*. The other six clones in this group, RB01, RB02, RB14, RB22, RB24, and RB31, form their own distinct cluster apart from the *V. spinosum*-MC III group (with a 100% bootstrap value). These clones range from only 84.8 to 87.7% identity to EA25 and from 83.5 to 85.6% identity to *V. spinosum*. The entire group forms a cluster that is unique from both the *Chlamydia* division and the *Planctomyces* division as manifested by the high bootstrap values at the nodes separating these taxa.

Gram-positive bacteria. Gram-positive 16S rDNA sequences were also represented in the library. Three clones (RB15, RB26, and RB34) clustered with members of the *Clostridium* and relatives subdivision (RDP designation, 2.15.2) (Fig. 5). RB26 is related to "*Clostridium saccharoperbutylacetonicum*," showing 98.5% identity in 927 bp, the highest such value observed in this study. "*C. saccharoperbutylacetonicum*" is a saccharolytic, solvent-producing bacterium originally isolated from soil in Japan and used for the industrial production of butanol and acetone (21). RB15 is related to *Clostridium pascui* (93.4% identity in 909 bp), and RB34 is related to

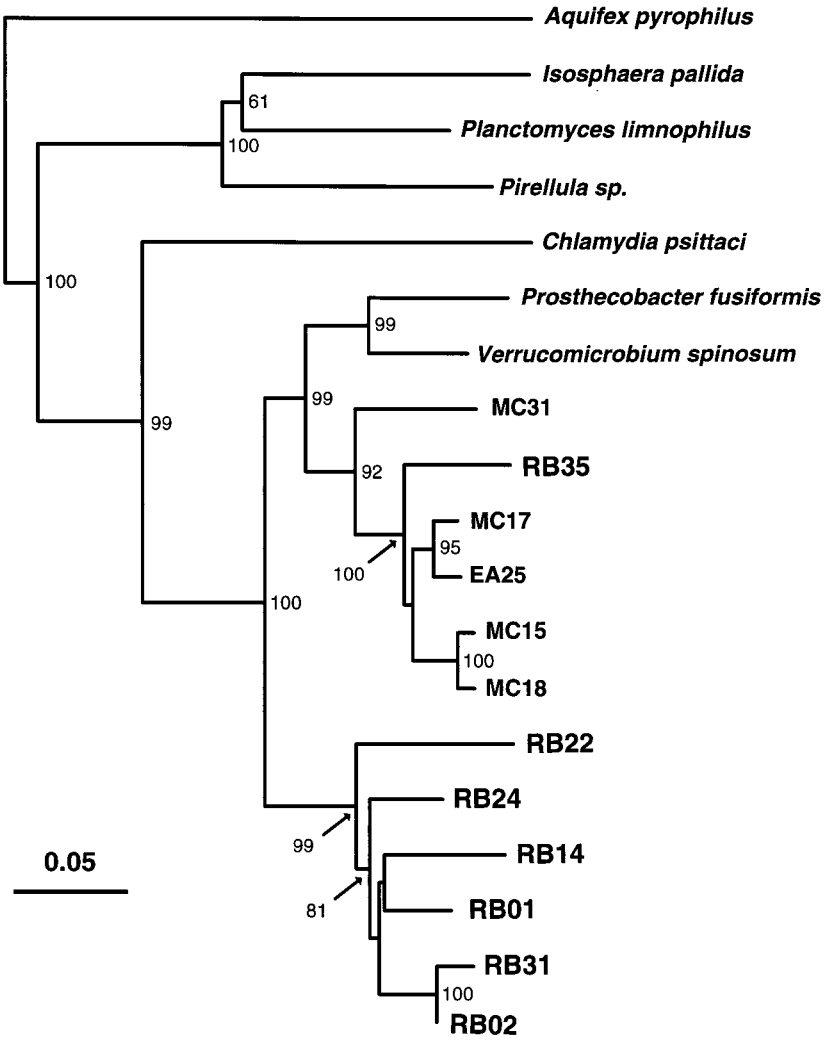

FIG. 4. Unrooted phylogenetic tree showing the relationship of selected Rainbow Bay 16S rDNA clones to representatives of the *Planctomyces* division, *Chlamydia psittaci*, *V. spinosum*, *P. fusiformis*, and environmental clones from Australian soil (cluster MC III) (28) and soil from Washington state (EA25) (25). The tree was constructed from a matrix of pairwise genetic distances by the neighbor-joining method (34). A total of 609 aligned positions, corresponding to *E. coli* positions 467 to 1065, were used in this analysis. *Aquifex pyrophilus* was used as the outgroup. The scale bar represents 0.05 substitutions per base position. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 50 are not shown).

Clostridium subterminale (90.6% identity in 967 bp). The Clostridia are anaerobic spore formers, and their presence in our collection (along with the genus *Geobacter*) indicates that the sediment sampled at Rainbow Bay contained some anaerobic microhabitats.

Green nonsulfur bacteria. Three more clones (RB03, RB21, and RB41) consistently clustered with the green nonsulfur division (RDP designation, 2.4), a branch consisting of mostly anoxygenic photosynthetic bacteria. Figure 6 shows the relationship between these clones and some members of this division. Phylogenetic analysis showed that the closest cultured relative to these three clones in this group is the nonphotosynthetic, obligately aerobic thermophile *Thermomicrobium roseum*. This relationship, however, is only tenuously supported, since the bootstrap value linking these clones to *T. roseum* is less than 50%. Since most members of this division are in fact thermophiles, the presence of Rainbow Bay clones related to this cluster is puzzling. Indeed, when FASTA searches were performed the most identical organisms in the database were often not from this division. RB03 is closest to unidentified bacterium clone SAR202 (GenBank accession no. U20797), from the Sargasso Sea, at 81.5% identity in 912 bp. RB41 is most identical to *Rubrobacter xylanophilus*, at 80.3% identity in 899 bp (*T. roseum* shows 77.1% identity in 951 bp). *Rubrobacter xylanophilus* is a newly described thermophilic, halotolerant species that belongs to the high-G+C-content (G+C%) gram-positive group (8). RB21 is 81.0% identical to *Clostridium aldrichii* in 917 bp, but it is 83.1% identical in 682 bp to the extreme thermophile *Dictyoglomus thermophilum*, a bacterium related to the thermophilic clostridia. Environmental clones related to the green nonsulfur bacteria have previously been obtained from the soil of paddy fields (PAD2 and PAD5 [GenBank accession no. D26189 and D26192, respectively]), but only ca. 250 bases from these clones have been reported, so it was not possible to include them on the phylogenetic tree.

RB39. One clone, RB39, branches deeply within the *Bacteria* and has no close relatives in the database (Fig. 1). FASTA searches showed that this sequence is most similar to a clone recovered from the Sargasso Sea, SAR307, but it was only 75.1% identical in 872 bp. Since chimeric rDNA clones can

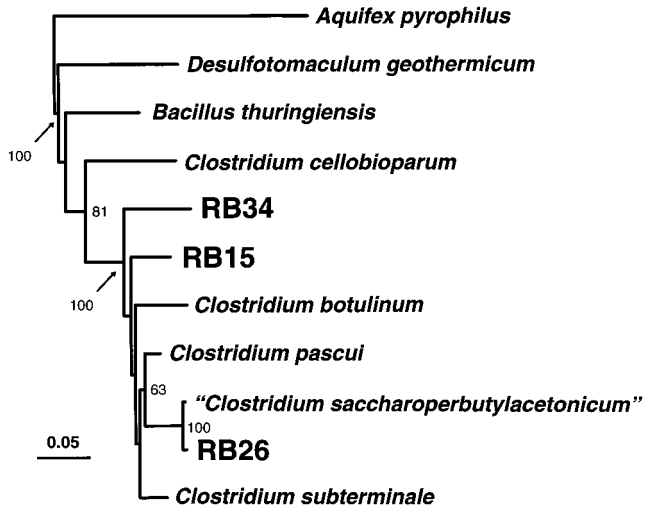

FIG. 5. Unrooted phylogenetic tree showing the relationship of selected Rainbow Bay 16S rDNA clones to representatives of the gram-positive (low G+C%) bacterial subdivision. The tree was constructed from a matrix of pairwise genetic distances by the neighbor-joining method (34). A total of 909 aligned positions, corresponding to *E. coli* positions 484 to 1378, were used in this analysis. *Aquifex pyrophilus* was used as the outgroup. The scale bar represents 0.05 substitutions per base position. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 50 are not shown).

arise during PCR amplification of mixed-population DNAs (28), all sequences were analyzed for chimeric structure by using the RDP's CHECK_CHIMERA program. We were unable to detect any firm evidence implicating RB39 as a chimeric sequence, primarily because similarity values were very low throughout the length of the gene. Since it is difficult to detect chimeras for novel 16S rDNA sequences with no close relatives in the database (23), the best confirmation of this sequence as a true 16S rDNA would be the recovery of this sequence in an independent sample.

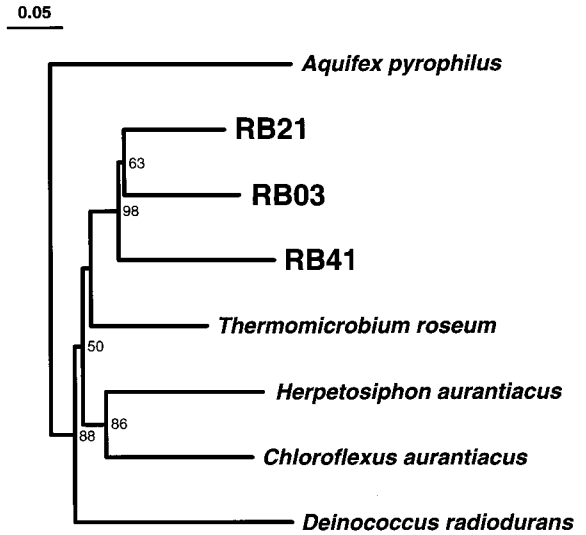

FIG. 6. Unrooted phylogenetic tree showing the relationship of selected Rainbow Bay 16S rDNA clones to representatives of the green nonsulfur bacterial subdivision. The tree was constructed from a matrix of pairwise genetic distances by the neighbor-joining method (34). A total of 946 aligned positions, corresponding to *E. coli* positions 445 to 1377, were used in this analysis. *Aquifex pyrophilus* was used as the outgroup. The scale bar represents 0.05 substitutions per base position. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 50 are not shown).

Possible chimeras. Two clones, however, looked suspiciously chimeric when examined with CHECK_CHIMERA. This program divided the 903 bp of clone RB36 into two fragments: the region from bp 1 to 500 had the highest binary association coefficient (S_{AB}), 0.503, (12) to environmental clone MC13, and the second segment, from position 501 to 895, showed a high S_{AB}, 0.607, to *Cytophaga fermentans*. Although the relationship between S_{AB} and percentage sequence similarity cannot be theoretically derived, a plot of S_{AB} versus percent similarity reveals that these values correspond to approximately 85 to 90% sequence similarity (48). Sequences that can be split into two fragments which show a high degree of similarity to two very different bacteria are a hallmark of chimeras. CHECK_CHIMERA divided clone RB09 into one fragment from position 1 to 640 that was most similar to *Selenomonas sputigena* (S_{AB} = 0.439), and a second fragment from position 641 to 875 that had a S_{AB} to environmental clone PAD39 of 0.797. *Selenomonas* spp. are relatives of the clostridia in the low-G+C% gram-positive group, and PAD39 is a clone from paddy fields that clusters with the *Myxobacteria* in the delta subdivision of the *Proteobacteria*. Although CHECK_CHIMERA is not a definitive method for detection of chimeras (33), the fact that these sequences tended to exhibit an unstable branching order when phylogenetic trees were produced by different algorithms could potentially be accounted for by the presence of chimeric artifacts.

Confirmation of a novel cluster. In order to confirm the presence of a novel cluster of sequences in our sample, we focused on a cluster of Rainbow Bay clones related to the *Verrucomicrobium* subdivision. As described above (and shown in Fig. 4), RB01, RB02, RB14, RB22, RB24, and RB31 form a clade separate from clones in the MC III soil group, EA25, RB35, *V. spinosum*, and *P. fusiformis*. The unique phylogenetic lineage of this cluster was exploited to design primers specific for this group. Primers were discovered by aligning these clones with 16S rDNA sequences from 17 bacterial species representing all major lineages, including *V. spinosum* and *P. fusiformis*, along with the clone sequences from the MC III group, EA25, and RB35. Using the BOX_SHADE program of the GCG package, two oligomers unique to the clade including RB01, RB02, RB14, RB22, RB24, and RB31 were identified. These oligomers are NRBV-1F (5′-CACGTTTGCTGTAAA AGG-3′, corresponding to *E. coli* positions 822 to 839), which hybridizes to the forward strand of the 16S rDNA, and NRBV-2R (5′-TTTCAYCCTTCTACTA-3′, corresponding to *E. coli* positions 1003 to 1014, where Y = C or T), which hybridizes to the reverse strand of the 16S rDNA. Both were tested against the all the sequences in the GenBank and EMBL databases by using the FASTA program of the GCG package and proved to be specific for this novel phylotype. These primers were used in PCR (using the same conditions previously described) to amplify DNA from our original environmental DNA sample from Rainbow Bay sediments. The expected amplification product from the members of this clade is a 198-bp fragment. The results of the PCR are shown in Fig. 7. A product of the expected size was found when the Rainbow Bay environmental DNA was used as a template. No such band was obtained with the negative control, which was the same *E. coli*-seeded sediment DNA used to calculate the PCR-sequencing error rate.

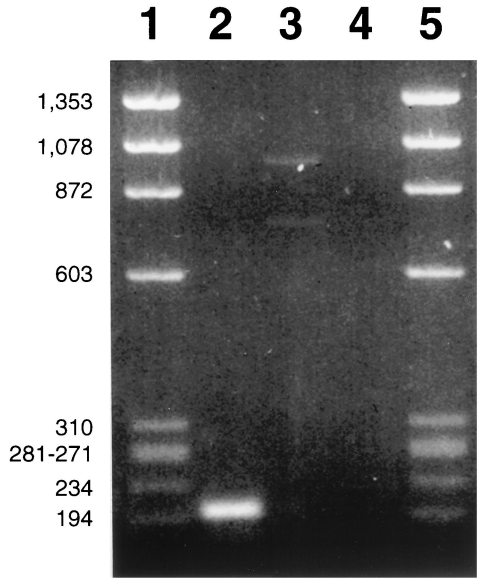

FIG. 7. Agarose (2%) gel showing PCR amplification products obtained by using primers targeted to members of a novel cluster of Rainbow Bay clones (RB01, RB02, RB14, RB22, RB24, RB31) related to the order *Verrucomicrobiales*. See the text for details on primer design and reaction conditions. Lanes 1 and 5, molecular weight standard (ϕX174 DNA-*Hae*III digest); lane 2, amplification products with Rainbow Bay environmental DNA as the template; lane 3, amplification products with DNA extracted from sterile sediment spiked with *E. coli* cells (10^{10} cells/g of sediment [wet weight]) as the template; lane 4, no template added.

DISCUSSION

The phylogenetic analysis of 16S rDNA clones from Rainbow Bay revealed a significant degree of microbial diversity; all the clones sequenced and used in this analysis seemed to represent novel bacterial species. One clone (RB26) was 98.5% identical to the sequence of a cultured organism ("*Clostridium saccharoperbutylacetonicum*"); considering the error rate of 0.7%, these sequences are significantly different from one another. No other clones were greater than 95% identical to known species. These results confirm the suspicions raised by previous studies, i.e., that the vast majority of microbial diversity is uncharacterized. Two groups of Rainbow Bay clones stand out as being especially interesting, and these warrant further discussion since, in both cases, (i) cultured relatives to these clones are particularly underrepresented in the 16S rDNA sequence databases, (ii) similar sequences have been recovered in molecular environmental surveys elsewhere, and (iii) both groups represent distinct radiations within the *Bacteria* that may be deserving of unique taxonomical status.

First, we retrieved clones which belong to a phylogenetic group that consists of only one known, cultured species, *A. capsulatum*, and the sequence of several environmental clones isolated from forest soil in Australia. *A. capsulatum* is a gram-negative, acidophilic, chemo-organotrophic, menaquinone-containing bacterium whose phylogeny remains controversial. Reports suggest that it belongs to a unique lineage that branches deeply from either the *Fibrobacter*, gram-positive, or *Planctomyces* line (19, 30). *A. capsulatum* shows the highest level of sequence similarity to *Heliobacterium chlorum* (a gram-positive bacterium), but this level is only 81% (19). We observed eight clones that grouped with *A. capsulatum*, which we were able to subdivide into two clades (Fig. 3). The first cluster contains three clones that are close relatives of *A. capsulatum*; the second cluster contains five Rainbow Bay clones that branch off with a 99% bootstrap value. Clones in this group are most similar to environmental clones discovered in Australia when 16S rDNAs were amplified from DNA extracted directly from forest soil with bacterium-specific and *Streptomyces*-specific primers (39). Members of the MC VI cluster, as this group was termed, were tentatively labeled as unknown actinomycetes, although the membership of this group within the actinomycetes was not reproducible by bootstrapping. The phylogenetic scheme presented by the RDP, however, groups the MC IV clones with *A. capsulatum*, their closest cultured relative, in the *Fibrobacter* division. Our analysis showed no evidence that these groups are related to the *Fibrobacter* division; instead our data suggest that the organisms represented by the Rainbow Bay clones and the MC VI clones from a distinct group deserving of their own taxonomical unit.

The Australian soil sampled by Stackebrandt et al. (39) was quite acidic (pH 4.2); although we did not measure the pH of our sample, sediments from Carolina bays are also known to be acidic, typically ranging from pH 5 to 6 (26). *A. capsulatum* strains were originally isolated from acid mineral environments (22), and they grow between pH 3.0 and 6.0. Clones recovered from Rainbow Bay and Mount Coot-tha soil could represent species descendent from *A. capsulatum* that have adapted to mildly acidic growth medium. The only other study that we are aware of that reported sequences related to *A. capsulatum* was carried out on slightly acidic (pH 6.5) soil in Wisconsin. Although it is not possible to state conclusively with such a small number of samples, this may be an example of a phylotype being correlated with an ecotype (acidic environments); it would be interesting to see whether RB clones from this group are recoverable from alkaline soil or sediment. Regardless, the fact that clones related to this group were retrieved in areas as geographically separated as South Carolina, Wisconsin, and Australia suggests that members of this group are widespread and presently overlooked, perhaps due to limitations of culturing techniques.

The second group of clones of interest presents a strikingly similar story. Seven Rainbow Bay clones cluster in a group that consists of but two known, cultured organisms, *V. spinosum* and *P. fusiformis*, and several clones retrieved from DNA extracted from environments as diverse as paddy and soybean fields, the Pacific Ocean, and the forest soil in Australia mentioned above. The MC III soil clones were the first such sequences reported, and they originally were believed to represent a novel phylum (27) with no close relatives among all cultured bacterial species. Recently, however, the full 16S rDNA of *V. spinosum* was sequenced and shown to be related to clones in this group (47). *V. spinosum* is a heterotrophic, fimbriate, prosthecate organism originally isolated from an alkaline eutrophic lake in Germany (37). *V. spinosum* is only distantly related to most of the other prosthecate bacteria and occupies a unique phylogenetic lineage (1, 47). On the basis of 16S rRNA analysis, *V. spinosum* has been placed in the *Planctomyces* division (30), although this relationship is remote at best. It shows a lower G+C% than members of the *Planctomyces* division, and its cell walls contain *meso*-diaminopimelic acid, unlike those of the *Planctomyces* division, which lack peptidoglycan (13). *V. spinosum* and related clones could represent a distinct division of the *Bacteria* (27, 47), to which a second appendaged bacterium, *P. fusiformis*, was recently added (17).

Phylogenetic analysis of our results showed that one clone, RB35, is closely related to the *V. spinosum*-MC III group, but a second cluster of six Rainbow Bay clones constitute a unique lineage, as evidenced by the 100% bootstrap value separating this clade from the *V. spinosum*-MC III group (Fig. 4). This

group seems to be independent of the *V. spinosum*-MC III group, constituting a distinct radiation. If we consider the *V. spinosum-P. fusiformis*-MC III-EA25-RB group as a whole, these bacteria seem to have a global distribution and are present in a diverse variety of habitat types. Members of this group (isolates or clones) have been collected from acidic soil (27), freshwater sediment (this study), eutrophic alkaline pond water (37), raw sewage (41), and even an oligotrophic saltwater subsurface open-ocean habitat (15). It seems unlikely, then, that this phylotype is correlated with an ecotype, but instead these microorganisms seem to enjoy a global distribution and are well adapted to a variety of environmental situations.

The major question remaining is whether these groups are indeed widespread and numerous in many environments or their retrieval is an artifact of selective recovery by the methodology employed here. PCR is known to be somewhat biased in its amplification (32); however, another member of our research group has been able to obtain 16S rRNA clones related to the *V. spinosum*-MC III group using a direct cloning method that does not rely on PCR (6). Reproducing the retrieval of some of the most unique sequences obtained here (i.e., RB39) in an independent sample would provide assurance that these clones do indeed represent novel microorganisms and are not artifactual. By using the information in sequence data, verification may ultimately be possible by isolating these novel bacterial species in pure culture. Although closely related phylogenetic groups often contain taxa of widely differing physiological types, environmental sequences similar to those of culturable strains may significantly enhance the chance of developing appropriate culture conditions for novel bacterial groups. Also, sequence data may allow one to develop group-specific nucleic acid probes that could prove useful in determining the abundance of such groups.

ACKNOWLEDGMENTS

We thank B. Hedlund for furnishing the 16S rDNA sequence of *P. fusiformis* and A. Buchan for proofreading an earlier version of the manuscript.

This research was supported by Financial Assistance Award DE-FC09-96SR18546 from the U.S. Department of Energy to the University of Georgia Research Foundation.

ADDENDUM IN PROOF

Subsequent to the most recent revision of this paper, 16S rDNA sequences related to the *Acidobacterium* subdivision have been deposited into the nucleic acid databases. These clone sequences (with accession numbers) are TM1 (X97097), TM2 (X97098), TM6 (X97099), TM13 (X97100), TM29 (X97101), TM21 (X97102), TM10 (X97103), TM44 (X97104), TM22 (X97105), TM84 (X97106), TM72 (X97107), TM255 (X97108), and TM200 (X97109) from DNA extracted from peat in Germany and Ep_T1.152 (Z73363), Ep_T1.153 (Z73364), Ep_T1.154 (Z73365), Ep_T1.172 (Z73366), Ep_T1.184 (Z73367), Ep_T1.185 (Z73368) and Ep_T1.186 (Z73369) from DNA extracted from forest soil in England.

REFERENCES

1. **Albrecht, W., A. Fischer, J. Smida, and E. Stackebrandt.** 1987. *Verrucomicrobium spinosum*, a eubacterium representing an ancient line of descent. System. Appl. Microbiol. **10:**57–62.
2. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
3. **Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace.** 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. USA **91:**1609–1613.
4. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935–1943.
5. **Britschgi, T. B., and S. J. Giovannoni.** 1991. Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. Appl. Environ. Microbiol. **57:**1707–1713.
6. **Brofft, J. E.** 1995. Unpublished data.
7. **Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli. Proc. Natl. Acad. Sci. USA **75:**4801–4805.
8. **Carreto, L., E. Moore, M. F. Nobre, R. Wait, P. W. Riley, R. J. Sharp, and M. S. da Costa.** 1996. *Rubrobacter xylanophilus* sp. nov., a new thermophilic species isolated from a thermally polluted effluent. Int. J. Syst. Bacteriol. **46:**460–465.
9. **DeLong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA **89:**5685–5689.
10. **DeLong, E. F., K. Y. Wu, B. B. Prezelin, and R. V. Jovine.** 1994. High abundance of Archaea in Antarctic marine picoplankton. Nature **371:**695–697.
11. **Felsenstein, J.** 1985. Confidence limits of phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
12. **Fox, G. E., K. J. Peckman, and C. R. Woese.** 1977. Comparative cataloging of 16S ribosomal ribonucleic acid: molecular approach to prokaryotic systematics. Int. J. Syst. Bacteriol. **27:**44–57.
13. **Fuerst, J. A.** 1995. The planctomycetes: emerging models for microbial ecology, evolution and cell biology. Microbiology (Reading) **141:**1493–1506.
14. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1992. Novel major archaebacterial group from marine plankton. Nature **356:**148–149.
15. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific oceans. Appl. Environ. Microbiol. **59:**1294–1302.
16. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature **345:**60–63.
17. **Hedlund, B. P., J. J. Gosink, and J. T. Staley.** 1996. Phylogeny of *Prosthecobacter*, the fusiform caulobacters: members of a recently discovered division of the *Bacteria*. Int. J. Syst. Bacteriol. **46:**960–966.
18. **Hillis, D. M., M. W. Allard, and M. M. Miyamoto.** 1993. Analysis of DNA sequence data: phylogenetic inference, p. 456–487. *In* E. A. Zimmer, T. J. White, R. L. Cann, and A. C. Wilson (ed.), Molecular evolution: producing the biochemical data. Academic Press, Inc., San Diego, Calif.
19. **Hiraishi, A., N. Kishimoto, Y. Kosako, N. Wakao, and T. Tano.** 1995. Phylogenetic position of the menaquinone-containing acidophilic chemo-organotroph *Acidobacterium capsulatum*. FEMS Microbiol. Lett. **132:**91–94.
20. **Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. Bergey's manual of determinative bacteriology, 9th ed. Williams & Wilkins, Baltimore, Md.
21. **Keis, S., C. F. Bennett, V. K. Ward, and D. T. Jones.** 1995. Taxonomy and phylogeny of industrial solvent-producing clostridia. Int. J. Syst. Bacteriol. **45:**693–705.
22. **Kishimoto, N., Y. Kosako, and T. Tano.** 1991. *Acidobacterium capsulatum* gen. nov., sp. nov.: an acidophilic chemo-organotrophic bacterium containing menaquinone from acidic mineral environment. Curr. Microbiol. **22:**1–7.
23. **Kopczynski, E. D., M. M. Bateson, and D. M. Ward.** 1994. Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. Appl. Environ. Microbiol. **60:**746–748.
24. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Ltd., Chichester, United Kingdom.
25. **Lee, S.-Y., J. Bollinger, D. Bezdicek, and A. Ogram.** 1996. Estimation of the abundance of an uncultivated soil bacterial strain by a competitive quantitative PCR method. Appl. Environ. Microbiol. **62:**3787–3793.
26. **Leff, L. G., J. L. Burch, and J. V. McArthur.** 1991. Bacterial use of dissolved organic carbon from Carolina bays. Am. Midl. Nat. **126:**308–316.
27. **Liesack, W., and E. Stackebrandt.** 1992. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol. **174:**5072–5078.
28. **Liesack, W., H. Weyland, and E. Stackebrandt.** 1991. Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microb. Ecol. **21:**191–198.
29. **Lovley, D. R., S. J. Giovannoni, D. C. White, J. E. Champine, E. J. P. Phillips, Y. A. Gorby, and S. Goodwin.** 1993. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch. Microbiol. **159:**336–344.
30. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The Ribosomal Database Project. Nucleic Acids Res. **22:**3485–3487.
31. **Pechmann, J. K., D. E. Scott, R. D. Semlitsch, J. P. Caldwell, L. J. Vitt, and J. W. Gibbons.** 1991. Declining amphibian populations: the problem of separating human impacts from natural fluctuations. Science **253:**892–895.
32. **Reysenbach, A.-L., L. J. Giver, G. S. Wickham, and N. R. Pace.** 1992. Differential amplification of rRNA genes by polymerase chain reaction.

Appl. Environ. Microbiol. **58:**3417–3418.
33. **Robinson-Cox, J. F., M. M. Bateson, and D. M. Ward.** 1995. Evaluation of nearest-neighbor methods for detection of chimeric small-subunit rRNA sequences. Appl. Environ. Microbiol. **61:**1240–1245.
34. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
36. **Schalles, J. F.** 1989. Comparative chemical limnology of Carolina Bay wetlands on the Upper Coastal Plain of South Carolina. DOE (U.S. Dep. Energy) Symp. Ser. **61:**89–111.
37. **Schlesner, H.** 1987. *Verrucomicrobium spinosum* gen. nov., sp. nov.: a fimbriated prosthecate bacterium. Syst. Appl. Microbiol. **10:**54–56.
38. **Schmidt, T. M., E. F. DeLong, and N. R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173:**4371–4378.
39. **Stackebrandt, E., W. Liesack, and B. M. Goebel.** 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. FASEB J. **7:**232–236.
40. **Stahl, D. A.** 1995. Application of phylogenetically based hybridization probes to microbial ecology. Mol. Ecol. **4:**535–542.
41. **Staley, J. T., J. A. M. De Bont, and K. De Joge.** 1976. *Prosthecobacter fusiformis* nov. gen. et sp., the fusiform caulobacter. Antonie Leeuwenhoek **42:**333–342.
42. **Taylor, B. E., and D. L. Mahoney.** 1990. Zooplankton in Rainbow Bay, a Carolina bay pond: population dynamics in a temporary habitat. Freshwater Biol. **24:**597–612.
43. **Torsvik, V., J. Goksøyr, and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56:**782–787.
44. **Tsai, Y.-L., and B. H. Olson.** 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. **57:**1070–1074.
45. **Ueda, T., Y. Suga, and T. Matsuguchi.** 1995. Molecular phylogenetic analysis of a soil microbial community in a soybean field. Eur. J. Soil Sci. **46:**415–421.
46. **Utaaker, J. B., L. Bakken, Q. Q. Jiang, and I. F. Nes.** 1995. Phylogenetic analysis of seven new isolates of the highly related ammonia-oxidizing bacteria based on 16S rRNA gene sequencing. Syst. Appl. Microbiol. **18:**549–559.
47. **Ward-Rainey, N., F. A. Rainey, H. Schlesner, and E. Stackebrandt.** 1995. Assignment of hitherto unidentified 16S rDNA species to a main line of descent within the domain *Bacteria*. Microbiology (Reading) **141:**3247–3250.
48. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
49. **Wyngaard, G. A., B. E. Taylor, and D. L. Mahoney.** 1991. Emergence and dynamics of cyclopoid copepods in an unpredictable environment. Freshwater Biol. **25:**219–232.
50. **Young, C. C., R. L. Burghoff, L. G. Keim, V. Minak-Bernero, J. R. Lute, and S. M. Hinton.** 1993. Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. Appl. Environ. Microbiol. **59:**1972–1974.