

Isolation of *Gemmata*-Like and *Isosphaera*-Like Planctomycete Bacteria from Soil and Freshwater

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New cultured strains of the planctomycete division (order *Planctomycetales*) of the domain *Bacteria* related to species in the genera *Gemmata* and *Isosphaera* were isolated from soil, freshwater, and a laboratory ampicillin solution. Phylogenetic analysis of the 16S rRNA gene from eight representative isolates showed that all the isolates were members of the planctomycete division. Six isolates clustered with *Gemmata obscuriglobus* and related strains, while two isolates clustered with *Isosphaera pallida*. A double-membrane-bounded nucleoid was observed in *Gemmata*-related isolates but not in *Isosphaera*-related isolates, consistent with the ultrastructures of existing species of each genus. Two isolates from this study represent the first planctomycetes successfully cultivated from soil.

The increasing significance of planctomycetes (i.e., members of the order *Planctomycetales*) has been reported within the areas of evolution, molecular ecology, and cell biology (6, 24, 27, 41). The planctomycetes form a distinctive division of the domain *Bacteria* (20, 31, 35) characterized by budding reproduction and the absence of peptidoglycan in their cell walls (14, 19). Four cultured genera so far have been described; all are aerobic chemoheterotrophs—*Planctomyces*, *Pirellula*, *Gemmata*, and *Isosphaera*. *Gemmata* and *Isosphaera* have been described only on the basis of a single species each: *Gemmata obscuriglobus* and *Isosphaera pallida* (5, 9, 38). The diversity of the group extends beyond these to include at least two other planctomycete genera at *Candidatus* status (“*Brocadia*” and “*Kuenenia*”) and consisting of anaerobic ammonium-oxidizing autotrophs (32, 40).

Planctomycetes were all originally isolated from aquatic habitats as diverse as acid bogs and sewage treatment plants, and direct molecular cloning and probe studies have extended this diversity (1, 5, 9, 12, 17, 27, 28, 29, 30, 33, 43, 44). Many of the original isolates have been confirmed as planctomycetes by phylogenetic analysis of rRNA sequences (11, 42). Seven clones related to planctomycetes were reported as soil cluster II in a study of soil from the Mt. Coot-tha region; this was one of the first molecular ecology investigations of the bacterial diversity in any soil microbial community (21, 37). Subsequently, several studies reported clones of planctomycetes from clone libraries prepared from different environments (2, 10, 15, 17, 18, 32, 40). Because of the increasing evidence for the ubiquitous distribution of planctomycetes in the environment from direct cloning retrieval, isolation of such organisms in pure cultures is needed to enrich the understanding of their

potential ecological role through study of their physiology and growth characteristics.

Samples used as inocula in isolation attempts were water obtained from University Lake, The University of Queensland St. Lucia Campus, Brisbane, Queensland, Australia, and the central library fountain of the same campus; soil collected from Mt. Coot-tha, Brisbane, Queensland, Australia, and Charlies Crossing Rd., Upper Coomera, Queensland, Australia; and a laboratory ampicillin solution (10 mg ml⁻¹) which had not been filter sterilized. Primary isolation and subculture media were M1 agar (30), R2A agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.), and PYGV agar (39), all with 1.5% agar, or M1 broth, 0.1% peptone in tap water, or sterile tap water; the media contained combinations of either ampicillin (200 µg ml⁻¹), streptomycin (1,000 µg ml⁻¹), and cycloheximide (20 to 100 µg ml⁻¹) or penicillin G (500 µg ml⁻¹), streptomycin (1,000 µg ml⁻¹), and amphotericin B (Fungizone) (0.25 or 0.5 µg ml⁻¹). Water samples were inoculated onto selective agar media; soil samples were suspended in sterile tap water before inoculation onto agar; and enrichments were inoculated by mixing an equal volume of water sample with a sterile deionized water solution of penicillin, streptomycin, and amphotericin B, each at double the concentration in selective media. The isolates have been deposited into the Australian Collection of Microorganisms (ACM) as ACM 5050 to ACM 5057. Micromanipulation was performed by using the methods and apparatus of Skerman (34) to separate a *Gemmata*-like isolate (strain Soil9) from a cocultured nonplanctomycete isolate after the conventional subculturing method failed to do so.

Cells of isolates were negatively stained with filtered (0.22-µm-pore-size membrane) 1% uranyl acetate containing 0.4% sucrose. Methods for cryofixation, cryosubstitution, and thin sectioning are fully described elsewhere (22).

DNA was extracted from cultures grown on M1 agar by one of the following methods: (i) direct heat lysis from colony growth deposited in a PCR reaction tube, done at 98°C for 2 to 10 min, with Taq buffer (Bresatec) and primers and Taq polymerase (Bresatec) subsequently added to form a complete

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PCR reaction mixture; (ii) boiling cells in 200 μ l of 5% Chelex 100 resin (Bio-Rad catalog no. 142-2832) and, after centrifugation, adding 4 to 5 μ l of supernatant per 100 μ l of PCR reaction mixture; or (iii) lysing cells in saline-EDTA with lysozyme (37°C for 30 min), followed by proteinase K-sodium dodecyl sulfate (50 to 55°C for 30 min), this step being followed by phenol-chloroform (1:1) protein denaturation and cleanup of the aqueous phase using a Prep-A-Gene kit (Bio-Rad) with 0.5 μ l of the resulting DNA used per 100 μ l of PCR reaction mixture.

Small-subunit (16S) ribosomal DNA (rDNA) was amplified using primer set 27f-1492r (16). PCR and cycle sequencing were performed as previously described (7, 13). The primers used for 16S rDNA sequencing of isolates were those described by Lane (16) or planctomycete-amplifying primers of our own design: 27f, 342r, 357f, 530f, 1114f, PLN945f (5'-GG GGCTCACACAAGCGGTGG), 1101r, 687r, PLN926r (5'-C CACCGCTTGTGTGAGCCCC), 1230fplanc [5'-CTGCACA CGT(G/C)CTACAATG], and 1492r (primers are based on *Escherichia coli* numbering [3]).

16S rDNA sequences of new isolates were imported into the ARB software package (26; www.biol.chemie.tu-muenchen.de/pub/ARB/) for alignment and phylogenetic analysis. The integrity of sequence data was checked by comparing complementary stem regions determined from the secondary structure of the 16S rRNA molecule. Variable regions of the alignment were excluded from the data set by using the bacterial mask of Lane (16) within ARB. Trees were constructed by using distance matrix method DNADIST and neighbor-joining tree generation within NEIGHBOR of the PHYLIP software implemented within 2D-ANGIS (Australian National Genomic Information Service; http://morgan.angis.su.oz.au/). Bootstrap analysis was performed by using SEQBOOT and CONSENSE within PHYLIP with 100 resamplings. A pairwise similarity matrix was generated by using the ae2 alignment editor (ftp://ftp.bio.indiana.edu/molbio/unix/) and the ARB alignment for the relevant phylotypes.

Eight planctomycete-like isolates were isolated from soil, freshwater, or a laboratory antibiotic solution by using enrichment in the presence of combinations of streptomycin as a protein synthesis inhibitor, penicillin or ampicillin as a peptidoglycan synthesis-inhibiting compound, and either amphotericin B or cycloheximide as an antifungal agent. Among the eight representative isolates studied, strains JW9-3f1, JW10-3f1, CJuq14, and CJuq11 are all from a eutrophic lake (on the St. Lucia Campus of The University of Queensland); JW11-2f5 is from water from an ornamental fountain (central library fountain); JW3-8s0 is from Mt. Coot-tha soil; strain Soil9 is from soil at another site (Charlies Crossing Rd.); and C2-3 is from a laboratory ampicillin solution. Another 17 isolates derived from Mt. Coot-tha soil were found by repetitive extragenic palindromic-PCR typing to be replicates indistinguishable by band patterns from strain JW3-8s0 (unpublished data). The new isolates are all pink or red pigmented, except for strain C2-3, which is nonpigmented. Strains C2-3 and CJuq11, unlike the *I. pallida* type strain, which needs incubation in enriched CO₂ at 42°C, can both grow in air not supplemented with CO₂ at 28°C, as can all the *Gemmata*-like isolates. All of the new isolates, both those with close relations to *Gemmata* and those with closest relations to *Isosphaera*, possess spherical

cells and reproduce by budding, as determined by phase-contrast microscopy. Morphological features are illustrated for some of these isolates in the electron micrographs of Fig. 1. Young cultures of pink *Gemmata*-like isolates are motile, but no swimming motility was observed in cultures of *Isosphaera*-like isolates. *Isosphaera*-like isolates did not exhibit filaments.

When viewed by transmission electron microscopy after negative staining with uranyl acetate, all of the isolates display crateriform structures on their cell walls, as illustrated for the *Gemmata*-like strain JW11-2f5 and the *Isosphaera*-like strain CJuq11 in Fig. 1A and B. These structures are electron-dense circular regions of negative stain accumulation characteristic of planctomycetes and were evenly distributed over the cell surface, whether the strain proved to be *Gemmata*-like or *Isosphaera*-like by 16S rRNA sequence analysis. Thin-sectioned cells of strains JW3-8s0, JW9-3f1, JW10-3f1, JW11-2f5, Soil9, and CJuq14 (*Gemmata*-like, as determined by 16S rRNA sequence relationships; see below) all display a nuclear body containing the nucleoid itself and surrounded by an envelope consisting of two membranes, as illustrated for representative strain JW3-8s0 in Fig. 1C. They also possess at the cell rim a peripheral electron-dense region bounded by an inner intracytoplasmic membrane, which divides the cell into two compartments, one with ribosomes and one without them. The central ribosome-containing region is the one which contains the nuclear body compartment. Even strain JW10-3f1, which is relatively distant from *G. obscuriglobus* but still clusters within the *Gemmata* group, possesses a nuclear body. Compartmentalization was seen for cells of C2-3 and CJuq11 (*Isosphaera*-like as determined by 16S rRNA sequence relationships; see below); however, their compartments differ in appearance from those of the *Gemmata* group, as illustrated for *Isosphaera*-like strain C2-3 in Fig. 1D. There are no double-membrane-bounded nuclear bodies in the *Isosphaera*-like strains. The nucleoid is contained within an internal compartment bounded by a single membrane only.

Nearly complete 16S rRNA gene sequences (1,447 to 1,511 bp) were obtained for the eight new isolates, and their phylogenetic relationship to existing planctomycete isolates and cloned sequences from uncultured members of native microbial communities was assessed. Six of the eight new isolates, JW11-2f5, JW9-3f1, Soil9, JW3-8s0, CJuq14, and JW10-3f1, clustered within the *Gemmata* group defined by *G. obscuriglobus*. Isolates C2-3 and CJuq11 clustered within the *Isosphaera* group defined by *I. pallida* (Fig. 2). Similarity values between the *Gemmata*-related strains and *G. obscuriglobus* and the *Isosphaera*-related strains and *I. pallida* are consistent with phylogenetic clusters. The *Gemmata*-like strain JW11-2f5 is the most similar to *G. obscuriglobus* at 96.4%, while JW9-3f1, Soil9, and JW3-8s0 are greater than 99% similar to each other and range from 94.3 to 95.1% in sequence similarity to *G. obscuriglobus*. Strains CJuq14 and JW10-3f1 display the lowest level of similarity to *G. obscuriglobus* at 92.2 and 86.6%, respectively. The *Isosphaera*-like strains C2-3 and CJuq11 display only 89.4 and 89.8% similarity to *I. pallida* but cluster with the Mt. Coot-tha soil clone MC25 and the northern Germany compost water strain 666, respectively. Most of the planctomycete signature nucleotides for 16S rRNA that are characteristic of a given genus group (7), either the *Gemmata* group or the *Isosphaera* group, are also present in the new isolates. Isolates

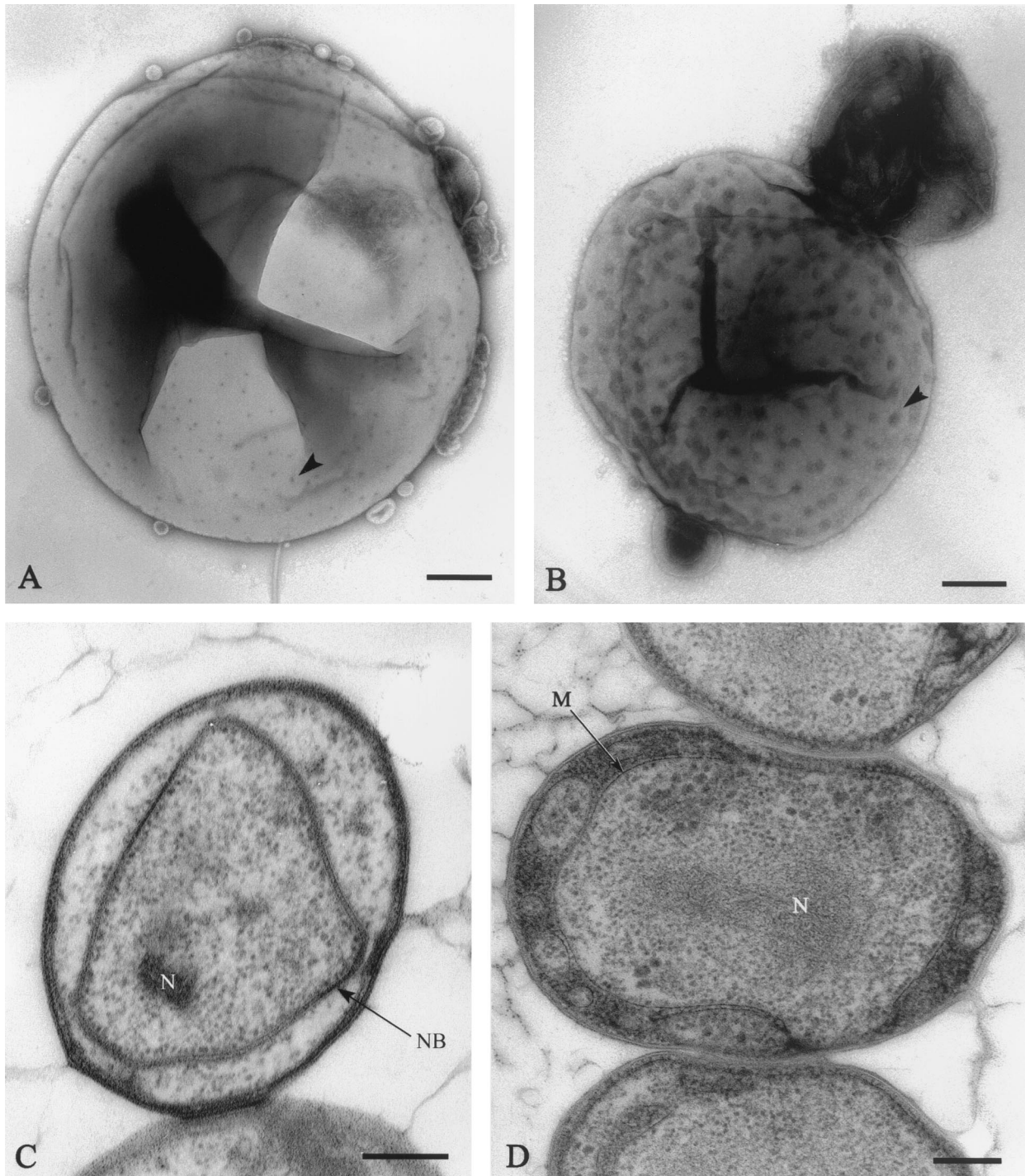


FIG. 1. Electron micrographs of cells of new *Gemmata*-like and *Isosphaera*-like isolates. (A) Negatively stained cell of the *Gemmata*-like strain JW11-2f5 showing crateriform structures (arrowhead) and coccoid cell morphology. Bar marker, 200 nm. (B) Negatively stained budding cell of *Isosphaera*-like strain CJuq1 showing uniform crateriform structures (arrowhead) on the mother cell and coccoid cell morphology. Bar marker, 200 nm. (C) Thin section of *Gemmata*-like cryosubstituted cell of strain JW3-8s0 showing the double-membrane-bounded nuclear body (NB) and nucleoid (N) enclosed within it. Bar marker, 200 nm. (D) Thin section of *Isosphaera*-like strain C2-3 possessing a fibrillar nucleoid (N) within a cytoplasmic compartment bounded by a single membrane (M) only. Bar marker, 200 nm.

clustered with the *Gemmata* group all possess in their 16S rRNA gene a 10-base sequence insertion between *E. coli* positions 998 and 999, characteristic of the group (21).

We have demonstrated for the first time that planctomyce-

tes, specifically *Gemmata*-like representatives of this division (as illustrated by strains JW3-8s0 and Soil9), can be isolated in pure cultures from a soil habitat. Planctomyces were previously isolated from soil only by use of 16S rRNA gene libraries

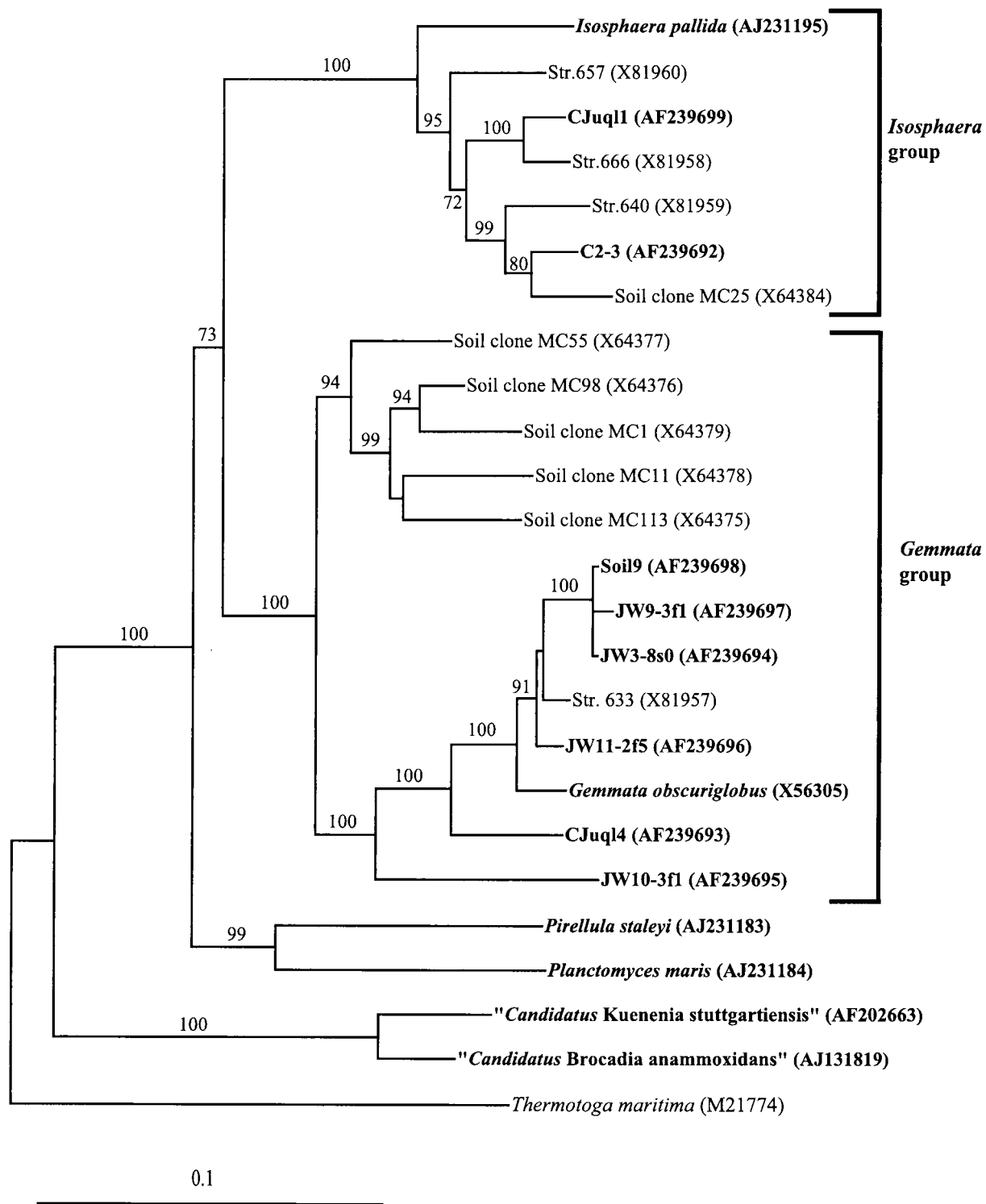


FIG. 2. Evolutionary distance tree derived from comparative analysis of 16S rDNAs from freshwater and soil isolates and reference strains of the order *Planctomycetales*. Database accession numbers are shown in parentheses after species, strain, or clone names. Bootstrap values of greater than 70% from 100 bootstrap resamplings from the distance analysis are presented at nodes. *Thermotoga maritima* was used as an outgroup. Isolates from this study and representative named species of the planctomycetes are indicated in bold. The scale bar represents 0.1 nucleotide substitution per nucleotide position.

(2, 15, 21) and in situ hybridization experiments (43). The type species of the genus, *G. obscuriglobus*, was isolated from freshwater (5), and other *Gemmata*-like isolates were isolated from leakage water from a compost heap (42). Of particular interest is the isolation of a *Gemmata*-related strain, JW3-8s0, from soil of Mt. Coot-tha. This isolation confirms the occurrence of culturable *Gemmata* strains in soil from the same site as was used in one of the first molecular 16S rRNA gene direct cloning studies of a soil habitat (21); in that study, several 16S rDNA clones were shown to be most closely related to *G. obscuriglobus* within the planctomycetes. These results are consistent with those of other studies using direct molecular ecology or hybridization methods to demonstrate that planctomycetes may be widely distributed soil inhabitants (4, 15, 17, 43, 44). Isolation in this study depended on the use of the β -lactam antibiotic penicillin or ampicillin, the aminoglycoside protein synthesis-inhibiting antibiotic streptomycin, and antifungal compounds; in one case, isolation occurred from an ampicillin solution habitat, consistent with known antibiotic sensitivities for planctomycetes and their cell wall composition (14, 19).

The planctomycete genera *Gemmata* and *Isosphaera* are at present described only on the basis of a single species each. In this study, we have isolated additional strains which are closely related to existing members of these genera. This study thus supplements previous work in which strains closely related to *Gemmata* and *Isosphaera* were isolated from various aquatic habitats (30, 42). The latter study (42) demonstrated the isolates within the Schlesner collection (30) to include a further *Gemmata*-like strain (strain 633) from leakage water from a compost heap and *Isosphaera*-like strains (strains 640, 657, and 666) also from leakage water from a compost heap. *Isosphaera*-like strains were also isolated from activated sludge in another study (25).

Of great interest also is the finding that six newly isolated strains with a close relationship to *Gemmata*, as demonstrated by 16S rDNA phylogenetic analysis and by the presence of *Gemmata*-specific signature nucleotide sequences in their 16S rDNAs, all possess membrane-bounded nuclear bodies, that is, nucleoid regions bounded by two membranes, analogous to that described for *G. obscuriglobus* (8). This finding is consistent with the apparent correlation between ultrastructural features and the phylogenetic position of a species within the planctomycetes, noted previously for *Pirellula* species (7, 22). Of particular interest from this perspective is the finding that even the isolate within the *Gemmata* group most distantly related to *G. obscuriglobus*, strain JW10-3f1, displays a clear nuclear body. The compartmentalization of the cells of the *Gemmata*-like and *Isosphaera*-like strains described here is also consistent with that which has been described for these genera in detail elsewhere (8, 23, 24).

According to 16S rRNA sequence analysis, six of the isolates clustered within the *Gemmata* group, while two clustered within the *Isosphaera* group. On the basis of the criteria of Stackebrandt and Goebel (36), according to which two organisms related at 16S rRNA sequence homology values of less than 97.5% are unlikely to be members of the same species, each of the new *Gemmata*-like isolates appears to represent a species separate from the reference strain, *G. obscuriglobus*. The *Gemmata* group and the *Isosphaera* group are clearly both quite phylogenetically diverse, perhaps to an even greater ex-

tent than indicated in a previous culture-based study (42). The new isolates are consistent with the 16S rRNA phylogenetic profile and diversity of the family *Planctomycetaceae* reported in previous studies (7, 42). The isolate from Mr. Coot-tha soil, JW3-8s0, clustered closer to *G. obscuriglobus* (UQM 2246) and strain 633, a *Gemmata*-like isolate from northern Germany (42), than to the *Gemmata*-like MC clones. Both the similarity matrix and the phylogenetic tree showed that JW9-3f1, Soil9, and JW3-8s0 may be members of the same species, as their similarity values are over 99%. The fact that sequences of these soil isolates differ from those of all MC clones may indicate that the diversity of planctomycetes in soil is wider than molecular cloning methods could detect and emphasizes the need for culturing as well as direct PCR retrieval for studying microbial diversity; however, this fact may also reflect differences in community composition at the same site at different times.

This study has established the validity of selective enrichment with antibiotics based on known properties of cultured planctomycetes for the isolation of planctomycetes from soil and water. In addition, it has demonstrated that culturable planctomycetes and members of the *Gemmata* group in particular exist in a soil habitat previously known to harbor planctomycetes only via evidence from direct cloning studies. It has also established (i) that significant 16S rRNA structural characteristics of the *Gemmata* group of planctomycetes known previously from only one cultured strain and cloned sequences are conserved throughout the group and (ii) that the ultrastructural feature of cell compartmentalization involving a membrane-bounded nucleoid is correlated with the phylogenetic distinction of the *Gemmata* group of planctomycetes and is present in *Gemmata* isolates from different habitats. The planctomycetes are now recognized as being ubiquitous in the environment. Furthermore, several studies indicate that these organisms may make up a significant proportion of the total microbial population (4, 27, 43).

The discovery of planctomycetes in new ecological niches and of new planctomycetes highlights the fact that the order *Planctomycetales* is a ubiquitously distributed and highly diverse order within the domain *Bacteria* that may harbor organisms which play a much more significant role in the environment than first imagined with regard to the cycling of organic and inorganic compounds. The development in this study of simple methods for the isolation of planctomycetes from soil should allow more extensive studies of the distribution, physiology, and ecological role of this distinct group of the domain *Bacteria* in microbial communities of agricultural and environmental significance.

Nucleotide sequence accession numbers. Nucleic acid sequences from this study have been deposited in the GenBank database under accession numbers AF239692 to AF239699.

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