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Phylogenetic and Metabolic Diversity of *Planctomycetes* from Anaerobic, Sulfide- and Sulfur-Rich Zodletone Spring, Oklahoma▽

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We investigated the phylogenetic diversity and metabolic capabilities of members of the phylum *Planctomycetes* in the anaerobic, sulfide-saturated sediments of a mesophilic spring (Zodletone Spring) in southwestern Oklahoma. Culture-independent analyses of 16S rRNA gene sequences generated using *Planctomycetes*-biased primer pairs suggested that an extremely diverse community of *Planctomycetes* is present at the spring. Although sequences that are phylogenetically affiliated with cultured heterotrophic *Planctomycetes* were identified, the majority of the sequences belonged to several globally distributed, as-yet-uncultured *Planctomycetes* lineages. Using complex organic media (aqueous extracts of the spring sediments and rumen fluid), we isolated two novel strains that belonged to the *Pirellula-Rhodopirellula-Blastopirellula* clade within the *Planctomycetes*. The two strains had identical 16S rRNA gene sequences, and their closest relatives were isolates from Kiel Fjord (Germany), Keauhou Beach (HI), a marine aquarium, and tissues of marine organisms (*Aplysina* sp. sponges and postlarvae of the giant tiger prawn *Penaeus monodon*). The closest recognized cultured relative of strain Zi62 was *Blastopirellula marina* (93.9% sequence similarity). Detailed characterization of strain Zi62 revealed its ability to reduce elemental sulfur to sulfide under anaerobic conditions, as well as its ability to produce acids from sugars; both characteristics may potentially allow strain Zi62 to survive and grow in the anaerobic, sulfide- and sulfur-rich environment at the spring source. Overall, this work indicates that anaerobic metabolic abilities are widely distributed among all major *Planctomycetes* lineages and suggests carbohydrate fermentation and sulfur reduction as possible mechanisms employed by heterotrophic *Planctomycetes* for growth and survival under anaerobic conditions.

Although microscopic observation of the rosette-forming *Planctomycetes* was reported as early as 1924, representatives of this group of microorganisms in pure cultures on dilute organic media were not obtained until 1973 (66, 75). Since then, aerobic heterotrophic *Planctomycetes* have been successfully isolated from brackish marine sediments (57, 59, 60), freshwater sediments (25, 35, 60), soil (74), hot springs (30), salt pits (58), and tissues and postlarvae of giant tiger prawns (27, 28). In addition, a special group of *Planctomycetes* ("*Candidatus*" genera "Anammoxoglobus," "Brocadia," "Kuenenia," and "Scalindua") has been implicated in the oxidation of ammonia under anaerobic conditions in wastewater plants, coastal marine sediments, and oceanic and freshwater oxygen minimum zones (16, 39, 43, 62, 63, 69).

In spite of the recent success in isolating members of the *Planctomycetes*, the phylum remains one of those underrepresented in microbial culture collections. Presently, only eight species and five genera have been fully characterized and validly described, and the total number of isolates reported represents a minor fraction of the *Planctomycetes* 16S rRNA gene sequences available in databases. *Planctomycetes* sequences available in the Ribosomal Database Project database (15) correspond to an isolate/clone ratio of 0.065 (as of November 2006), compared to ratios of 0.5, 1.9, and 0.75 for the *Firmicutes*, *Actinobacteria*, and *Proteobacteria*, respectively. Also, the majority of *Planctomycetes* available in pure cultures have been obtained in relatively few studies (59, 60), all of which used similar enrichment and isolation strategies based on an *N*-acetylglucosamine as a substrate or dilute complex media, all supplemented with antibiotics and antifungal agents, as a substrate.

The ubiquity of *Planctomycetes* has been extensively documented in culture-independent 16S rRNA gene-based surveys of marine (18, 46, 53–55, 71) and terrestrial (12, 20, 21, 32, 61, 73) environments, including soil (6, 9, 42). In addition, culture-independent analyses have indicated that the phylogenetic diversity of this phylum is not restricted to its cultured representatives, since these studies have described several as-yet-uncultured lineages within the phylum (12, 20, 71). This geographical ubiquity and broad phylogenetic diversity argue for a similarly high level of metabolic versatility. However, all cultured *Planctomycetes* so far appear to be aerobes specializing in sugar metabolism. *Planctomycetes*-affiliated sequences have been identified in surveys of anaerobic environments such as rice paddies, wastewater treatment plants, and hydrocarbon-contaminated environments (12, 20, 21, 71), suggesting that

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this group may have other as-yet-unidentified metabolic capabilities. Previous work has documented the presence of *Planctomycetes* in the anaerobic sediments at the source of Zodletone Spring, a sulfide- and sulfur-rich spring in southwestern Oklahoma, by using 16S rRNA surveys (24) as well as metagenomic libraries (22). In this study, the goal was to determine the level of phylogenetic diversity as well as possible metabolic pathways utilized by members of the *Planctomycetes* in this anaerobic, sulfide-saturated, hydrocarbon-impacted environment. We present evidence for the extreme diversity of *Planctomycetes* thriving in this environment and suggest sulfur reduction and sugar fermentation as two possible survival and growth strategies for heterotrophic *Planctomycetes* in anaerobic environments.

MATERIALS AND METHODS

Site description and sampling. Zodletone Spring emerges near Zodletone Mountain in the Anadarko Basin in southwestern Oklahoma. The spring was first described by Havens (34), and the geological and hydrological characteristics were subsequently described in detail (56, 65). The source is a contained area, approximately 1 m^2, filled with biomass and soft sediments to a depth of at least 15 cm. The dissolved-sulfide concentration in the emergent spring water is high (8 to 10 mM) and maintains anoxic conditions at the spring source (24, 65). Samples for phylogenetic analysis were collected from the spring source by using a sterile spatula, frozen immediately on dry ice, and transferred within 3 h of sampling to the laboratory, where they were stored at −20°C. For enrichment and isolation experiments, a combination of source sediments and sulfide-saturated source water was collected by immersing 100-ml sterile bottles into the source sediments and then screwing the caps onto the bottles while the bottles were still submerged. The bottles were then stored on ice while being transferred to the laboratory, where they were stored at 4°C.

DNA extraction, PCR amplification, cloning, and sequencing. DNA isolation was carried out using a lysis bead-beating protocol (21). *Planctomycetes*-affiliated 16S rRNA genes were amplified using two primer pairs: Pln46F/U1390R (12) and Eub338F-0-III/Pla930R (4) (Invitrogen Corp., Carlsbad, CA). 16S rRNA genes were amplified from the bulk community DNA in a 50-μl reaction mixture containing (at the indicated final concentrations) 2 μl of a 1:10 dilution of extracted DNA, 1× PCR buffer (Invitrogen), 2.5 mM $MgSO_4$, a 0.2 mM mixture of deoxynucleoside triphosphates, 2.5 U of platinum *Taq* DNA polymerase (Invitrogen), and 2 μl of a 10 μM solution of each of the forward and reverse primers. PCR amplification was carried out according to the following protocols: initial denaturation for 5 min at 94°C followed by 30 cycles of denaturation for 30 s at 94°C, annealing at 58°C for 1 min, and elongation at 72°C for 1.5 min with primer pair Pln46F/U1390R and for 1 min with primer pair Eub338F-0-III/Pla930R. A final elongation step at 72°C for 20 min was included in both protocols. The PCR products obtained were cloned into a TOPO-TA cloning vector and sequenced as previously described (24).

For the identification of PsrC gene homologs in *Planctomycetes* strain Zi62, we aligned putative PsrC genes from the *Blastopirellula marina* (ZP_01093647) and *Rhodopirellula baltica* (NP_870639) genomes. Primer pair 262F and 1162R (CC GATYGTCAACTTCGTGTT and AACCACAKTCCGATGTTCAC; primer designations are based on *R. baltica* PsrC gene numbering) was designed to theoretically amplify the PsrC genes in both *B. marina* and *R. baltica*, and the primer pair was tested with Zi62 genomic DNA. The PCR protocol used an initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing at 50°C for 30 s, and elongation at 72°C for 1.5 min and a final elongation step at 72°C for 5 min. The product obtained was directly sequenced without cloning.

Phylogenetic analysis. Sequences initially were compared to entries in the GenBank nr database and checked using BLAST (1). Sequences were aligned using the CLUSTAL_X program (72), and the aligned sequences were exported to PAUP. A pairwise distance matrix was generated using PAUP (version 4.01b10; Sinauer Associates, Sunderland, MA), and the distances were used to define operational taxonomic units (OTUs) based on a 98% similarity cutoff. The presence of chimeric sequences in our data set was checked by screening all sequences with the Bellerophon Web interface (http://foo.maths.uq.edu.au/~huber/bellerophon.pl; 36). Overall, eight chimeric sequences (six from the Pln46F/U1390R clone library and two from the Eub338F-0-III/Pla930R clone library) in the data sets were identified and removed from further analysis. OTUs from Zodletone Spring samples and GenBank-downloaded sequences were aligned using the CLUSTAL_X program. The program ModelTest (52) was used to choose the optimum model of DNA substitution for each data set. Phylogenetic trees were constructed using representatives of closely related reference sequences to highlight the phylogenetic affiliation of clones obtained in this study. Distance neighbor-joining trees were constructed using PAUP.

Isolation and characterization of aerobic and anaerobic heterotrophs from Zodletone Spring source sediments. *Planctomycetes* isolates were obtained during a culture-based survey of heterotrophic microorganisms at the spring source. The media contained (in grams per liter) K_2HPO_4 (5), $MgCl_2 \cdot 6H_2O$ (3.3), NaCl (4), NH_4Cl (4), $CaCl_2 \cdot 2H_2O$ (0.5), vitamins, and a trace-metal solution (49), in addition to a complex carbon source (5% [vol/vol] rumen fluid or aqueous sediment extract). Aqueous sediment extract was prepared by boiling 20 g of Zodletone Spring source sediments in 50 ml of NANOpure water. The mixture was centrifuged, and the supernatant was then filter sterilized and used as a nutrient source at a concentration of 50 ml/liter. The *Planctomycetes* isolates Zi62 and Zi142 were obtained using soil extract- and rumen fluid-based media, respectively. Zi62 was further purified by restreaking onto *N*-acetylglucosamine-based medium amended with ampicillin and cycloheximide (59, 67). A near-full-length sequence of the 16S rRNA gene of isolate Zi62 was obtained using the U8F and U1492R primer pair (44).

Physiological and biochemical characterization of strain Zi62. Physiological and biochemical characterization of strain Zi62 was carried out with the growth medium described by Staley et al. (67), except that sucrose instead of *N*-acetylglucosamine was used and ampicillin and cycloheximide were omitted. Detailed protocols for the biochemical tests conducted were obtained from reference 29. The ability of strain Zi62 to grow anaerobically was tested with media prepared under anaerobic conditions (2) with sucrose or yeast extract as the carbon source and SO_4^{2-}, $S_2O_3^{2-}$, or NO_3^- (30 mM each) as the electron acceptor. The ability to reduce elemental sulfur under anaerobic conditions was tested using a 1% sulfur slurry and ferrous ammonium sulfate as previously described (23). Sulfur reduction was followed by the quantification of sulfide production from elemental sulfur by the methylene blue assay (14). The level of SO_4^{2-}, $S_2O_3^{2-}$, or NO_3^- was determined using ion chromatography (10). Sugar levels were quantified using the phenol-sulfuric acid method with a 96-well-plate format (48). Acids produced during sugar metabolism were identified following the acidification of culture supernatants and the extraction of acids with ethyl acetate. The extract was concentrated under a stream of N_2 and derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemicals, Rockford, IL). Trimethylsilyl derivatives were identified by gas chromatography-mass spectroscopy using a 6890N network gas chromatography system and a 5973 network mass selective detector (Agilent Technologies, Wilmington, DE). The detected acids were then quantified using a System Gold high-performance liquid chromatography (HPLC) system (Beckman, Fullerton, CA) equipped with a Prevail organic acid 5-μm column (Alltech, Nicholasville, KY). The mobile phase was 25 mM KH_2PO_4 (pH 2.5) at a flow rate of 1 ml/min. CO_2 was quantified using a gas chromatograph equipped with a thermal conductivity detector (Varian) and a Porapak Super Q column (Alltech).

Cell wall amino acids were quantified by first purifying the cell envelopes according to the previously outlined procedure (41). Amino acid composition was determined at the University of Oklahoma Health Sciences Center proteomics facility, Oklahoma City (http://wmriokc001.ouhsc.edu/amino.htm; 77). The G+C content of genomic DNA was determined using the services of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Lipids were extracted by ultrasonication of freeze-dried cell pellets by a modification of the Bligh and Dyer extraction method (5) for intact polar lipids (IPLs) as described by Sturt et al. (70). The lipid classes were separated on 2-g silica columns (5% deactivated with water) using 15 ml of *n*-hexane, 18 ml of *n*-hexane–dichloromethane (2:1), 18 ml of dichloromethane-acetone (9:1), and 20 ml of methanol to yield hydrocarbons, ketones and esters, alcohols, and polar lipids, respectively. The polar lipids were saponified with a 6% methanolic KOH solution at 80°C for 3 h. The polar lipid fatty acids (PLFAs) were extracted four times with hexane and derivatized with 14% BF_3 in methanol at 70°C for 1 h to form fatty acid methyl esters.

HPLC-mass spectroscopy analysis was performed at the University of Bremen, Bremen, Germany (70). Relative concentrations of IPLs were calculated based on the mass spectroscopy responses of molecular ions relative to that of known amounts of the internal standard (1-*O*-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine).

Fatty acid methyl esters were analyzed on a Trace MS gas chromatograph-mass spectrometer (ThermoFinnigan, San Jose, CA) with a fraction of the column effluent diverted to a flame ionization detector for quantification. The

gas chromatograph was operated at 310°C in the split/splitless mode and equipped with a Varian VF5-ms capillary column (length, 30 m; internal diameter, 0.25 mm; film thickness, 0.25 μm; carrier gas, He; flow rate, 1 ml min^{-1}). The column temperature was programmed as follows: 60°C for 1 min; an increase at 10°C min^{-1} to 150°C; and an increase at 4°C min^{-1} to 310°C for 15 min.

Microscopy. Light microscopy was performed using an Olympus CX41 system microscope set up with a Diagnostic Instruments Insight camera and the SPOT software. Transmission electron microscopy was done in the Samuel Noble Electron Microscopy Laboratory of the University of Oklahoma. Briefly, 3 ml of a Zi62 culture was harvested by centrifugation in a microcentrifuge for 5 min and the cell pellet was washed three times by centrifugation with a 2% $NaHCO_3$ solution under (4:1) N_2-CO_2 (pH 7.8). Cells were fixed using 1 ml of 2.5% glutaraldehyde in 2% $NaHCO_3$ at room temperature for 2 h and were then washed twice with 2% $NaHCO_3$ buffer, followed by overnight incubation at 4°C. The cell suspension was fixed with 1 ml of 2% OsO_4 in 2% $NaHCO_3$ buffer (under air) at room temperature for 1 h. Cell pellets were resuspended and fixed with a saturated uranyl acetate solution (pH 5.2) and washed with 2% $NaHCO_3$. Cells were then dehydrated and embedded as described previously (http://ou.edu/research/electron/bmz5364/fix-mbio.html) except that no agarose was used, cells were not resuspended following the dehydration step, and cell pellets were embedded in Epon 812. Transmission electron microscopy images were obtained from a JEOL 2000-FX intermediate-voltage (200,000-V) scanning transmission electron microscope.

Nucleotide sequence accession numbers. Sequences obtained in this study were deposited in GenBank under accession numbers EF602462 to EF602549.

RESULTS

Culture-independent analysis of the *Planctomycetes* community in Zodletone Spring sediments. A total of 186 clones from clone libraries generated using primer pairs Pln46F/U1390R and Eub338F-0-III/Pla930R were fully sequenced. After the exclusion of chimeric sequences ($n = 8$) and non-*Planctomycetes* sequences, a total of 34 *Planctomycetes* OTUs were identified.

Based on data from previous studies, isolates and clone sequences belonging to the phylum *Planctomycetes* could be broadly grouped into three putative classes (Fig. 1) (38), as follows: (i) the cultured *Planctomycetes* (class *Planctomycetacia*), which contains all previously described heterotrophic cultured representatives of this phylum (genera *Planctomyces*, *Pirellula*, *Blastopirellula*, *Rhodopirellula*, *Isosphaera*, and *Gemmata*) as well as isolates (59, 60) and 16S rRNA sequences (8, 20, 24, 40, 42, 50, 54, 73) from a variety of environments; (ii) a collection of as-yet-uncultured microorganisms represented by 16S rRNA gene sequences with a global distribution (7, 9, 12, 20, 21, 46, 53), which is hereinafter referred to as candidate class WPS-1 (51), although some sequences belonging to these lineages have been previously referred to as BD2-16 (20); and (iii) deeply branching *Planctomycetes*, detected in a wide array of environments and including several widespread, independent lineages with high levels of sequence divergence, in addition to the anammox group. Members of the latter group have previously been referred to as Pla3, Pla4 (20), and group VI *Planctomycetes* (12).

In spite of the fact that all *Planctomycetes* sequences from Zodletone previously identified using general 16S bacterial primers belonged to the group of cultured *Planctomycetes* and related sequences (OTUs designated ZB in Fig. 1) (24), only a small fraction (three OTUs) of the clones in this study belonged to this group. The majority of sequences (27 OTUs) described here using the *Planctomycetes*-biased primers were associated with candidate class WPS-1. These OTUs belonged to three different monophyletic lineages within this class (groups A, B, and C in Fig. 1). Interestingly, many of the clones most similar to Zodletone OTUs have been reported to occur in anaerobic environments (e.g., a methanogenic digester clone [32], *p*-toluate-degrading consortium clones [76], anaerobic deep-sea-volcano mud and subseafloor clones [37, 46], and anoxic bulk soil clones [20]). Finally, three OTUs belonging to the deeply branching *Planctomycetes* were identified, one of which was affiliated with the anaerobic ammonia-oxidizing group of *Planctomycetes* (Fig. 1).

Non-*Planctomycetes* clones detected in Zodletone Spring. As previously observed (4, 11, 20), neither of the primer pairs used is exclusively specific for members of the *Planctomycetes*, and as a result, several non-*Planctomycetes* type rRNA clones were identified. Interestingly, most of the phylotypes obtained using the two primer pairs belonged to as-yet-uncultured bacterial candidate divisions. In addition to the *Planctomycetes*, primer pair Eub338F-0-III/Pla930R amplified sequences that belonged to novel candidate divisions OP11 (17 clones; 7 OTUs) and OD1 (4 clones; 4 OTUs) (Fig. 2), while primer pair Pln46F/U1390R amplified sequences belonging to candidate divisions WW1 (61 clones; 22 OTUs), WW2 (2 clones; 2 OTUs), WS3 (9 clones; 6 OTUs), and OP3 (1 clone; 1 OTU), in addition to *Chlorobia* (1 clone) and *Verrucomicrobia* (2 clones; 2 OTUs).

Isolation and characterization of novel *Planctomycetes* from Zodletone Spring source sediments. Two isolates, strains Zi62 and Zi142, were obtained during a survey of heterotrophic bacteria in Zodletone Spring on plates containing either sediment aqueous extracts (Zi62) or 5% rumen fluid as a sole carbon source. The two strains were shown to have identical 16S rRNA gene sequences. Strain Zi62 was successfully purified by restreaking three times onto medium with *N*-acetylglucosamine as the sole carbon and nitrogen source and with ampicillin and cycloheximide (50 μg/ml each) to inhibit the growth of other bacteria and fungi (67).

Zi62 formed opaque, white-beige colonies with mucoid surfaces and a viscous texture. Cells were ovoid to pear shaped and occurred as singles or pairs or in rosette formations attached at the smaller cell pole. Cells were motile, with a single flagellum, and motility was most visible during the log growth phase. Negatively stained cells showed dense crateriform-like structures occurring at only one pole of the cell (Fig. 3a) and covering one-fourth to one-third of the cell surface. Thin-sectioned cells showed the presence of an intracellular membrane dividing the cells into the pirellulosome and the paryphoplasm (26). A dense nucleoid structure that covered 20 to 30% of the pirellulosome was also observed (Fig. 3b). There was no evidence of small, prosthecate projections.

Phylogenetic analysis placed strain Zi62 within the *Pirellula-Rhodopirellula-Blastopirellula* (PRB) clade within the family *Planctomycetaceae.* Strain Zi62 formed a monophyletic lineage with strong bootstrap support (Fig. 1) with a group of 10 different *Planctomycete*s isolates. The levels of similarity of the 16S rRNA gene sequences of Zi62 and these isolates ranged between 98.8 and 96.7%. Interestingly, all 10 strains were isolated from marine habitats: five strains were obtained from infected and uninfected postlarvae of the giant tiger prawn *Penaeus monodon* (strains AGA/M12, AGA/M41, ACM 3181, ACM 3180, and AGA/C41) (27), two strains were obtained from the tissue of *Aplysina* sp. sponges (strains 81 and 16) (Fig. 1), and the remaining strains

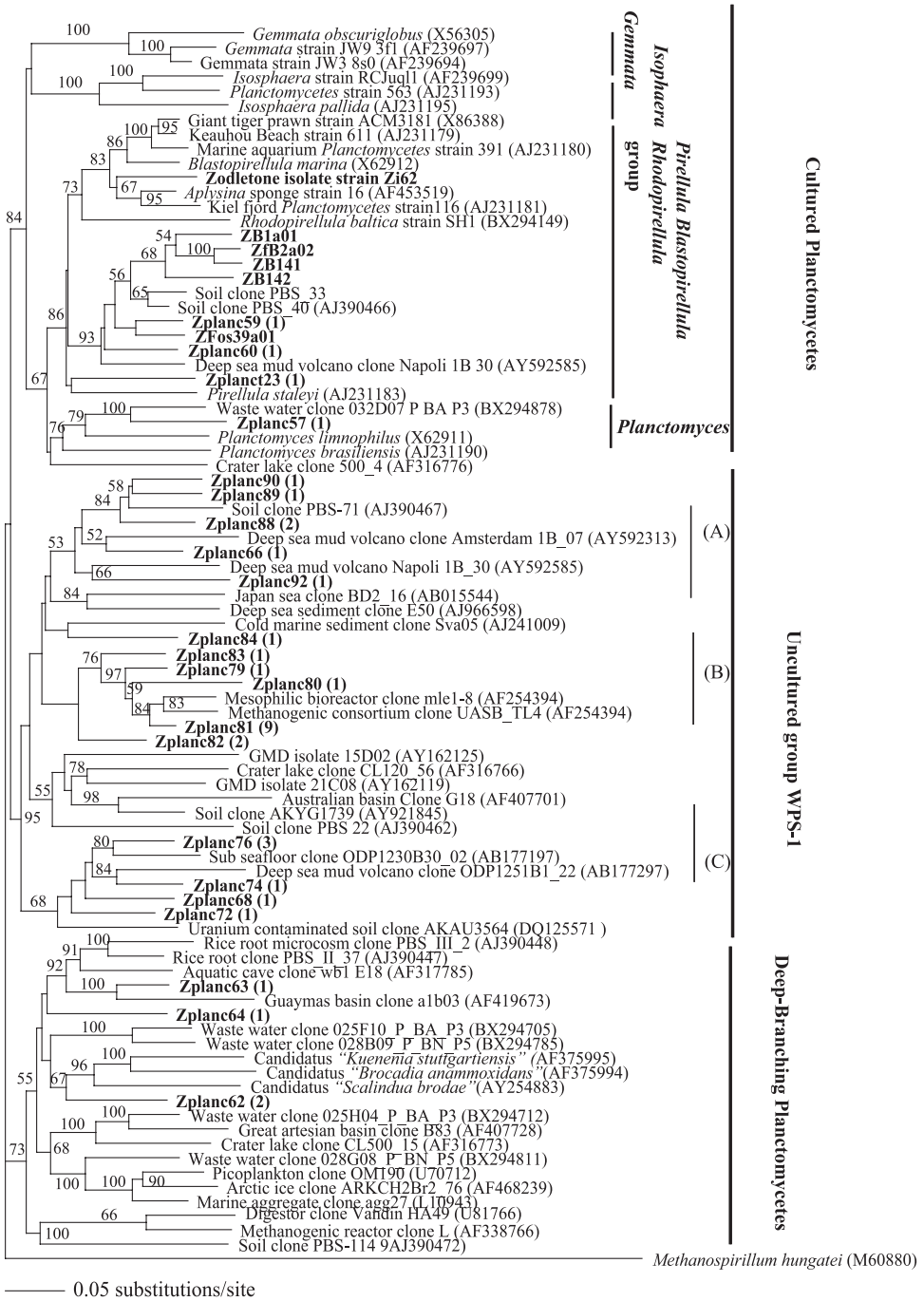

FIG. 1. Distance neighbor-joining tree based on the 16S rRNA gene sequences of *Planctomycetes* OTUs encountered in Zodletone Spring source sediments. Bootstrap values (expressed as percentages) are based on 1,000 replicates and are shown for branches with more than 50% bootstrap support. All designations for *Planctomycetes* sequences from the Zodletone Spring are in boldface. Sequences generated in this study using *Planctomycetes*-specific primers are designated Zplanc, and the frequency of occurrence of each OTU is reported in parentheses. Sequences identified in previous studies using either *Bacteria*-specific primers (24) or metagenomic analysis (22) are designated ZB or ZFos, respectively. GMD, grand canonical molecular dynamics.

were isolated from Kiel Fjord, Germany, a marine aquarium, and Keauhou Beach, HI (33, 60). The recognized species closest to strain Zi62 is *B. marina* (60) (93.9% 16S rRNA gene sequence similarity). The 16S rRNA gene of strain Zi62 was 87.3 and 86.8% similar, respectively, to the 16S rRNA genes of *Pirellula staleyi* and *R. baltica*, the other two recognized species within the PRB clade, and was distantly (80.5 to 85% sequence similarity) related to those of all PRB-affiliated *Planctomycetes* clones from Zodletone Spring identified in this as well as in previous studies (22, 24).

Physiological characterization of strain Zi62 indicated that the strain can grow at a wide range of temperatures and pHs compared to *B. marina*, *R. baltica*, and *P. staleyi* (Table 1). The

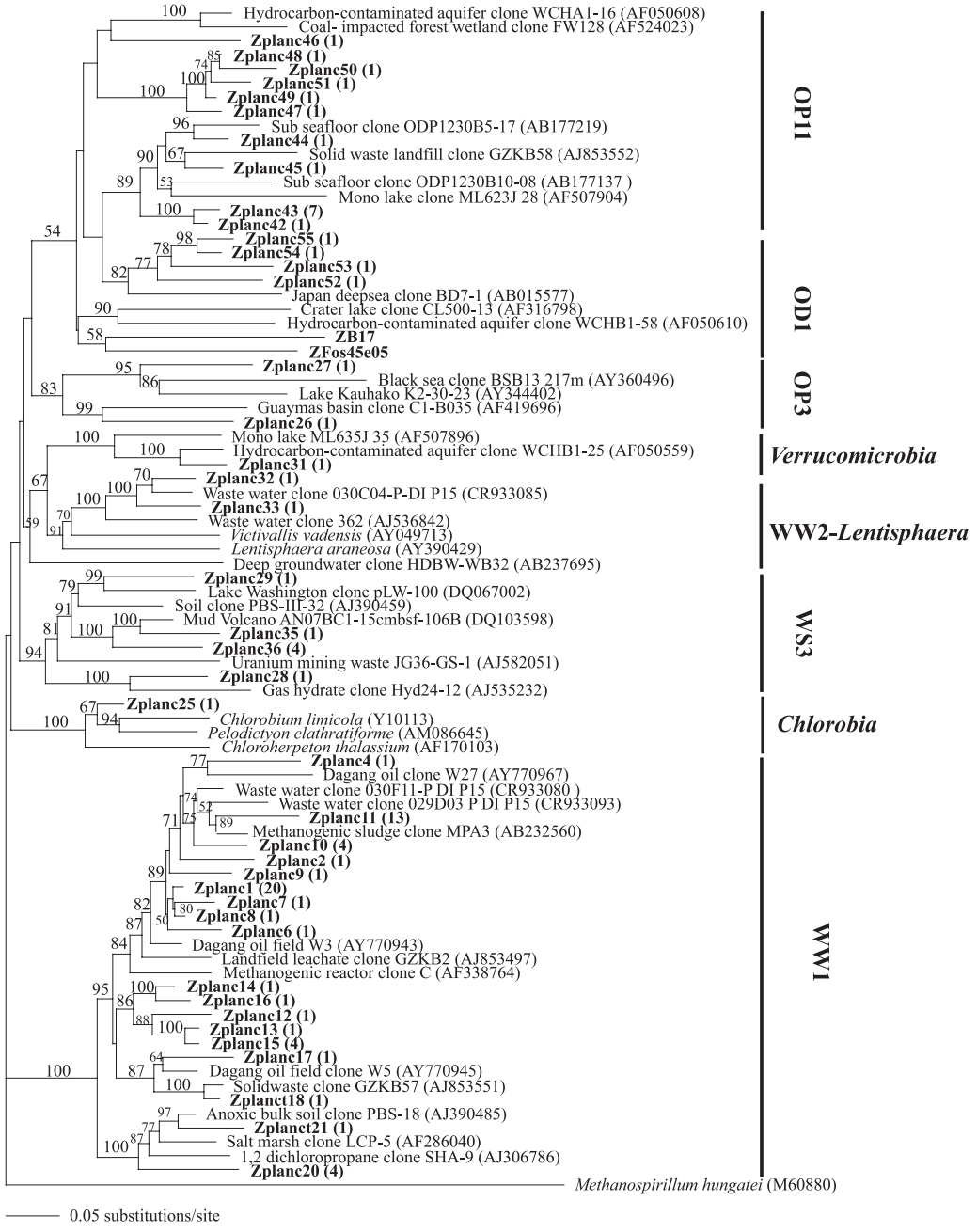

FIG. 2. Distance neighbor-joining tree based on the 16S rRNA gene sequences of non-*Planctomycetes* OTUs encountered in Zodletone Spring source sediments. Bootstrap values (expressed as percentages) are based on 1,000 replicates and are shown for branches with more than 50% bootstrap support. All designations for sequences from the Zodletone Spring are in boldface. Sequences generated in this study are designated Zplanc, and the frequency of occurrence of each OTU is reported in parentheses. Sequences identified in previous studies using either *Bacteria*-specific primers (24) or metagenomic analysis (22) are designated ZB or ZFos, respectively.

isolate also exhibited a similarly wide range of salt tolerance comparable to that of marine species but not to that of the freshwater species *P. staleyi*. The G+C content of strain Zi62 was 61.2%, considerably higher than those of the other three recognized type strains within the PRB group. However, such high values in several *Planctomycetaceae* isolates (e.g., strains SH 479, SH 241, SH 269, SH 217, SH 240, SH 292, SH 293, and SH 295) have been reported previously (60).

PLFA analysis demonstrated that, as in *B. marina*, *R. baltica*, and *P. staleyi*, $C_{16:0}$ and $C_{18:1\Delta9}$ are the major PLFAs in strain Zi62 (Table 1). Surprisingly, analyses of the IPLs indicated that the major lipid components in strain Zi62 are betaine-type glycerolipids (19) (95.8% of total IPLs), followed by phosphatidylcholine (0.5%), phosphoethanolamine (0.2%), phosphatidylglycerol (0.06%), and some unknown IPLs (3.4%). The presence of betaine glycerolipids as a major IPL component in the *Planctomycetes* has not been previously reported. In fact, betaine-type glycerolipids are abundant mainly in eukaryotic

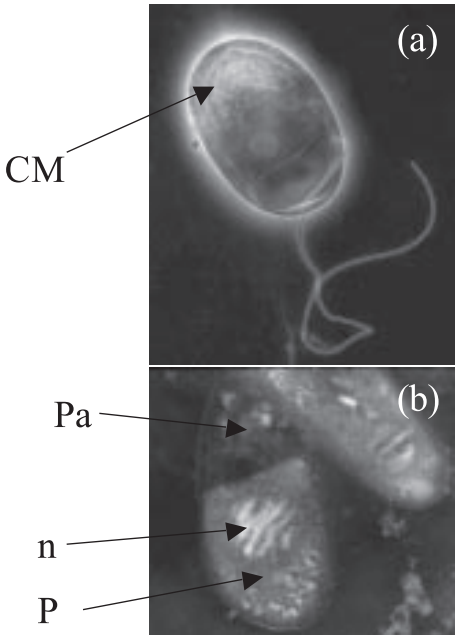

FIG. 3. (a) Negatively stained electron micrograph of strain Zi62, showing crateriform-like structures (CM) covering one-third of the cell and a single flagellum. (b) Thin-sectioned micrograph of strain Zi62 showing the pirellulosome (P), the paryphoplasm (Pa), and the nucleoid structure (n).

organisms such as algae, lower plants, bryophytes, fungi, and some protozoa (19) and, with few exceptions (3), are not usually encountered in *Bacteria*.

The cell wall amino acid composition was similar to those of the three recognized species within the PRB group. Six amino acids (glutamate, proline, alanine, glycine, threonine, and lysine) constituted 70.8% of the cell wall amino acids. The percentages of glutamic acid and lysine in Zi62 were the highest among those in described *Planctomycetes*, while the percentages of cysteine and serine were the lowest (Table 1).

Potential adaptations of strain Zi62 to anaerobic environments. The ability of strain Zi62 to grow under anaerobic conditions was tested using both complex (yeast extract) and defined (sucrose) carbon and energy sources. Strain Zi62 did not utilize NO_3^-, SO_4^{2-}, or $S_2O_3^{2-}$ as an electron acceptor. However, it reduced elemental sulfur (supplied as sulfur slurry) into sulfide (Fig. 4) under strict anaerobic conditions, and the production of sulfide (up to 2.4 mM within 60 days) was coupled to the disappearance of the sulfur precipitate in active incubations. The abundance of zerovalent sulfur in Zodletone Spring (65) argues for the potential importance of anaerobic sulfur respiration for the survival of the spring's microbial community. Anaerobic sulfur respiration has previously been shown to be mediated by polysulfide reductase, a molybdopterin oxidoreductase that catalyzes the reduction of polysulfide into sulfide (64). Analyses of the *R. baltica* (31) and *B. marina* genomes (accession numbers NC_005027.1 and NZ_AANZ00000000, respectively) indicated the presence of putative genes encoding the three different enzyme subunits (PsrA, PsrB, and PsrC) in both microorganisms (accession numbers NP_870639, NP_870637, and NP_870640 for *R. baltica* and ZP_01093647, ZP_01093648, and ZP_01093646 for *B. marina*). A set of primers designed to target conserved sequences in the *psrC* genes of *R. baltica* and *B. marina* (Psr262F and Psr1162R) was used to test for the presence of a polysulfide reductase gene homolog in strain Zi62. A phylogenetic analysis of the translated amino acid sequence corresponding to the PCR product obtained using Zi62 genomic DNA indicated that this peptide is most closely related to *B. marina* and *R. baltica* PsrC subunits (Fig. 5).

In addition, strain Zi62, unlike *B. marina*, *R. baltica*, and *P. staleyi*, produced acids from all carbohydrates tested (glucose, fructose, sucrose, trehalose, mannose, and xylose), lowering the pH of the medium to 5.1 to 5.2 in all cases. Using gas chromatography-mass spectroscopy and HPLC, we identified succinate and acetate as the major products accumulating, with minor amounts of lactate, propionate, and formate produced as well (Table 2). However, repeated attempts to grow strain Zi62 under anaerobic fermentative conditions, either in complex (rumen fluid or yeast extract) or defined (glucose, sucrose, or *N*-acetylglucosamine) medium, were not successful.

DISCUSSION

The aim of this study was to investigate the phylogenetic diversity and the metabolic capabilities of members of the phylum *Planctomycetes* in the permanently anaerobic, sulfide-saturated source sediments of Zodletone Spring. The16S rRNA clone library analysis described here indicated the presence of *Planctomycetes* living under strictly anaerobic conditions. At the spring source, cloned sequences corresponding to the three major classes and candidate classes of this phylum (as defined by Janssen [38]) were recovered. This finding is in accordance with results of previous culture-independent studies documenting a broad diversity of *Planctomycetes* sequences in anaerobic environments by using general (21, 24, 32, 50, 71) or *Planctomycetes*-specific (12, 20, 40) 16S rRNA gene primers. Whether this broad phylogenetic diversity among sequences within the phylum *Planctomycetes* retrieved from anaerobic ecosystems reflects a similar diversity in metabolic strategies utilized for growth under anaerobic conditions remains to be determined.

The results of this study point to anaerobic sulfur reduction and sugar fermentation as two possible metabolic processes that may enable *Planctomycetes* to grow in anaerobic environments. Previous field studies have shown a high concentration of zerovalent sulfur at Zodletone Spring (65), and 16S rRNA gene-based analyses have indicated that sulfur-metabolizing anaerobes (e.g., sulfur reducers of the genus *Desulfuromonas*, sulfur disproportionators of the genus *Desulfocapsa*, and various groups of anaerobic sulfur- and sulfide-oxidizing phototrophs) are important components of the microbial community in the spring (24). Strain Zi62 slowly reduced elemental sulfur into sulfide in a laboratory medium prepared under anaerobic conditions (Fig. 4). However, the low rate of the process in laboratory incubations and the low energy yield associated with sulfur reduction did not allow us to determine whether strain Zi62 could rely solely on sulfur reduction for growth and biomass production in the spring. In addition, it is doubtful that sulfur reduction is the sole global process used by anaerobic het-

TABLE 1. General characteristics that distinguish strain Zi62 from *B. marina*, *R. baltica*, and *P. staleyi*[a]

Characteristic	Value for or feature of:			
	Strain Zi62	*B. marina*	*R. baltica*	*P. staleyi*
Cell size (μm)	0.6–1.2 by 1.6–2.2	0.7–1.5 by 1.0–2.0	1.0–2.5 by 1.2–2.3	0.9–1.0 by 1.0–1.5
Pigmentation	Unpigmented	Unpigmented	Pink to red	
Temp range (°C)	13–37	ND–38	ND–32	17.7–29.6
Temp optimum (°C)	33	27–33	28–30	24
Range of NaCl concn (%)	0–8	0.4–6.0	0.4–6.9	0–1.7
Presence of:				
Caseinase	+	−	−	+
Lipase	+	+	−	−
Urease	−	−	−	−
Ability to reduce elemental sulfur	+	ND	ND	ND
G+C content (%)	61.9	53.6–57.4	55	57.1
% Similarity of 16S rRNA sequence to sequence from Zi62	100	93.9	86.8	87.3
Utilization of substrate:				
Amygdalin	+	ND	+	−
Pyruvate	+	+	−	+
Fructose	+	+	+	−
Glucuronic acid	+	+	+	−
Glutamic acid	+	+	−	−
Glycerol	+	+	+	−
Chondroitin sulfate	+	+	+	−
Lyxose	+	+	+	−
Fatty acid content (%)				
$C_{14:0}$	0.7	NP	0.5	4.9
$C_{15:0}$	0.9	5.9	0.5	NP
$C_{16:0}$	0.4	4.9	NP	NP
$C_{16:1\Delta9}$	7.6	4.1	8.0	3.5
$C_{16:0}$	38.0	27.5	39.2	33.8
$C_{17:1\Delta9}$	1.5	NP	4.0	14.4
$C_{17:0}$	0.9	NP	1.2	5.3
$C_{18:1\Delta9}$	48.0	26.6	40.8	26.6
$C_{18:1\Delta11}$	NP	2.3	1.6	2.0
$C_{18:0}$	0.5	2.5	4.3	3.3
$C_{19:1\Delta11}$	NP	2.6	NP	NP
$C_{19:0}$	NP	2.7	NP	NP
$C_{20:1\Delta11}$	1.4	1.2	NP	15.7
Molar ratio for cell wall amino acid[b]:				
Glutamate	15.6	15.53	15.5	9
Proline	10.4	ND	12.6	6.5
Glycine	7.7	10	9.8	6
Threonine	6.0	5.0	9.0	3.0
Lysine	6.0	4.0	3.1	2.5
Cysteine	1.2	1.6	9.2	3.6
Serine	2.8	4.74	4.3	5.5

[a] Data for *B. marina*, *R. baltica*, and *P. staleyi* were obtained from previous studies (41, 47, 57, 60, 66). ND, not determined; NP, not present.
[b] As determined relative to the molar ratio for alanine, which was 10.

erotrophic *Planctomycetes* for growth and energy production since *Planctomycetes*-affiliated sequences were observed under anaerobic conditions where zerovalent sulfur is probably absent or present in very low concentrations (e.g., wastewater treatment plants, anaerobic soil, and methanogenic consortium habitats) (12, 20, 45, 76). The enzyme catalyzing polysulfide reduction, the polysulfide reductase, is a molybdopterin oxidoreductase. The identification of a putative operon encoding the three subunits in the genomes of *R. baltica* and *B. marina* and the *psrC* gene in strain Zi62 suggests that sulfur reduction capability is widespread among members of the family *Planctomycetaceae*, regardless

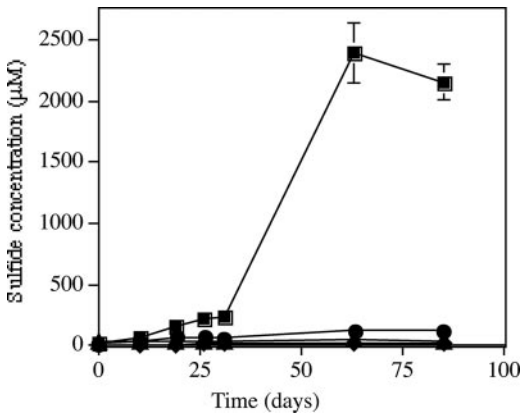

FIG. 4. Plot of sulfide production over time in anaerobic medium after inoculation and the addition of elemental sulfur and a substrate (sucrose, 0.1%; ■), after inoculation and the addition of elemental sulfur but no substrate (●), after no inoculation and the addition of elemental sulfur and a substrate (◆), and after no inoculation and with no elemental sulfur and no substrate (▲). All values shown are averages of results for triplicate tubes.

of the source of isolation. However, testing of *R. baltica* and *B. marina* for their abilities to reduce elemental sulfur has not been reported.

Another possible strategy for the anaerobic growth of *Planctomycetes* is sugar fermentation. Since this process does not require a terminal electron acceptor, it could explain the ubiquity of *Planctomycetes* in anaerobic habitats. Strain Zi62 thus differs from the three recognized species within the PRB clade, all of which are unable to produce acid from sugars. However, note that the genomes of *R. baltica* and *B. marina* contain the genes necessary for lactic acid fermentation (including the L-lactate dehydrogenase gene; GenBank accession numbers NP_868582, and ZP_01090380, respectively), although the expression of these genes has not yet been reported (31, 60). Strain Zi62 produced succinic, acetic, lactic, propionic, and formic acids as end products of sugar metabolism under aerobic conditions. Acid production from sugars indicates that strain Zi62 is capable of partially disposing of reducing equivalents via substrate-level phosphorylation. The pattern of product formation suggests a mixed acid fermentation pathway in which phosphoenolpyruvate is converted into both pyruvate (resulting in the formation of acetate, lactate, and formate) and oxaloacetate (resulting in the formation of succinate and propionate) (13). Physiological factors (e.g., the CO_2 concentration, pH, and redox potential) controlling the proportion of sugar metabolized into acid, as well as the pattern of product formation in strain Zi62, remain to be determined.

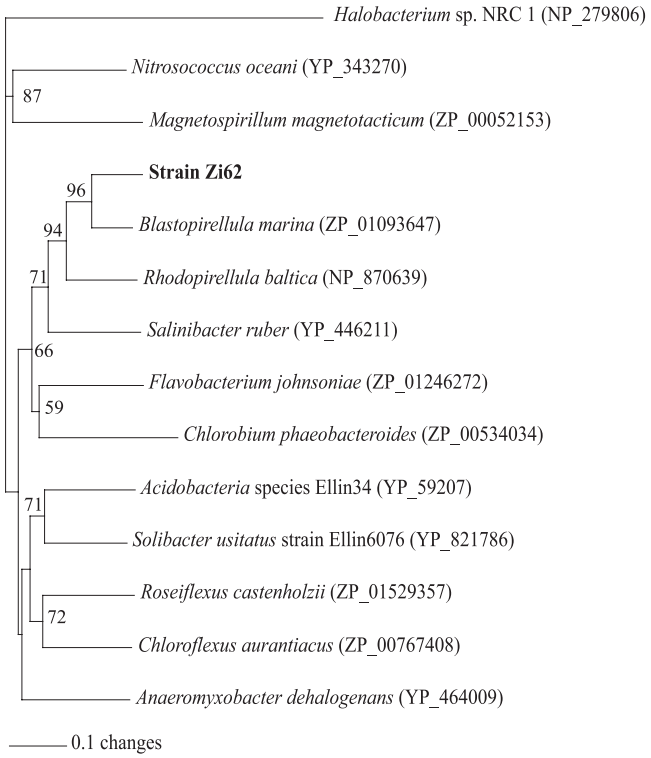

FIG. 5. Distance neighbor-joining tree constructed using translated amino acid sequences corresponding to the putative *psrC* gene identified in Zi62 using PCR. The tree was constructed using a partial (920-bp) sequence of putative *psrC* amplified using primer pair 262F and 1162R (*R. baltica*; PsrC gene numbering). Bootstrap values (expressed as percentages) were determined based on 1,000 replicates and are shown for branches with more than 50% bootstrap support.

TABLE 2. Products of strain Zi62 metabolism under aerobic conditions

Product or substrate	Amt (μmol) produced or utilized	Amt (μmol) produced/ 100 μmol of sucrose	% of substrate recovered as indicated product
Sucrose (consumed)	79.29	NA[a]	NA
Succinate	64.2	80.97	26.99
Acetate	126.3	159.29	26.55
Lactate	18.45	23.27	5.82
Propionate	12.3	15.15	3.88
Formate	6.6	8.23	0.86
CO_2	400.7	505.54	42.11
C			106.0

[a] NA, not applicable.

In spite of its fermentative capability, repeated attempts to grow strain Zi62 under strict anaerobic conditions in laboratory incubations using a complex carbon source (yeast extract) or a defined carbon source (glucose or sucrose as the substrate) were not successful (data not shown). Equally unsuccessful were our attempts to isolate *Planctomycetes* strains in anaerobic incubations with 5% soil extract or 5% rumen fluid, supplemented with ampicillin and cycloheximide, as a carbon source. Under these conditions, only isolates belonging to the *Bacteroidetes*, *Firmicutes*, *Actinomycetes*, and *Spirochetes* were obtained. With a more defined medium, with *N*-acetylglucosamine as a substrate and ampicillin and cycloheximide, only isolates belonging to the *Actinomycetes* were obtained. Until truly fermentative *Planctomycetes* that are capable of anaerobic growth in the absence of electron acceptors are isolated, the ecological significance of this process will remain uncertain.

Strain Zi62 belongs to the PRB lineage within the *Planctomycetaceae.* The fact that only members of this class of the *Planctomycetaceae* have been isolated renders our view of metabolic capabilities within the phylum incomplete. This work, therefore, highlights the need for additional efforts towards

isolating *Planctomycetes* belonging to the remaining two candidate classes. One possible strategy may involve using complex and defined media similar to media previously used to isolate *Planctomycetes* (59, 60) while applying high-throughput isolation and screening approaches (68, 78). It is worth mentioning that some cells belonging to candidate lineage WPS-1 have successfully been encapsulated in gel microdroplets from soil using grand canonical molecular dynamics methodologies (78). Alternatively, if as-yet-unspecified nutritional requirements or growth conditions are necessary for the growth of such isolates, prior elucidation of the physiological capabilities and metabolic potentials of these as-yet-uncultured *Planctomycetes* (e.g., by metagenomic analysis) may be required for the design of an effective isolation medium.

16S rRNA gene sequence divergence among strain Zi62, *B. marina*, *R. baltica*, and *P. staleyi*, as well as several of the physiological, nutritional, and biochemical differences reported above (differences in patterns of acid production from sugars, temperature and pH ranges, and substrate utilization profiles), suggests that strain Zi62 does not belong to any of the three recognized genera within the PRB lineage. Strain Zi62 thus probably represents a novel genus, together with closely related marine isolates (Fig. 1). Unfortunately, within this group, only strain Zi62 is fully characterized, and a more thorough characterization of the other isolates, coupled with DNA-DNA hybridization studies of isolates within this group, is probably required to determine the defining biochemical, metabolic, and chemotaxonomic characteristics of this novel lineage.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32:**781–791.
3. **Benning, C., Z. H. Huang, and D. A. Gage.** 1995. Accumulation of a novel glycolipid and a betaine lipid in cells of Rhodobacter sphaeroides grown under phosphate limitation. Arch. Biochem. Biophys. **317:**103–111.
4. **Blackwood, C. B., A. Oaks, and J. S. Buyer.** 2005. Phylum- and class-specific PCR primers for general microbial community analysis. Appl. Environ. Microbiol. **71:**6193–6198.
5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
6. **Borneman, J., and E. W. Triplett.** 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. **63:**2647–2653.
7. **Bowman, J. P., and R. D. McCuaig.** 2003. Biodiversity, community structural shifts, and biogeography of prokaryotes within Antarctic continental shelf sediment. Appl. Environ. Microbiol. **69:**2463–2483.
8. **Brummer, I. H. M., A. D. M. Felske, and I. Wagner.** 2004. Diversity and seasonal changes of uncultured *Planctomycetales* in river biofilms. Appl. Environ. Microbiol. **70:**5094–5101.
9. **Buckley, D. H., V. Huangyutitham, T. A. Nelson, A. Rumberger, and J. E. Thies.** 2006. Diversity of *Planctomycetes* in soil in relation to soil history and environmental heterogeneity. Appl. Environ. Microbiol. **72:**4522–4531.
10. **Caldwell, M. E., R. M. Garret, R. C. Prince, and J. M. Suflita.** 1998. Anaerobic biodegradation of long chain n-alkanes under sulfate-reducing conditions. Environ. Sci. Technol. **32:**2191–2195.
11. **Chouari, R., D. Le Paslier, C. Dauga, P. Daegelan, J. Weissenbach, and A. Sghir.** 2005. Novel major bacterial candidate division within a municipal anaerobic sludge digestor. Appl. Environ. Microbiol. **71:**2145–2153.
12. **Chouari, R., D. L. Paslier, P. Daegelan, P. Ginestet, J. Weissenbach, and A. Sghir.** 2003. Molecular evidence for novel planctomycete diversity in a municipal wastewater treatment plant. Appl. Environ. Microbiol. **69:**7354–7363.
13. **Clark, D. P.** 1989. The fermentation pathways of *Escherichia coli*. FEMS Microbiol. Lett. **63:**223–234.
14. **Cline, J. D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. **145:**454–458.
15. **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje.** 1 January 2005, posting date. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. **33**(Database issue)**:**D294–D296. doi: 10.1093/nar/gki038.
16. **Dalsgaard, T., D. E. Canfield, J. Peterson, B. Thamdrup, and J. Acuna-Gonzalez.** 2003. N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. Nature **422:**606–608.
17. **Damsté, J. S. S., W. I. C. Rijpstra, J. A. J. Geenevasen, M. Strous, and M. S. M. Jetten.** 2005. Structural identification of ladderane and other membrane lipids of planctomycetes capable of anaerobic ammonium oxidation (anammox). FEBS J. **272:**4270–4283.
18. **DeLong, E. F., D. G. Franks, and A. L. Alldredge.** 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. **38:**924–934.
19. **Dembitsky, V. M.** 1996. Betaine ether-linked glycerolipids: chemistry and biology. Prog. Lipid Res. **35:**1–51.
20. **Derakshani, M., T. Lukow, and W. Liesack.** 2001. Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. Appl. Environ. Microbiol. **67:**623–631.
21. **Dojka, M. A., P. Hugenholtz, S. K. Haack, and N. R. Pace.** 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. **64:**3869–3877.
22. **Elshahed, M. S., F. Z. Najar, M. Aycock, C. Qu, B. A. Roe, and L. R. Krumholz.** 2005. Metagenomic analysis of the microbial community at Zodletone Spring (Oklahoma): insights into the genome of novel candidate division OD1. Appl. Environ. Microbiol. **71:**7598–7602.
23. **Elshahed, M. S., F. Z. Najar, B. A. Roe, A. Oren, T. A. Dewers, and L. R. Krumholz.** 2004. Survey of archaeal diversity reveals an abundance of halophilic *Archaea* in a low-salt, sulfide- and sulfur-rich spring. Appl. Environ. Microbiol. **70:**2230–2239.
24. **Elshahed, M. S., J. M. Senko, F. Z. Najar, S. M. Kenton, B. A. Roe, T. A. Dewers, J. R. Spear, and L. R. Krumholz.** 2003. Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. Appl. Environ. Microbiol. **69:**5609–5621.
25. **Franzmann, P. D., and V. B. D. Skerman.** 1984. *Gemmata obscuriglobus*, a new genus and species of budding bacteria. Antonie Leeuwenhoek **50:**261–268.
26. **Fuerst, J. A.** 2005. Intracellular compartmentation in planctomycetes. Annu. Rev. Microbiol. **59:**299–328.
27. **Fuerst, J. A., H. G. Gwilliam, M. Lindsay, A. Lichanska, C. Belcher, J. E. Vickers, and P. Hugenholtz.** 1997. Isolation and molecular identification of planctomycete bacteria from postlarvae of the giant tiger prawn, *Penaeus monodon*. Appl. Environ. Microbiol. **63:**254–262.
28. **Fuerst, J. A., K. S. Sharan, J. L. Paynter, J. A. Hawkins, and J. G. Atherton.** 1991. Isolation of a bacterium resembling *Pirellula* species from primary tissue culture of the giant tiger prawn (*Panaeus monodon*). Appl. Environ. Microbiol. **57:**3127–3134.
29. **Gerhardt, P., R. G. E. Murray, W. A. Wood, and N. R. Krieg.** 1994. Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC.
30. **Giovannoni, S. J., J. E. Schabtach, and R. W. Castenholz.** 1987. *Isosphaera pallida*, gen. nov., and comb. nov., a gliding, budding eubacterium from hot springs. Arch. Microbiol. **147:**276–284.
31. **Glockner, F. O., M. Kube, M. Bauer, H. Teeling, T. Lombardot, W. Ludwig, D. Gade, A. Beck, K. Borzym, K. Heitmann, R. Rabus, H. Schlesner, R. Amann, and R. Reinhardt.** 2003. Complete genome sequence of the marine planctomycete *Pirellula* sp. strain 1. Proc. Natl. Acad. Sci. USA **100:**8298–8303.
32. **Godon, J. J., E. Zumstein, P. Dabert, F. Habouzit, and R. Moletta.** 1997. Molecular microbial diversity of an anaerobic digester as determined by small-subunit rDNA sequence analysis. Appl. Environ. Microbiol. **63:**2802–2813.
33. **Griepenburg, U., N. Ward-Rainey, S. Mohamed, and H. Schlesner.** 1999. Phylogenetic diversity, polyamine pattern and DNA base composition of members of the order Planctomycetales. Int. J. Syst. Evol. Microbiol. **49:**689–696.
34. **Havens, J. S.** 1983. Oklahoma geological survey circular, vol. 85. Reconnaissance of ground water in the vicinity of the Wichita Mountains, southwestern Oklahoma. Oklahoma Geological Survey, Norman, OK.
35. **Hirsch, P., and M. Muller.** 1985. Planctomyces limnophilus sp. nov., a stalked and budding bacterium from fresh water. Syst. Appl. Microbiol. **6:**276–280.

36. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20:**2317–2319.
37. **Inagaki, F., T. Nunoura, S. Nakagawa, A. Teske, M. Lever, A. Lauer, M. Suzuki, K. Takai, M. Delwiche, F. S. Colwell, K. H. Nealson, K. Horikoshi, S. D'Hondt, and B. B. Jφrgensen.** 2006. Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. Proc. Natl. Acad. Sci. USA **103:**2815–2820.
38. **Janssen, P. H.** 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. **72:**1719–1728.
39. **Kartal, B., J. Rattray, L. A. van Niftrik, J. van de Vossenberg, M. C. Schmid, R. I. Webb, S. Schouten, J. A. Fuerst, J. S. S. Damsté, M. S. M. Jetten, and M. Strous.** 2007. *Candidatus* "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. **30:**39–49.
40. **Kirkpatrick, J., B. Oakley, C. Fuchsman, S. Srinivasan, J. T. Staley, and J. W. Murray.** 2006. Diversity and distribution of *Planctomycetes* and related bacteria in the suboxic zone of the black sea. Appl. Environ. Microbiol. **72:**3079–3083.
41. **Koning, H., H. Schlesner, and P. Hirsch.** 1984. Cell wall studies on budding bacteria of the Planctomyces/Pasteuria group and on a Prosthecomicrobium sp. Arch. Microbiol. **138:**200–205.
42. **Kuske, C. R., S. M. Barns, and J. D. Busch.** 1997. Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. Appl. Environ. Microbiol. **63:**3614–3621.
43. **Kuypers, M. M. M., G. Lavik, D. Woebken, M. Schmid, B. M. Fuchs, R. Amann, B. B. Jφrgensen, and M. S. M. Jetten.** 2005. Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. Proc. Natl. Acad. Sci. USA **102:**6478–6483.
44. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–174. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Chichester, United Kingdom.
45. **LaPara, T. M., C. H. Nakatsu, L. Pantea, and J. E. Alleman.** 2000. Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. Appl. Environ. Microbiol. **66:**3951–3959.
46. **Li, L. N., C. Kato, and K. Horikoshi.** 1999. Bacterial diversity in deep-sea sediments from different depths. Biodivers. Conserv. **8:**659–677.
47. **Liesack, W., H. Koning, H. Schlesner, and P. Hirsch.** 1986. Chemical composition of the peptidoglycan-free envelopes of budding bacteria of the Pirella/Planctomyces group. Arch. Microbiol. **145:**361–366.
48. **Masuko, T., A. Minamib, N. Iwasakib, T. Majimab, S.-I. Nishimura, and Y. C. Leea.** 2005. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal. Biochem. **339:**69–72.
49. **McInerney, M. J., M. P. Bryant, and N. Pfennig.** 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. **122:**129–135.
50. **Miskin, I. P., P. Farrimond, and I. M. Head.** 1999. Identification of novel bacterial lineages as active members of microbial populations in a freshwater sediment using a rapid RNA extraction procedure and RT-PCR. Microbiology **145:**1977–1987.
51. **Nogales, B., E. R. B. Moore, E. Llobet-Brossa, R. Rosello-Mora, R. Amann, and K. N. Timmis.** 2001. Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl. Environ. Microbiol. **67:**1874–1884.
52. **Posada, D., and K. A. Crandall.** 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817–818.
53. **Rappe, M. S., P. F. Kemp, and S. J. Giovannoni.** 1997. Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. Limnol. Oceanogr. **42:**811–826.
54. **Ravenschlag, K., K. Sahm, J. Pernthaler, and R. Amann.** 1999. High bacterial diversity in permanently cold marine sediments. Appl. Environ. Microbiol. **65:**3982–3989.
55. **Reed, D. W., Y. Fujita, M. E. Delwiche, D. B. Blackwelder, P. P. Sheridan, T. Uchida, and F. S. Colwell.** 2002. Microbial communities from methane hydrate-bearing deep marine sediments in a forearc basin. Appl. Environ. Microbiol. **68:**3759–3770.
56. **Sanders, W. E.** 1998. Rate and mechanism of barite mineralization at Zodletone Mountain, southwestern Oklahoma. University of Oklahoma, Norman.
57. **Schlesner, H.** 1986. *Pirella marina* sp. nov., a budding peptidoglycan-less bacterium from brackish water. Syst. Appl. Microbiol. **8:**177–180.
58. **Schlesner, H.** 1989. *Planctomyces brasiliensis* sp. nov., halotolerant bacterium from a salt pit. Syst. Appl. Microbiol. **12:**159–161.
59. **Schlesner, H.** 1994. The development of media suitable for the microorganisms morphologically resembling *Planctomyces* spp., *Pirellula* spp., and other *Planctomycetales* from various aquatic habitats using dilute media. Syst. Appl. Microbiol. **17:**135–145.
60. **Schlesner, H., C. Rensmann, B. J. Tindall, D. Gade, R. Rabus, S. Pfeiffer, and P. Hirsch.** 2004. Taxonomic heterogeneity within the Planctomycetales as derived by DNA-DNA hybridization, description of *Rhodopirellula baltica* gen. nov., sp. nov., transfer of *Pirellula marina* to the genus *Blastopirellula* gen. nov. as *Blastopirellula marina* comb. nov. and emended description of the genus *Pirellula*. Int. J. Syst. Evol. Microbiol. **54:**1567–1580.
61. **Schlotelburg, C., F. von Wintzingerode, R. Hauck, W. Hegemann, and U. B. Gobel.** 2000. Bacteria of an anaerobic 1,2-dichloropropane-dechlorinating mixed culture are phylogenetically related to those of other anaerobic dechlorinating consortia. Int. J. Syst. Evol. Microbiol. **50:**1505–1511.
62. **Schmid, M., K. Walsh, R. Webb, W. I. C. Rijpstra, K. van de Pas-Schoonen, M. J. Verbruggen, T. Hill, B. Moffett, J. Fuerst, S. Schouten, J. S. S. Damsté, J. Harris, P. Shaw, M. Jetten, and M. Strous.** 2003. Candidatus "*Scalindua brodae*", sp. nov., Candidatus "*Scalindua wagneri*", sp. nov., two new species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. **26:**529–538.
63. **Schmid, M., U. Twachtmann, M. Klein, S. Strous, S. Juretschko, M. S. M. Jetten, J. W. Metzger, K.-H. Schleifer, and M. Wagner.** 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. **23:**93–106.
64. **Schroder, I., A. Kroger, and J. M. Macy.** 1998. Isolation of the sulfur reductase and reconstitution of the sulfur respiration of *Wolinella succinogenes*. Arch. Microbiol. **149:**572–579.
65. **Senko, J. M., B. S. Campbell, J. R. Henricksen, M. S. Elshahed, T. A. Dewers, and L. R. Krumholz.** 2004. Barite deposition mediated by phototrophic sulfide-oxidizing bacteria. Geochim. Cosmochim. Acta **68:**773–780.
66. **Staley, J. T.** 1973. Budding bacteria of the *Pasteuria-Blastobacter* group. Can. J. Microbiol. **19:**609–614.
67. **Staley, J. T., J. Fuerst, and S. Giovannoni.** 1992. The order Planctomycetales and the genera Planctomyces, Pirellula, Gemmata, and Isophera. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes: a handbook on the biology of bacteria—ecophysiology, isolation, identification, applications. Springer, New York, NY.
68. **Stevenson, B. S., S. A. Eichorst, J. T. Wertz, T. M. Schmidt, and J. A. Breznak.** 2004. New strategies for cultivation and detection of previously uncultured microbes. Appl. Environ. Microbiol. **70:**4748–4755.
69. **Strous, M., J. A. Fuerst, E. H. M. Kramer, S. Logemann, G. Muyzer, K. T. van de Pas-Schoonen, R. Webb, J. G. Kuenen, and M. S. M. Jetten.** 1999. Missing lithotroph identified as new planctomycete. Nature **400:**446–449.
70. **Sturt, H. F., R. E. Summons, K. Smith, M. Elvert, and K.-U. Hinrichs.** 2004. Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry: new biomarkers for biogeochemistry and microbial ecology. Rapid Commun. Mass Spectrom. **18:**617–628.
71. **Teske, A., K.-U. Hinrichs, V. Edgcomb, A. D. Gomez, D. Kysela, S. P. Sylva, M. L. Sogin, and H. W. Jannasch.** 2002. Microbial diversity of hydrothermal sediments in the Guaymas Basin: evidence for anaerobic methanotrophic communities. Appl. Environ. Microbiol. **68:**1994–2007.
72. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
73. **Urbach, E., K. L. Vergin, L. Young, and A. Morse.** 2001. Unusual bacterioplankton community structure in ultra-oligotrophic Crater Lake. Limnol. Oceanogr. **46:**557–572.
74. **Wang, J., C. Jenkins, R. I. Webb, and J. A. Fuerst.** 2002. Isolation of *Gemmata*-like and *Isosphaera*-like planctomycete bacteria from soil and freshwater. Appl. Environ. Microbiol. **68:**417–422.
75. **Ward, N., J. T. Staley, J. A. Fuerst, S. Giovannoni, H. Schlesner, and E. Stackebrandt.** 10 October 2006, posting date. The order Planctomycetales, including the genera *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera* and the Candidatus genera *Brocadia*, *Kuenenia* and *Scalindua*, p. 757–793. *In* M. Dworkin et al. (ed.), The prokaryotes: a handbook on the biology of bacteria, 3rd ed. Springer, New York, NY. doi:10.1007/0-387-30747-8_31.
76. **Wu, J. H., W. T. Liu, I. C. Tseng, and S. S. Cheng.** 2001. Characterization of 4-methylbenzoate-degrading methanogenic consortium as determined by small-subunit rDNA sequence analysis. J. Biosci. Bioeng. **91:**449–455.
77. **Youssef, N. H., K. E. Duncan, and M. J. McInerney.** 2005. Importance of 3-hydroxy fatty acid composition of lipopeptides for biosurfactant activity. Appl. Environ. Microbiol. **71:**7690–7695.
78. **Zengler, K., G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short, and M. Keller.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. USA **99:**15681–15686.