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# Isolation and Molecular Identification of Planctomycete Bacteria from Postlarvae of the Giant Tiger Prawn, *Penaeus monodon*

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**Bacteria phenotypically resembling members of the phylogenetically distinct planctomycete group of the domain *Bacteria* were isolated from postlarvae of the giant tiger prawn, *Penaeus monodon*. A selective medium designed in the light of planctomycete antibiotic resistance characteristics was used for this isolation. Planctomycetes were isolated from both healthy and monodon baculovirus-infected prawn postlarvae. The predominant colony type recovered from postlarvae regardless of viral infection status was nonpigmented. Other, less commonly observed types were pink or orange pigmented. A planctomycete-specific 16S rRNA-directed probe was designed and used to screen the isolates for their identity as planctomycetes prior to molecular phylogenetic characterization. 16S rRNA genes from nine prawn isolates together with two planctomycete reference strains (*Planctomyces brasiliensis* and *Gemmata obscuriglobus*) were sequenced and compared with reference sequences from the planctomycetes and other members of the domain *Bacteria*. Phylogenetic analyses and sequence signatures of the 16S rRNA genes demonstrated that the prawn isolates were members of the planctomycete group. Five representatives of the predominant nonpigmented colony type were members of the *Pirellula* group within the planctomycetes, as were three pink-pigmented colony type representatives. Homology values and tree topology indicated that representatives of the nonpigmented and pink-pigmented colony types formed two discrete clusters within the *Pirellula* group, not identical to any known *Pirellula* species. A sole representative of the orange colony type was a member of the *Planctomyces* group, virtually identical in 16S rDNA sequence to *P. brasiliensis*, and exhibited distinctive morphology.**

Planctomycetes constitute a discrete phylum of the domain *Bacteria* (48), forming the order *Planctomycetales*, with coherent and distinctive phenotypic and molecular characteristics such as budding division, peptidoglycanless cell walls displaying crater-like pits, unusually short 5S rRNA, and distinct 16S rRNA sequences (13, 19, 21, 39). They have been isolated from diverse aquatic ecosystems, including aquatic freshwater, brackish water, marine, and hypersaline habitats (33). Culture-independent rRNA-based molecular phylogenetic studies indicate their presence in marine organic aggregates (8) and even in soil (23).

Bacteria conforming to the phenotypic characteristics expected of planctomycetes had been isolated in a previous study from primary tissue culture initiated from the hepatopancreas (midgut gland) of the giant tiger prawn, *Penaeus monodon* (14). The occurrence of these unusual bacteria in this habitat is relevant to studies of diseases of aquatic invertebrates, since they may be encountered during attempts to establish tissue culture for virus isolation and may be observed in investigations of bacterial microflora of aquacultured crustaceans. However, their membership in the planctomycetes had not been established by analysis of molecular sequences. The present study was aimed to resolve this question by examining both previously obtained and new isolates by 16S rRNA gene (16S rDNA) sequencing and phylogenetic analysis. We have confirmed their membership in the planctomycetes by such methods, revealed their distinctiveness in relation to named planctomycete species, and established an increased range of phylogenetic diversity for microorganisms culturable from invertebrates and for organisms related to genera within the order *Planctomycetales*.

The prawns used to establish the tissue culture in the original study (14) were known to have been infected with monodon baculovirus (most commonly referred to as MBV, this virus has recently been renamed *Penaus monodon* single nuclear polyhedrosis virus [25]). MBV has been found in wild and/or cultured prawns in Australia, Southeast Asia, the Middle East, and southern Europe and can occur at high levels in aquaculture facilities, particularly in postlarvae (PL), which are weakened and probably become more susceptible to other diseases when infected by the virus (11). It was therefore of interest to isolate further planctomycete strains from MBV-infected PL to investigate the diversity of planctomycetes recoverable from such PL and to establish if similar planctomycetes also occurred in uninfected PL. We approached this by experimentally infecting healthy PL with MBV to supply infected PL for direct comparison with healthy specimens with respect to phylogenetic relationships of their planctomycete populations.

## MATERIALS AND METHODS

**Bacterial strains.** Prawn isolates from a previous study (14), ACM 3180 (PRPL-2) and ACM 3181 (PRPL-1), were obtained from the Australian Collection of Microorganisms (ACM), University of Queensland. *Gemmata obscuriglobus* ACM 2246, *Planctomyces brasiliensis* ATCC 49424, *Escherichia coli* ACM 1803, *Agrobacterium tumefaciens* ACM 2429, *Flavobacterium breve* ACM 2384, *Pirellula marina* ACM 3344, and *Planctomyces limnophilus* ATCC 43996 were used as reference cultures in probe hybridizations. All other strains used were new isolates from the present study (see Results).

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**Infection of PL with MBV.** Modifications of published methods were employed for viral infection of prawn PL (46). For experiments which yielded additional nonpigmented and orange planctomycete strains, *Penaeus monodon* PL of stage 15 (26) were stocked at 100 PL per liter of seawater, with 5,000 to 10,000 per container, and fed four to five *Artemia* organisms per ml of seawater twice daily. Furazolidone (Rhone-Poulenc Animal Nutrition Pty. Ltd., Brisbane, Australia) was added at 5 mg per liter of seawater. Furazolidone [3-(5-nitrofurfurylideneamino)-2-oxazolidone] is a nitrofuran derivative with antiprotozoan and antibacterial activity (29). Frozen MBV-infected stage 15 *Penaeus monodon* PL (1 g per 1,000 PL to be infected) were homogenized in a buffer consisting of 0.14 M NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, and 1.0 mM EDTA, and the homogenate was added to aquaria containing PL. PL were checked daily for MBV infection by an impression smear technique (45). Hepatopancreases were dissected out on days 4 to 10 postinfection and placed immediately into 1.5-ml polypropylene tubes on dry ice. Material was stored at −70°C until used. In a separate series of experiments, those in which pink isolates happened to be obtained, MBV-infected prawn PL had been derived as follows: stage 5 PL of *Penaeus monodon* were collected from the Gold Coast Marine Hatchery, stocked at ca. 500 PL per 3 liters of seawater in 5-liter conical flasks, and fed commercial prawn feed once a day. Seawater was changed every day just prior to feeding. No antibiotics were added. For MBV infection of these PL, 1 g of MBV-infected frozen stage 15 PL per 10,000 PL to be infected was homogenized in 3 ml of homogenizing buffer (10× phosphate-buffered saline plus 0.037 g of EDTA) with a hand-held homogenizer on ice. The resulting homogenate was added directly to aquaria containing the PL (PL were not fed on the day of infection). MBV infection was confirmed by histology.

**Media.** Nonselective half-strength marine agar (HSMA) comprised half-strength Difco marine broth containing 1.5% Bitek agar (Difco), sterilized by autoclaving. Planctomycete selective agar (PSA) was HSMA supplemented with filter-sterilized penicillin G (500 μg $ml^{-1}$) and streptomycin (1000 μg $ml^{-1}$). Planctomycete selective broth was PSA without the agar.

**Isolation of planctomycetes from noninfected and infected PL prawns.** Approximately 0.2 g of noninfected or infected prawn PL was weighed out, washed three times with sterile artificial seawater, and ground in a sterile mortar and pestle or in a stomacher to produce a crude homogenate. The homogenate was streaked directly onto PSA or preenriched prior to being plated onto PSA. For enrichment, aliquots (0.25 ml) of the liquid fraction were inoculated into 50 ml of planctomycete selective broth (in 250-ml flasks), shaken at 28°C at ca. 100 rpm for 6 days, and then streaked onto PSA plates. All PSA plates were incubated in the dark at 28°C for 4 to 10 days prior to subculturing of selected colonies. Representative colony types were initially purified on PSA plates and subsequently subcultured onto nonselective HSMA plates incubated at 28°C. Strains were grown on HSMA for 4 to 7 days at 28°C to obtain biomass for PCR.

**Electron microscopic characterization.** Cells were grown on HSMA plates for 7 to 8 days at 28°C and then negatively stained with filtered (0.22-μm-pore-size membrane) 1% uranyl acetate containing 0.4% sucrose. A suspension of cells in sterile water was prepared on a glass slide with sterile plastic loop; a drop of suspension was placed on a Formvar-coated specimen support grid, then mixed with a drop of uranyl acetate-sucrose stain, and left for ca. 30 s; and the drop was removed with filter paper. Grids were examined with a Hitachi H-800 transmission electron microscope operated at 100-kV accelerating voltage.

**Design and preparation of planctomycete-specific probe.** 16S rRNA gene sequences for planctomycete species from the GenBank database, X62911 (*Planctomyces limnophilus*), X62912 (*Pirellula marina*), X56305 and X54522 (*G. obscuriglobus*), X54372 (*Isosphaera pallida*), and M34126 (*Planctomyces staleyi* [sic: *Pirellula staleyi*]), were manually aligned with Eyeball Sequence Editor version 1.04 software (6). The sequence for *Pirellula staleyi* was compared with 16S rRNA sequences for other bacteria in the alignment of Lane (20), and a probe (sequence 5′-CCACCGCTTGTGTGAGCCCC-3′) was designed based on positions 945 to 926 (*E. coli* numbering) and conservation within the aligned planctomycete species. The oligonucleotide probe was chemically synthesized by the Centre for Molecular Biology and Biotechnology, University of Queensland. This oligonucleotide was labelled with digoxigenin (DIG) by using the 3′ DIG labelling kit (Boehringer Mannheim) according to the manufacturer's instructions.

**Hybridizations of amplified 16S rDNA with planctomycete-specific probe.** 16S rDNA from each strain examined was PCR amplified from genomic DNA purified from cells via InstaGene matrix (Bio-Rad). PCR was performed via 30 cycles: annealing for 45 s at 48°C, elongation for 2 min at 72°C, and denaturation for 1 min at 94°C with final extension for 5 min at 72°C, using universal 16S rDNA primers 27f and 1492r (20). PCR product DNA (20 μl) was blotted onto a positively charged nylon membrane (Boehringer Mannheim) with a slot blot apparatus (Hoefer PR600) and treated with UV for 4 min. Prehybridization was performed in standard prehybridization solution (Boehringer Mannheim) in hybridization bottles at 46°C for 2 h in a hybridization oven (Hybaid). Hybridization was performed at 46°C for 2 h in a hybridization oven (Hybaid) after addition of 1 μl of labelled probe per ml of prehybridization solution, so that a final labelled probe concentration of $5 \times 10^{-3}$ pM $\mu l^{-1}$ was achieved. Washes were performed at low stringency (twice with 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 46°C for 10 min each and twice with 2× SSC at 56°C for 15 min each) or at high stringency (twice with 2× SSC at 46°C for 5 min each, twice with 1× SSC at 60°C for 15 min each, once with 0.1× SSC at 66°C for 15 min, and once with 0.1× SSC at 68°C for 15 min). The DIG-labelled probe was detected by a colorimetric reaction with nitroblue tetrazolium plus 5-bromo-4-chloro-3-indolyl-phosphate and anti-DIG–alkaline phosphatase conjugate from the DIG detection kit (Boehringer Mannheim) according to the manufacturer's instructions, with development for only 1 h. *A. tumefaciens*, *E. coli*, and *F. breve* were used as nonplanctomycete reference negative control strains. An 18-mer universal probe complementary to a sequence in all cellular 16S-like rRNAs with the sequence 5′-GWATTACCGCGGCKGCTG-3′ (W, A or T; K, G or T) (519r), equivalent to a 519r 16S rRNA sequencing primer and to the universal probe of Giovannoni et al. (16), was also DIG labelled and used in hybridizations as a positive control for 16S rDNA presence and reactivity in the PCR products.

**PCR amplification and cycle sequencing of 16S rDNA.** A modification of methods previously described by us (18) was used for PCR amplification and cycle sequencing of 16S rDNA. Small-subunit (16S) rDNAs were amplified directly from bacterial cell lysates with a 27f and 1492r or 27f and 1525r primer set designed for most eubacteria (20). Strains were grown on HSMA for 4 to 7 days at 28°C to obtain biomass for PCR. A tiny quantity of agar plate culture (ca. 1 $mm^3$) was deposited at the base of a 500-μl PCR Eppendorf tube and lysed in *Taq* buffer (Boehringer Mannheim; 10 μl of 10× stock) and sterile water (ca. 85 μl) overlaid with mineral oil at 98°C for 15 min by using a Perkin-Elmer Cetus thermocycler.

Amplification primers (200 ng of each), 50 μM (each; final concentration) deoxynucleoside triphosphates (dNTPs) (Promega), and 2 U of *Taq* polymerase (Boehringer Mannheim) were then added as a concentrated mix (ca. 5-μl total volume) under the mineral oil to each tube for a final reaction volume of 100 μl. Thirty cycles were conducted on a Perkin-Elmer Cetus thermocycler with the cycling profile described above. Amplified products were purified by using the Wizard DNA Clean-up System (Promega) according to the manufacturer's instructions.

The double-stranded PCR products were sequenced with the Taq Dye Deoxy Terminator Cycle Sequencing kit of Applied Biosystems, which employs fluorescently labelled ddNTPs rather than labelled primers. Briefly, 20-μl reaction mixtures contained 25 to 30 ng of sequencing primer, 1 μl of Terminator Ammonium Cycle Sequencing dNTP mix, 1 μl of each fluorescently labelled dideoxynucleotide chain terminator, 50 to 100 ng of amplified 16S rDNA template, and 0.25 μl of *Taq* polymerase in 1× Terminator Ammonium Cycle Sequencing buffer. The cycling profile used was 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min, for 25 cycles. Following PCR, the reaction volume (20 μl) was made up to 100 μl with sterile Milli-Q grade water, and the cycle sequencing products were then extracted twice with equal volumes of a phenol-water-chloroform (68:18:14) mixture and precipitated with sodium acetate and ethanol. Electrophoresis and laser fluorimeter detection of purified sequencing products were performed on an Applied Biosystems 373A DNA sequencer. Near-complete or partial 16S rDNA sequences of both strands were obtained with primers modified from those described by Lane (20), some of which were modified to optimize annealing to planctomycete template; forward and reverse primers specific for planctomycete amplification (PLN) designed from the planctomycete probe sequence were also used. The primers used were as follows (where no modification was employed, sequences are as described by Lane [20]; M, C or A; K, G or T; W, A or T): 27f, 342r (modified to 5′-CTGCWGCCWCCCGTAG), 519r, 530f, 687r (modified to 5′-ACGCATTTCACCGCT), PLN926r (5′-CCAC CGCTTGTGTGAGCCCC), PLN945f (5′-GGGGCTCACACAAGCGGTGG), 1101r (modified to 5′-GGGTTKCGCTCGTT), 1114f (modified to 5′-AACGA GCGMAACCC), 1406f (modified to 5′-TGTACACACCGCCCGT) and 1492r.

**Phylogenetic analyses.** The resulting 16S rDNA sequences were initially compiled with Eyeball Sequence Editor version 1.09 (6) or SeqEd (Applied Biosystems). Compiled sequences were compared against the sequence databases with the Basic Local Alignment Search Tool (BLAST) (1) to determine closest phylogenetic neighbors. A data set containing the prawn isolate sequences and closest neighbors estimated from BLAST, sequences of which were obtained from the Ribosomal Database Project (24), was aligned with Clustal W (43) and then checked manually with sequence editor ae2 available within the Ribosomal Database Project. The integrity of the 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 according to the bacterial mask of Lane (20) by using the ae2 sequence editor, prior to analysis. N's were excluded from the data set. The final data set comprising 46 taxa and 740 homologous nucleotide positions included the following regions of the 16S rDNA: 322 to 450, 481 to 836, 850 to 1003, 1037 to 1128, and 1145 to 1209 (*E. coli* numbering [5]).

Trees were constructed by either distance or parsimony methods. For distance trees, pairwise evolutionary distances of unambiguously alignable regions of the sequences were computed with the Kimura two-parameter model and DNADIST within the PHYLIP package (12). A rooted phylogenetic tree was then constructed from the distance matrix by the neighbor-joining method of Saitou and Nei (30), using the NEIGHBOR program within PHYLIP. *Thermotoga maritima* was used as the out-group for this tree. Parsimony analysis was performed with PAUP (42). Relative support for tree topology was determined by bootstrap resampling (100 resamplings) of the masked data set by distance and parsimony methods. For parsimony using PAUP, a heuristic bootstrap analysis

employing default settings was used with *T. maritima* again as the out-group. Bootstrap values less than 75% were considered to provide no support for an inferred branching point (50).

**Nucleotide sequence accession numbers.** Nucleic acid sequences from this study have been deposited in the EMBL database under accession numbers X85247 to X85249 and X86387 to X86394.

## RESULTS

**Isolation and morphological characterization of planctomycetes from PL prawns.** A selective medium employing antibiotics for isolation of planctomycetes from crustaceans was developed. This medium was designed in the light of the isolation of such bacteria from tissue culture media with concentrations of the ribosome-targeting antibiotic streptomycin and the antibiotic resistance spectrum of the first two planctomycete strains so isolated (14). High streptomycin concentrations were necessary to avoid nonplanctomycete bacterial enrichment when broth enrichments were employed. PSA was used successfully to isolate putative planctomycete strains from homogenized PL of *Penaeus monodon*. Isolates could be recovered directly from colonies on dilution plates of PSA from prawn homogenate. Seven strains were isolated in the course of the present study: strains AGA/M12, AGA/M18, HGG/MP1, PH/CP1, and PH/CP2 (isolated directly on PSA) and AGA/M41 and AGA/C41 (isolated with an initial broth enrichment); in the code behind the slash, "M" designates strains from MBV-infected prawns and "C" designates strains from uninfected control prawns. Representatives of three colony types were isolated from both MBV-infected prawn PL and uninfected control prawn PL. The predominant colony type recovered from prawn PL exhibited small nonpigmented, opaque, glistening colonies with an entire edge (e.g., ca. 90 to 95% of total colonies observed on plates receiving a primary dilution of homogenized healthy PL). Pink-pigmented colonies comprised ca. 5 to 10% of total colonies, and in one instance, a single orange colony was observed and subcultured. Two strains with a nonpigmented colony type, ACM 3180 and ACM 3181, had been isolated in a previous study. Of the three new strains with a nonpigmented colony type isolated in this study, one, AGA/C41, was isolated from control prawns not infected with MBV and two, AGA/M12 and AGA/M41, were isolated from prawns infected with MBV. Three strains with pink-pigmented colony type were isolated: HGG/MP1, from MBV-infected PL, and PH/CP1 and PH/CP2, from control uninoculated PL. One isolate from MBV-infected PL, strain AGA/M18, produced orange-pigmented colonies.

Crateriform structures (negative-stain-accumulating pits in the cell wall) indicating a planctomycete morphotype occurred on the cell surface of all fresh isolates examined for the first time in this study (AGA/M12, AGA/M41, AGA/C41, HGG/MP1, PH/CP1, and AGA/M18) by negative staining and transmission electron microscopy (see Fig. 1). Cells of isolates AGA/M12, AGA/M41, AGA/C41, HGG/MP1, and PH/CP1 displayed a polar distribution of such structures and an ovoid or teardrop cell morphology consistent with their possible membership in the genus *Pirellula*. In contrast, AGA/M18 demonstrated a uniform distribution of crateriform structures and spherical single-cell morphology more consistent with membership in either the genus *Planctomyces* or the genus *Gemmata*. A distinctive feature of AGA/M18 was the presence of polar unicorn prostheca-like projections on one cell pole in many cells (Fig. 1c). This feature was also found on negative-stain examination of *Planctomyces brasiliensis* (Fig. 1d).

**Design and application of a planctomycete-specific probe.** In order to confirm membership within the planctomycetes of the first four prawn isolates (AGA/M12, AGA/M18, AGA/M41, and AGA/C41) from this study and two isolates (ACM 3180 and ACM 3181) from a previous study (14), a planctomycete-specific probe was designed from existing database comparisons of 16S rRNA sequences and a sequence from the region from 926 to 945 (*E. coli* numbering system) was chosen as the target region. A nonradioactive DIG-labelled 20-mer planctomycete-specific probe was designed to recognize this target and applied to dot blot membrane hybridizations against PCR-amplified 16S rDNA from reference planctomycetes, nonplanctomycete reference strains, and prawn isolates from a previous study and the present study.

When employed with high-stringency wash conditions, the probe proved specific for known reference planctomycetes relative to nonplanctomycete reference bacteria and also reacted positively with prawn isolates ACM 3180, ACM 3181, AGA/M12, AGA/M18, AGA/M41, and AGA/C41 (Fig. 2b). No hybridization of the probe was observed with 16S rDNA from representative nonplanctomycete members of the domain *Bacteria*, such as strains from the $\alpha$ and $\gamma$ subdivisions of the class *Proteobacteria* (*A. tumefaciens* and *E. coli*) or the *Bacteroides-Flavobacterium* subdivision (*F. breve*), as long as high-stringency wash conditions were applied (Fig. 2b), while such 16S rDNA did hybridize with the universal probe (Fig. 2a). The planctomycete-specific probe should prove useful for preliminary identification of any planctomycete strain, for example, when screening isolates during preliminary identification of bacteria from prawns. A BLAST analysis using the probe sequence demonstrated a perfect match only with planctomycete sequences or environmental clones known to cluster with planctomycetes in phylogenetic analysis. A match with one base pair mismatch occurred with the 16S rRNA of the environmental clone AGG27 from marine phytodetrital macroaggregates with a distant planctomycete relationship and with 16S rRNAs of several obligate intracellular pathogen *Rickettsia* species. It is unlikely that confusion in identity by using the probe even in unmodified form could arise from this latter homology when applied to culturable bacteria.

**16S rRNA sequence characteristics and phylogenetic analysis.** 16S rDNAs from nine prawn-derived planctomycete-like isolates, including two isolates, ACM 3180 (PRPL-2) and ACM 3181 (PRPL-1), from a previous study (14) and the seven new isolates from the present study, were sequenced. Near-complete 16S rDNA sequences (1,377 to 1,500 bases) were determined for strains ACM 3180, ACM 3181, AGA/C41, AGA/M12, AGA/M41, AGA/M18, and PH/CP1, while 878- to 1,185-base stretches were determined for PH/CP2 and HGG/MP1. Some standard 16S rDNA sequencing primers were slightly modified to optimize annealing to planctomycete rDNA templates which as a group differ from the rest of the domain *Bacteria* in some nucleotide positions at priming sites. To ensure complete sets of reference strain sequences, 16S rDNA of *G. obscuriglobus* was resequenced (1,438 nucleotides) due to ambiguities in existing database sequences and *Planctomyces brasiliensis* was sequenced due to an absence of available database sequence for that species. Only 1,092 nucleotides of the 16S rDNA of the latter species could be determined due to problems sequencing the first 370 bases. All strains isolated from prawn PL, except AGA/M18, were most closely related to each other (87.9 to 100.0% sequence similarity) and to other members of the *Pirellula* group (81.8 to 94.2% sequence similarity). Strain AGA/M18 was most closely related to *Planctomyces brasiliensis* (99.4% similarity).

The nonpigmented isolates appear closest to *Pirellula marina* among known planctomycetes (93.7 to 93.9% sequence similarity). Nonpigmented *Pirellula*-like isolates not only cluster together in phylogenetic analysis, their 16S rDNA sequences

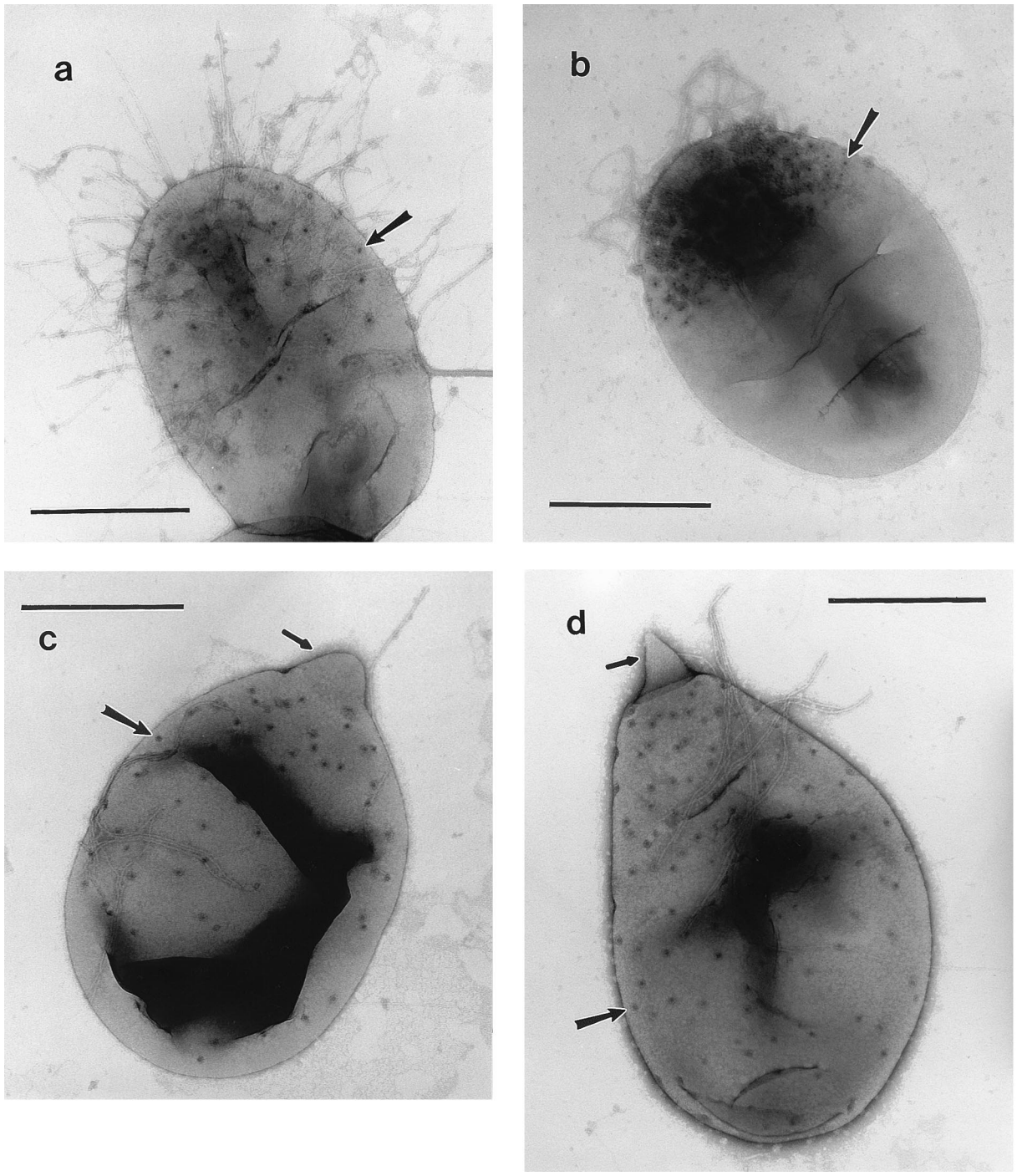


FIG. 1. Electron micrograph of negatively stained cells of strains isolated from *Penaeus monodon* PL. (a) Nonpigmented strain AGA/M12, displaying crateriform structures (arrow) with polar distribution; (b) pink-pigmented strain HGG/MP1, displaying polar crateriform structures (arrow) and polar fibrillar appendages; (c) orange-pigmented strain AGA/M18, displaying uniform distribution of crateriform structures (large arrow) and a polar unicorn prostheca-like projection (small arrow); (d) *Planctomyces brasiliensis* ATCC 49424, displaying uniform distribution of crateriform structures (large arrow) and a polar unicorn prostheca-like projection (small arrow). Bars, 0.5 μm.

exhibiting 99.8% similarity or above with each other, but also exhibit sequence signatures specific to their own cluster, for example, at positions 73 to 91 (*E. coli* numbering system) in the V1 variable region with the sequence CCAAGAAAGCUUG CUUU, at positions 591 to 600, and at position 916, displaying an A instead of the G present in all other planctomycetes, including the named *Pirellula* species (it should be noted that an A is also present at this position in chlamydiae). However, they share a unique stretch within the V3 variable region (*E. coli* positions 450 to 483) with *Pirellula marina*. Nonpigmented isolates were 87.9 to 91.0% similar in sequence to the pink-pigmented colony type isolates. Four of the representatives of

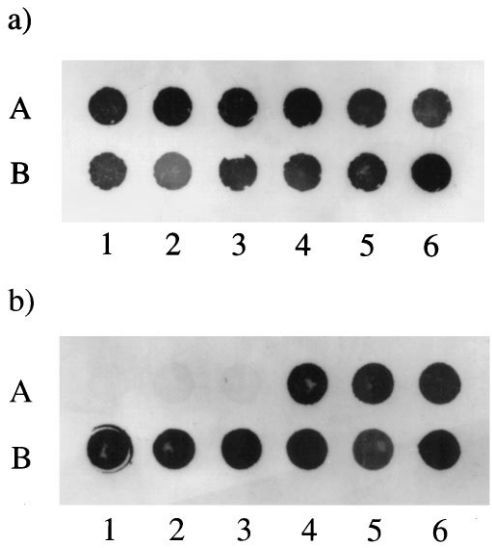


FIG. 2. Dot blot hybridization of PCR products from amplification of 16S rDNA hybridized with the DIG-labelled universal probe (a) or DIG-labelled planctomycete-specific probe (b). (A) Spots: 1, *A. tumefaciens*; 2, *F. breve*; 3, *E. coli*; 4, ACM 3180; 5, ACM 3181; 6, *Pirellula marina*. (B) Spots: 1, AGA/M12; 2, AGA/M18; 3, AGA/M41; 4, AGA/C41; 5, *G. obscuriglobus*; 6, *Planctomyces limnophilus.*

the nonpigmented colony type isolates from prawn PL, strains ACM 3180, ACM 3181, AGA/C41, and AGA/M41, possessed identical 16S rDNA sequences, and AGA/M12 displayed only three positions which differed from the sequences for the other four nonpigmented strains (when regions of 1,384 to 1,500 bases were compared depending on the sequence pair), two of which were the paired bases at 824 and 876 (C and G in AGA/M12 compared to U and A in the other strains).

The pink-pigmented colony type isolates were most similar (90.2 to 94.2%) to three pink- or red-pigmented planctomycete strains, 140, 384, and 1, sequenced by Ward et al. (47); strains 140 and 384 were from Kiel Fjord, and strain 1 was from an aquarium. The partial sequence for pink-pigmented strain PH/CP2 was not included in the phylogenetic analysis, since it was identical in sequence to the two other pink-pigmented prawn PL strains, which were also identical in sequence to each other. The pink prawn isolates and strains 140, 384, and 1 of Ward et al. (47) have characteristic nucleotides at positions 559 (U) (shared with strains 158, 516, 139, and 302 of Ward et al.) and 678 and 712 (U and G), where other planctomycetes have A and have U and A, respectively. The pink prawn isolates, together with strains 1, 158, and 678 of Ward et al. (47) and marine organic macroaggregate clone sequence env. AGG8, differ at positions 1354 and 1368 (U and A) from most other planctomycetes (C and G). The pink prawn isolates, together with strains 140, 384, and 1 of Ward et al. (47) as well as strains 158, 516, 139, and 302 of Ward et al. (47) and env. AGG8, also differ at positions 1421 and 1479 (U and G) from most other planctomycetes. Strains 1 and 158 of Ward et al. sharing U and A with the pink prawn isolates at positions 1354 and 1368 are reported to be both pink and of *Pirellula*-like morphology, while strains 1, 140, 384, 158, 516, 139, and 302 sharing a U and G at 1421 and 1479 with the pink prawn isolates are all red or pink and except for the prosthecate 516 exhibit *Pirellula*-like morphology (47).

16S rRNA sequences from all five nonpigmented prawn isolates display all the signature nucleotides and nucleotide pairs characteristic of planctomycetes noted by Liesack and Stackebrandt (23) except for position 811 (U rather than C), while available sequences for pink *Pirellula*-like prawn isolates also match with signatures of planctomycetes. All prawn isolates exhibit the 16S rRNA planctomycete phylum signatures listed in Table 3 of Woese (48), with the exceptions of positions 812, 976, and 1,415. Both the nonpigmented and pink-pigmented isolates from prawn PL share the signature nucleotides exhibited by all members of the *Pirellula* group within the planctomycetes, which in the case of nucleotides at positions 114 and 313, 680 and 710, 948 and 1233, and 1420 and 1480 are signatures exclusive to the *Pirellula* group (Table 1).

A representative of the orange colony type from prawn PL, strain AGA/M18, exhibited all planctomycete-specific signature positions (23) possessed by nonpigmented *Pirellula*-like isolates except for that at position 538 (U in AGA/M18) and displayed near identity in available homologous sequence to that of *Planctomyces brasiliensis* (99.4% similarity), with only six nucleotide differences at paired base positions in known variable regions V6 and V7 (numbering system of Neefs et al. [28]), at positions 1009 and 1020, 1010 and 1019, and 1121 and 1152 (U and A, C and G, and U and A, respectively, in AGA/M18 versus G and C, G and U, and C and G in *Planctomyces brasiliensis*). Strain AGA/M18 shares with *Planctomyces brasiliensis* the signature nucleotide pair C and G at positions 682 and 708 (G and C or sometimes U in all other planctomycetes except the *Planctomyces*-clustering strain 658 of Ward et al. [47], where it is A and U) and a U at position 819 (A or C in all other planctomycetes). However, it is perhaps significant that this comparison does not include the first 370 bases, positions which were able to be derived from AGA/M18 but not from *Planctomyces brasiliensis*. The difference in difficulty of sequencing this region between these otherwise very similar strains may indicate important differences between the strains with respect to primer binding to the target priming site on the template or to local secondary structure. Similar difficulty with sequencing this region has also been found in *Planctomyces brasiliensis* by other authors (47). The longer available sequence from strain AGA/M18 enables us to state that it possesses signature nucleotides displayed by available sequence for all known members of the *Planctomyces* group within the planctomycetes, including nucleotides U and A at positions 115 and 312, exclusive to the *Planctomyces* group (Table 1).

Comparative analysis of 16S rDNA sequences from ACM 3180, ACM 3181, and six isolates from the present study via

TABLE 1. 16S rRNA signature nucleotides for the subdivisions of the order *Planctomycetales* and the outlier sequence for marine aggregate clone AGG27[a]

| Position(s) | *Pirellula* group | *Planctomyces* group | *Gemmata* group | *Isosphaera* | AGG27 |
|---|---|---|---|---|---|
| 114-313 | A-U | G-C | G-C | U-A | U-A |
| 115-313 | G-C | U-A | C-G | G-C | G-C |
| 668-738 | U (a)-A (u) | U-A | C-G | G-C | U-A |
| 680-710 | A-U | C-G | C (u)-G (a) | C-G | C-G |
| 812 | G | G | C | G | G |
| 822-878 | A (u)-U (a) | A-U | G-C | G-C | G-C |
| 948-1233 | G-C | C-G | C-G | C-G | C-G |
| 976 | G | G | G | A | G |
| 1100 | U | G or A | U | U | U |
| 1361-1 | | C | C | C | C |
| 1415-1485 | G-U | G-U | G-U | C-G | G-N |
| 1420-1480 | U-G (a) | G (c)-C (g) | A-U | G-C | C-N |

[a] The signature nucleotides were derived from oligonucleotide catalogs presented in Tables 5 and 4 of references 36 and 38, respectively. Bases in a minority of strains are represented in lowercase letters.

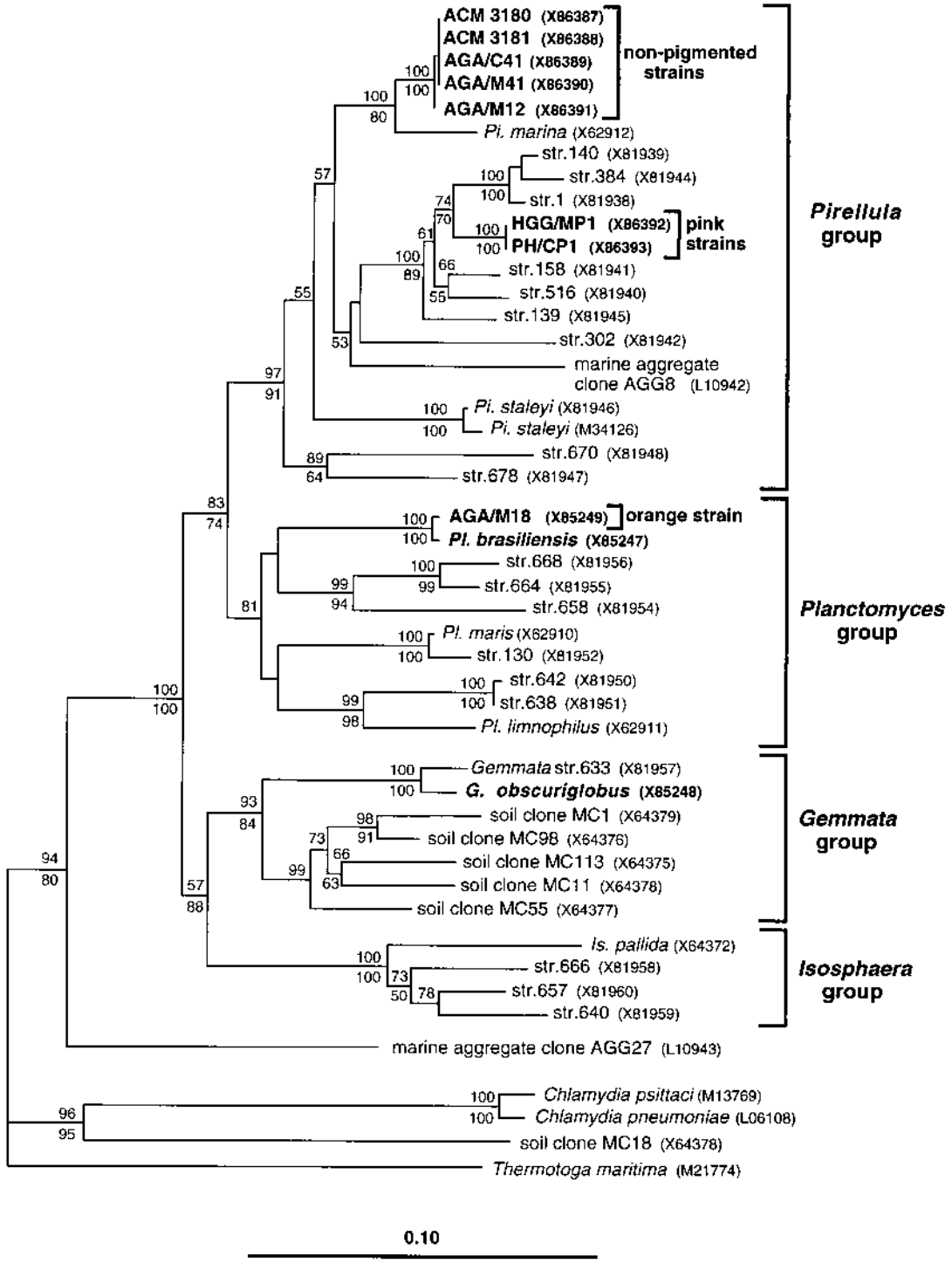


FIG. 3. Evolutionary distance tree derived from comparative analysis of 740 nucleotide positions of 16S rDNA from prawn isolates and reference strains of the order *Planctomycetales*. Database accession numbers are shown in parentheses after species, strain, or clone names. Bootstrap values greater than 50% from 100 bootstrap resamplings from distance (upper) and parsimony (lower) analyses are presented at nodes. *T. maritima* was used as an out-group. *Pi.*, *Pirellula*; *Pl.*, *Planctomyces*; *Is.*, *Isosphaera.*

evolutionary distance and parsimony phylogenetic inference methods showed that both the isolates from the previous study and the isolates from the present study cluster in the resulting tree with known reference sequences in the order *Planctomycetales* (Fig. 3). As found in other studies (3, 22), the planctomycete phylum comprises four groups or clusters, those including reference representatives of the genera *Pirellula*, *Planctomyces*, *Gemmata*, and *Isosphaera*. Signature nucleotide sets are consistent with the phylogenetic coherence of these four groups (Table 1). Only 740 homologous nucleotides were

used for comparative analyses due to missing sequence data from some of the sequences at the 5′ and 3′ ends of the 16S rDNA, such as those for *Planctomyces brasiliensis* and HGG/MP1 (missing data at the 5′ end) and environmental Mt. Cootha (env. MC) clones (missing data at the 3′ end). Bootstrap resampling of the data revealed significant support (97% in distance analysis; 91% in parsimony analysis) for the *Pirellula* group containing the nonpigmented and pink *Pirellula*-like prawn isolates as well as *Pirellula marina*, *Pirellula staleyi*, env. AGG8, and a number of *Pirellula*-like isolates from northern Germany (Fig. 3). Strain AGA/M18, together with its closest relative *Planctomyces brasiliensis*, was a member of the *Planctomyces* group. Monophyly for this group as a whole was (moderately) supported only by distance bootstrap resampling (81% [Fig. 3]). Bootstrap values of less than 75% are considered to give no support to a phylogenetic grouping (50). Further sequences and analyses may show the *Planctomyces* group to be a heterogeneous one.

Phylogenetic analysis supports the valid inclusion of the five nonpigmented strains and the two analyzed pink-pigmented strains (together with the identical third strain) within the *Pirellula* cluster and the orange-pigmented strain within the *Planctomyces* cluster. However, both the nonpigmented strains and the pink-pigmented strains form discrete clusters within the *Pirellula* group supported by bootstrap analysis (Fig. 3). This separation is also supported by signature sequences (e.g., in positions 73 to 91 and 591 to 600). The pink-pigmented prawn isolates form a cluster with a series of pink or red isolates from northern Germany of Ward et al. (47). The branching order of the pink-pigmented strains, *Pirellula staleyi*, and marine aggregate clone AGG8 is unresolved within the *Pirellula* cluster according to bootstrap resampling. However, the nonpigmented strains and *Pirellula marina* form a cluster strongly supported by a distance bootstrap of 100%. Phylogenetic analysis confirms the close evolutionary relationship between the orange-pigmented strain AGA/M18 and *Planctomyces brasiliensis*, consistent with their sequence similarity of 99.4% (based on available homologous sequence, which is of course missing some 370 bases which could not be sequenced from *Planctomyces brasiliensis*).

Planctomycetes of similar phylogenetic relations were isolated from both MBV-infected and control uninoculated PL. Thus, in isolations during experiments involving MBV infection, strain AGA/C41, from uninoculated PL from the same batch as that used for infection, and AGA/M41, from infected PL from the same experiment, were found to be members of the same nonpigmented *Pirellula* cluster, and in fact they are identical in 16S rDNA sequence. Compared 16S rDNA sequences for strains ACM 3180 and ACM 3181, isolated on a separate occasion from MBV-infected PL, are identical to each other and to those of AGA/C41 and AGA/M41. Similarly, the pink *Pirellula* cluster strains PH/CP1 and HGG/MP1 are from uninoculated and MBV-infected PL, respectively and are also identical to each other in 16S rDNA sequence.

## DISCUSSION

Members of the phylogenetically distinctive planctomycete phylum of the domain *Bacteria* can be isolated from both MBV-infected and control uninoculated *Penaeus monodon* PL, as determined by phylogenetic analysis of 16S rRNA sequences. Discovery of such organisms increases our knowledge of the diversity of the microorganisms associated with invertebrates, especially the range of divisions of the domain *Bacteria*, and also of the diversity of cultured members of the order *Planctomycetales*. These organisms include those with a planctomycete morphotype isolated from prawn tissue culture attempts in an earlier study (14). Identification of such organisms found as contaminants of future attempts at prawn tissue culture or as isolates from aquacultured prawns should be considerably assisted by comparisons with sequence data and analyses presented here. In addition, the selective culture medium used for isolation of the planctomycetes described is effective for retrieval of pure cultures from prawn-derived inoculum for use in such identification. The medium takes advantage of the fact that planctomycetes possess proteinaceous cell walls without peptidoglycan (19, 21). In addition, at least marine planctomycetes from prawns appear to be relatively resistant to the protein synthesis inhibitor streptomycin.

Most of the prawn isolates (all but AGA/M18), including the two strains ACM 3180 and ACM 3181 described in a previous report (14), are related most closely to the planctomycete species *Pirellula marina*, but the pink strains are closer to the strains 1, 140, and 384 from Germany than they are to *Pirellula marina*. Based on evolutionary distances between the two described species of *Pirellula*, *Pirellula marina* and *Pirellula staleyi*, the nonpigmented *Pirellula*-like prawn isolates are not separated from *Pirellula marina* by as great a distance as separates the two existing species. Nonpigmented *Pirellula*-like isolates not only cluster together in phylogenetic analysis of their 16S rDNA sequences but also exhibit sequence signatures specific to their own cluster, as outlined in Results. Phenotypic data and the apparent host relationship with a crustacean species therefore must be taken into account to decide whether these isolates constitute a new species of *Pirellula* or are merely a coherent subgroup of *Pirellula marina*. The former possibility is most likely since they are only 93.7 to 93.9% related to *Pirellula marina* in their 16S rDNA sequence, and this is less than the 97% level of 16S rDNA similarity considered equivalent to $\leq$60% DNA similarity (homology). It is therefore a level of dissimilarity at which species can be distinguished clearly (37). Polar crateriform structure distribution and nonspherical cell shape are consistent with a close relationship to members of the genus *Pirellula* (34).

The pink-pigmented strains from PL are markedly distinguishable from known *Pirellula* species and other named planctomycetes on the basis of 16S rRNA signatures. However, they share a number of nucleotide signatures with some pink- or red-pigmented *Pirellula*-like or prosthecate planctomycete isolates from northern Germany, with which they also cluster in phylogenetic analysis. They are most closely related to three of these, pink strains 1 and 140 from Kiel Fjord and red strain 384 from an aquarium (47). The prawn isolates share signature nucleotide pairs at positions 1354 and 1368 and position 1421 and 1479 with the marine organic macroaggregate clone AGG8, which also clusters with the northern German pink or red *Pirellula*-like isolates. In fact, the pink or red strains in this cluster including the Australian prawn isolates and the northern German isolates constitute the closest known relatives of clone AGG8 of all the planctomycetes in culture. These data combined with the low 86.7 to 89.1% similarity of the pink or red strains of that cluster with *Pirellula marina* suggest there are even stronger reasons to believe that these pink strains constitute another, separate new species of *Pirellula*. The taxonomic significance of pigmentation for grouping of related *Pirellula* strains has been previously noted, as supported by unpublished observations of Bartels and Schlesner (cited by Staley et al. [39]) concerning homology groups described from DNA-DNA hybridization experiments containing either pigmented or unpigmented strains.

An orange-pigmented strain isolated from MBV-infected PL, AGA/M18, is most closely related to the genus *Plancto-*

*myces* but branches deeply within the *Planctomyces* cluster. Consistent with its evolutionary relationship revealed by phylogenetic analysis of 16S rRNA sequences, AGA/M18 shares a spherical cell shape and uniform crateriform structure distribution with *Planctomyces* species, in contrast with cell shape and crateriform structure distribution in *Pirellula* species. All *Planctomyces* signatures listed in Table 1 are present in AGA/M18. This strain exhibits an extremely close phylogenetic relationship to *Planctomyces brasiliensis*, differing only in a few base pairs in the variable regions of the 16S rDNA (see Results). *Planctomyces brasiliensis* was isolated from a hypersaline lake in Brazil (32), so we have here an example of bacteria with virtually identical 16S rDNA sequences being isolated from vastly different geographical regions. Habitats of the strains also differ, since *Planctomyces brasiliensis* was originally isolated from the hypersaline water of a salt pit, not invertebrates with an estuarine to marine salinity range. Strain AGA/M18 is much more closely related to *Planctomyces brasiliensis* than to the only previously known marine *Planctomyces* species, *Planctomyces maris*, isolated from neritic waters of Puget Sound, United States (2). AGA/M18 may thus constitute another strain of *Planctomyces brasiliensis*, but it should be noted that strains such as these with greater than 97% similarity in 16S rDNA sequence are not necessarily members of the same species (37). *Planctomyces brasiliensis* was classified originally on the basis of phenotype, and the present clustering in the *Planctomyces* group confirms this species as a member of the *Planctomyces* genus by molecular phylogenetic criteria. Difficulty in sequencing 16S rDNA amplified from *Planctomyces brasiliensis* was also found in a recent study where positions 1 to 372 could not be analyzed and where it was suggested to be caused by microheterogeneity in multiple *rrn* operons (47). Strain AGA/M18 displays unusual cell morphology in negatively stained preparations viewed by transmission electron microscopy, namely, the unicorn, a single polar spur or hornlike projection on one pole of many cells, possibly representing a prostheca. The existence of freshwater prosthecate planctomycetes has been observed in another study (33). Reexamination of the morphology of *Planctomyces brasiliensis* demonstrated that this unusual appendage is also present on the type strain of this species. This constitutes an interesting case of a morphological similarity successfully predicted from phylogenetic analysis of sequence data.

The phylogenetic distinction of both clusters of nonpigmented and pink-pigmented *Pirellula*-like prawn isolates from *Pirellula marina*, together with the repeated isolation of phylogenetically similar and even identical organisms from different batches of *Penaeus monodon*, suggests that *Pirellula*-like planctomycetes are normally associated with *Penaeus monodon* in a specific manner, perhaps as hepatopancreas- or hemolymph-inhabiting commensals. *Pirellula marina* was originally isolated (as *Pirellula marina*) from brackish water of Kiel Fjord of the Baltic Sea as an apparently free-living bacterium (31), though the samples of surface water used may have contained crustaceans, and there is mentioned no specific filtration which might have excluded small zooplankton, so that *Pirellula marina* might also be associated normally with crustaceans.

Bacteria are well-known to be associated with digestive tracts of invertebrates (10, 35, 40, 41), and bacteria are also known to be harbored within the hemolymph from several species of crustaceans (4, 44). Prolific colonization of crustacean hindguts with bacteria with apparent monocultures may be common (17). The digestive tracts of *Penaeus* species have been reported to harbor a diverse commensal bacterial flora, including *Vibrio*, *Pseudomonas*, and *Aeromonas* species (49), and in paddy field-cultured prawns, including *Penaeus monodon*, non-O1 *Vibrio cholerae* appears to constitute normal microflora (27). The present study increases the scope of the known phylogenetic diversity of crustacean microflora beyond the class *Proteobacteria* to a group within the domain *Bacteria* of separate phylum status as based on 16S rRNA analysis, the planctomycetes (family *Planctomycetaceae*, order *Planctomycetales*). This is consistent with our increasing appreciation of the wide extent of phylogenetic diversity in prokaryotes of marine habitats, encompassing in some cases diverse members of the *Archaea* (7, 9, 15) as well as of the domain *Bacteria.*

On phytodetrital macroaggregates composed of dense populations of *Rhizosolenia* sp. and *Phaeocystis* sp. collected off the coast of southern California (5 km offshore in the Santa Barbara Channel, at ca. 10-m depth), a molecular ecology approach analyzing cloned bacterial 16S rDNA sequences showed that planctomycetes were the second most abundant aggregate-associated clonal class, including clone AGG8, sharing highest similarity with *Pirellula* sp. strain IFAM1310 (8). One of the cloned sequences from that study of marine organic aggregates, AGG8, clusters with *Pirellula* species and the *Pirellula*-like prawn isolates from the present study and is most closely related to the cluster of pink or red isolates, including those from Australian prawns and those from aquatic sources in northern Germany, among the *Pirellula* group. A phylogenetically related guild of planctomycetes may occupy marine microniches which are relatively rich in organic nutrients, whether free living or associated with invertebrates.

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