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Isolation of a Bacterium Resembling *Pirellula* Species from Primary Tissue Culture of the Giant Tiger Prawn (*Penaeus monodon*)

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During attempts to establish tissue cultures from hepatopancreas, heart, and hemolymph of the giant tiger prawn (*Penaeus monodon*), using a medium including penicillin, streptomycin, and amphotericin B, bacterial contamination in the form of a sheet of growth attached to the tissue culture vessel was a persistent problem. Contaminant bacteria were teardrop-shaped cells arranged in rosettes, and electron microscopy revealed buds, crateriform structures, and the absence of a peptidoglycan layer in the cell wall, features characteristic of bacteria in the *Planctomyces-Pirellula* group, a phylogenetically distinct group of eubacteria. Two strains of contaminant bacteria were isolated in pure culture. Both exhibited morphology and antibiotic resistance consistent with their membership in the *Planctomyces-Pirellula* group (order *Planctomycetales*) of eubacteria. Tissue culture media for marine invertebrates may select for such bacteria if high concentrations of cell wall synthesis-inhibiting antibiotics are included.

Viral infections may cause morbidity and mortality to the marine invertebrates used in intensive commercial aquaculture (20). However, rapid and efficient methods for the detection of such infections depend on the development of cell cultures supporting multiplication of invertebrate viruses. The virus infections of penaeid prawns, including baculovirus penaei, hepatopancreatic parvolike virus, plebejus baculovirus, and monodon baculovirus may be responsible for increased mortality rates in prawn hatcheries and farms (10, 12, 14–20, 23). However, prawn cell lines from *Penaeus* species have not yet been established in continuous culture for the growth and study of such viruses in vitro. A major problem in establishing such tissue cultures has been microbial contamination. Such contamination has been reported to involve chytrid protists when *Penaeus japonicus* cell culture was attempted (22) and bacteria of possible *Vibrio* affinities when *P. semisulcatus* cell culture was attempted (24). In the latter case, the hemolymph of the prawn harbored three distinct bacterial strains. The association of bacteria with digestive tracts of marine invertebrates is well established (6, 7, 9, 32), and hemolymph from several species of marine crustacea, including lobsters, blue crabs, and horseshoe crabs, has been reported to harbor bacteria (3, 5, 36, 39). Contaminants may inevitably accompany microbially colonized prawn tissue inocula in some cases, posing severe problems for successful cell culture establishment. Contamination may be accompanied by toxic and competitive effects on prawn cell growth, and for prawn cell cultures to be used to study prawn viruses, cytopathic effects due to other microorganisms must be eliminated. Understanding the exact nature of such contamination is important for achieving progress in prawn cell culture, since such understanding may lead to prevention of such problems. During our attempts to establish such cell lines from tissues of *P. monodon* (25), a morphologically unusual bacterium persistently contaminated some cultures derived from hepatopancreas, heart, and hemolymph. The combination of marine crustacean tissue inoculum and antibiotic-supplemented tissue culture medium resulted in enrichment for an unusual bacterium of the *Planctomyces-Pirellula* group, two strains of which were isolated in pure culture. Similar bacteria may be encountered as contaminants in future prawn tissue culture attempts if antibiotic supplementation is not designed to inhibit such peptidoglycanless bacteria. Recognition of their special nature and their possible occurrence should be useful for design of future prawn cell culture attempts. The descriptions of these bacteria and measures to prevent their occurrence given here may help recognition of these contaminants in future prawn tissue culture and prevent their occurrence.

MATERIALS AND METHODS

Media for prawn tissue culture. For prawn primary tissue culture experiments, a medium adapted from Chen et al. (4) was used; it was composed of the following: 2 × Leibovitz-15 (L-15) medium (Commonwealth Serum Laboratories, Melbourne, Australia), 50 ml; prawn muscle extract, 30 ml; Hybriserum fetal calf serum, inactivated at 56°C for 30 min (Commonwealth Serum Laboratories), 20 ml; 7.5% (wt/vol) sodium bicarbonate solution, 2 ml; NaCl, 0.7 g; penicillin G (Glaxo, Boronia, Australia), 10,000 U; streptomycin sulfate (Glaxo), 10,000 μg; amphotericin B (Fungizone; Squibb), 250 μg. The pH was adjusted to 7.0, and the medium was sterilized by filtration through a 0.45- and 0.22-μm membrane filter stack. Prawn muscle extract was prepared as follows: abdominal muscle tissue of fresh prawns (*Penaeus* sp.) was cleaned with distilled water and homogenized in sterile half-strength artificial seawater (3 ml/g of tissue); homogenate was sonicated and centrifuged at 17,000 × *g* for 30 min, and the supernatant was clarified by filtration and sterilized by membrane filtration. The resulting osmolarity of the tissue culture medium was 800 mosmol liter^{-1}. Prawn muscle extract agar was prawn tissue culture medium agar

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(i.e., the tissue culture broth mixed with one-fourth volume of 6% agar to yield 1.5% agar).

Media for bacterial isolation and culture. Prawn muscle extract agar was prawn tissue culture medium agar (broth mixed with 6% agar to yield a final agar concentration of 1.5%). M14 medium was that of Schlesner (26) modified by using Tropic Marin-New Sea Salt (Dr. Biener GmbH Aquarientechnik, Wartenberg-Angersbach, Germany) to make up the artificial seawater, used either as a broth or with the addition of 1.8% Bacto-Agar (Difco, Detroit, Mich.). For carbon substrate utilization tests, the following media were used as basal media: BMMV [Hutner's basal salts, 20 ml; Staley's vitamin solution, 10 ml; $(NH_4)_2SO_4$, 0.25 g; KH_2PO_4, 2.0 g; distilled water to 1 liter (pH adjusted to 7.0 to 7.1 before autoclaving)] and M9 medium of Schlesner (26).

Primary prawn tissue culture experiments. Small juvenile (1 to 1.5 cm long) prawns (*P. monodon*) collected from a prawn farm in North Queensland, Australia, were killed by exposure to −60°C for 5 min in a metal canister, rinsed with distilled water, and surface sterilized by submerging in a 1% sodium hypochlorite solution for 10 min. After being rinsed with distilled water, they were swabbed with 70% ethanol four to five times and placed in a disinfected dissecting tray. Hepatopancreas and heart were obtained by cutting away the dorsal part of the carapace with sterile instruments, taking care to avoid disruption of the digestive system. Hemolymph was obtained by using a sterile 1-ml syringe and needle (0.45 by 13 mm) containing 0.5 ml of cold tissue culture medium; the needle was inserted into the soft epidermis of the cephalothorax (preswabbed with 70% ethanol) to withdraw hemolymph from the heart and surrounding sinus; the needle was then removed with sterile forceps, and the hemolymph was diluted with culture medium. Dissected organs were washed for 45 min in an antibiotic solution (1,000 U of benzylpenicillin, 1,000 μg of streptomycin sulfate, 20 μg of neomycin, 40 U of polymyxin B, and 5 μg of amphotericin B ml^{-1}). Tissues were then minced in tissue culture medium, large fragments were removed by filtration, and the suspension was adjusted to 10^6 cells per ml and distributed into 25-cm^2-surface-area tissue culture flasks, each containing 5 ml of medium. Tissue culture vessels were incubated in a 5% CO_2 incubator at 28°C.

Isolations of pure bacterial cultures from contaminated tissue culture media. Strains of bacteria were isolated from contaminated prawn tissue culture flasks by using several passages of colony selection on M14 agar, with incubation aerobically at 28°C in the dark, without CO_2 enrichment. In one case, M14 agar was streaked directly with tissue culture medium which had been originally inoculated with prawn hepatopancreas, while for isolation of the second bacterial strain, colonies grown on prawn muscle extract agar inoculated from contaminated prawn tissue culture medium derived from a hepatopancreas-inoculated primary tissue culture via six passages in tissue culture medium were used as inoculum for M14 agar plates. Pure cultures were derived from three successive single-colony streakings on M14 agar. The two strains isolated were designated PRPL-1 and PRPL-2. These strains have been deposited in the University of Queensland Department of Microbiology Culture Collection as UQM 3181 and UQM 3180, respectively.

Biochemical reactions. Hayward's modification of Hugh and Leifson's oxidation and fermentation medium supplemented with 1% (wt/vol) glucose was used to determine metabolism of glucose (8). Tubes were stab inoculated with growth from a 2- to 3-day culture, incubated at 28°C, and read after 5 and 7 days.

Carbon substrate utilization tests. Strains PRPL-1, PRPL-2, and *Pirellula marina* UQM 3344 (DSM 3645) were inoculated by addition of 1 drop of washed suspension in sterile 0.15 M NaCl to an agar plate of M-9 basal medium (Schlesner [26]) containing filter-sterilized carbon substrate at 0.1% (wt/vol) except for glucose and lactose, which were used at 1% (wt/vol). Growth after 7 days of incubation at 28°C was compared with that on an inoculated control plate of M-9 basal medium without added substrate. The carbon substrate utilization pattern of *Pirellula staleyi* UQM 2488 (ATCC 27377) was tested by using microtiter plates and BMMV basal medium. In this method, 180 μl of each filter-sterilized substrate at 0.1% (wt/vol) final concentration in BMMV minimal medium was added to separate wells of a 96-well microtiter tray. Twenty microliters of a washed inoculum in BMMV medium was added to each well, and the Parafilm-sealed tray was incubated at 28°C for 7 days. Wells were scored for the presence or absence of growth after 5 and 7 days.

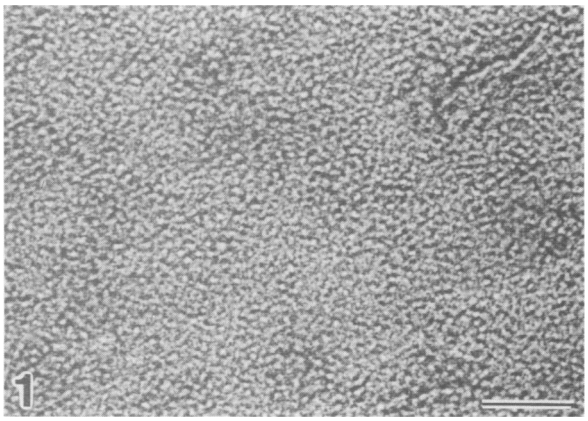
Antibiotic sensitivities. Five-day cultures of strains PRPL-1 and PRPL-2 were used to inoculate a dilution series of each antibiotic in M14 broth (in glass test tubes) at a final concentration of antibiotic of 1.25 to 1,000 μg ml^{-1}. Growth was determined after incubation for 1 week at 28°C by visual comparison of turbidity with an inoculated blank to which formalin had been added.

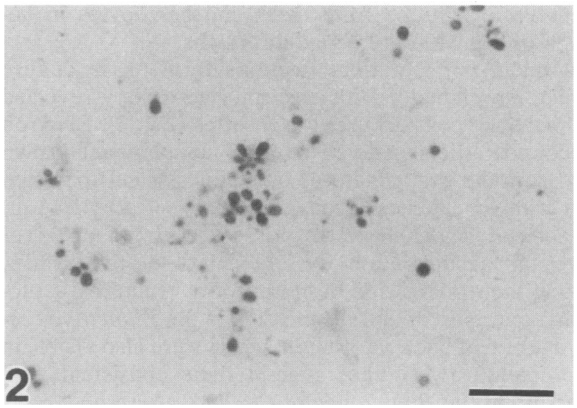
Electron microscopy. (i) Thin sectioning. Pure cultures of bacterial isolates were grown for 5 days at 28°C on Staley PYG agar (34) made up in half-strength artificial seawater of Lyman and Fleming (21). Cells were harvested in sterile distilled water, fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, and enrobed in agarose, and cubes of the agarose-embedded cells were fixed with 1% osmium tetroxide in cacodylate buffer for 1.5 h, dehydrated in a graded ethanol series, and embedded in LR White resin (medium grade), polymerized for 20 h at 50°C. Thin sections were stained with uranyl acetate and Reynolds lead citrate.

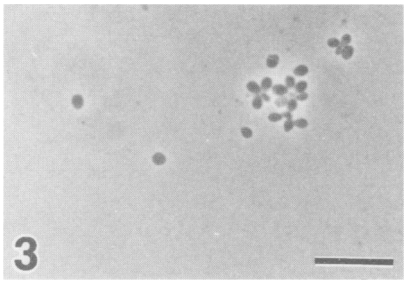
(ii) Negative staining. In the case of bacterial isolates in pure culture, two protocols were used. In the first protocol, used for strain PRPL-1, cells were grown in M14 broth for 14 days at 28°C and a drop of broth culture was placed on a carbon-stabilized, nitrocellulose-filmed specimen support grid for 1 min; the grid was then washed three times with drops of saline and stained with membrane-filtered 1% uranyl acetate containing 0.4% sucrose. In the second protocol, used for strain PRPL-2, cells were grown on M14 agar for 7 days at 28°C, and growth was suspended in sterile membrane-filtered distilled water on a microscope slide. A carbon-stabilized grid was inverted on the suspension for 1 min; after removal of the grid with forceps, a drop of the suspension was placed on the grid and mixed with a drop of 1% uranyl acetate. Excess fluid was then removed immediately, and the grid was air dried. All grids were examined in an Hitachi H-800 transmission electron microscope operated at 100 kV.

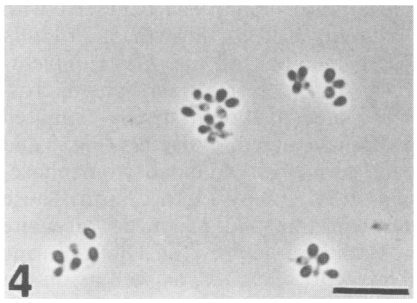
RESULTS

In the course of attempts to establish a primary prawn tissue culture from hepatopancreas of juvenile *P. monodon* (≤2 cm long), a novel bacterial contaminant was first noticed as a dense sheet of growth, similar to the appearance of eukaryotic cells in tissue culture, which was attached to the entire surface of the culture vessel (Fig. 1). Obvious turbidity of the medium was not prominent, but in old cultures the sheets of growth tended to detach. Growth of similar contaminants as tissuelike sheets was also observed in at-









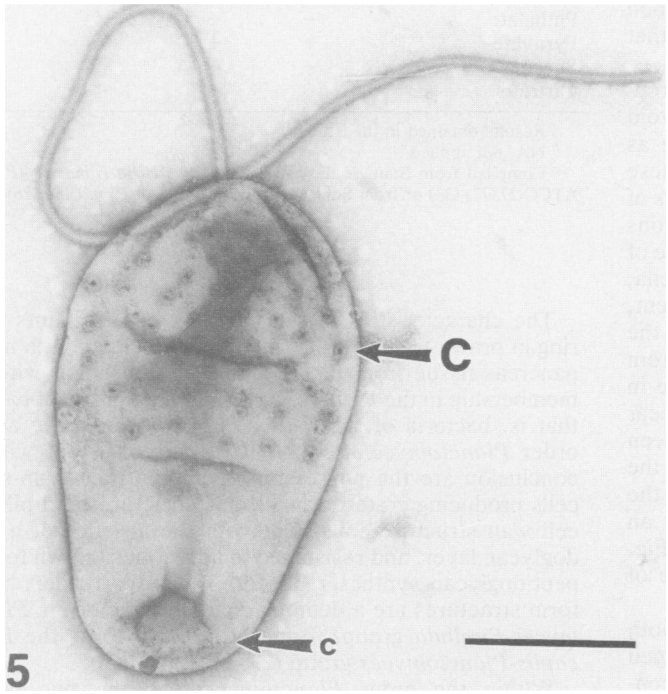


FIG. 1–5. FIG. 1. Inverted microscope photomicrograph of cells of bacterial contaminant in prawn hepatopancreas-inoculated tissue culture medium. A continuous layer is visible growing on the bottom of the flask, resembling the appearance of layers formed by adherent eukaryotic cells in tissue culture. Bar, 20 μm.

FIG. 2. Photomicrograph of toluidine blue-stained thick section of contaminant cells in tissue culture medium inoculated with prawn hepatopancreas. Budding ovoid cells and cell rosettes are visible. Bar, 10 μm.

FIG. 3. Phase-contrast photomicrograph of strain PRPL-1 isolated from contaminated prawn tissue culture. Rosettes and the teardrop shape of individual cells are visible. Bar, 10 μm.

FIG. 4. Phase-contrast photomicrograph of strain PRPL-2 isolated from contaminated prawn tissue culture. Rosettes and the teardrop shape of individual cells are visible. Bar, 10 μm.

FIG. 5. Electron micrograph of strain PRPL-2 isolated from contaminated prawn tissue culture; negatively stained with 1% uranyl acetate. Shown is a teardrop-shaped cell with a single sheathed flagellum and large crateriform structures (C) covering half of the cell surface. A cluster of small crateriform structures (c) is visible at the narrower pole. Bar, 0.5 μm.

tempted tissue cultures from heart and hemocytes in hemolymph of 8- to 10-cm-long adult prawns.

Light microscopy of thick sections from tissue culture contamination embedded for electron microscopy revealed teardrop-shaped cells arranged in rosettes (Fig. 2). Electron microscopy of the negatively stained contaminant grown directly from the tissue cultures on stainless-steel specimen support grids revealed cells consistent in size (0.8 by 1.1 μm) with bacteria. These bacteria possessed round areas of negative stain accumulation over one-half of the cell surface, suggesting their possession of crateriform structures, structures characteristic of and diagnostic for the *Planctomyces-Pirellula* group of bacteria. Budding cells were also visible in these preparations, another characteristic consistent with membership in the *Planctomyces-Pirellula* group.

Isolation of pure cultures and their nutritional and biochemical characteristics. Isolation of two strains of bacteria from the contaminated tissue culture media was achieved with M14 agar medium, a medium used for growth of *Pirellula marina*, a brackish-water member of the *Planctomyces-Pirellula* group of eubacteria. Phase-contrast light microscopy of these isolates revealed that both strains exhibited tear-drop-shaped cells, often arranged in rosettes (Fig. 3 and 4). The cell size of each strain as estimated from phase-contrast preparations was 1.3 to 2.0 by 1.0 to 1.6 μm. These strains were both gram variable and produced off-white colonies on M14 agar. Both strains grew on Difco Marine agar with salinity equivalent to seawater, as well as on the one-fourth-strength seawater concentration in M14. Both strains exhibited a fermentative glucose metabolism. Carbon substrate utilization patterns for 31 substrates indicated that the strains had identical patterns (Table 1).

Electron microscopy of isolates. In negatively stained preparations, the isolates displayed teardrop-shaped or ovoid cells with many large crateriform structures appearing as electron-dense circular areas (see Fig. 5, 6, 7, and 9). These were distributed over one-half of the cell surface. Clusters of small crateriform structures were also present in regions distant from the large structures, such as the opposite pole of the cell (Fig. 5). Both strains also produced sheathed flagella, similar to that seen in Fig. 5. Fimbriae were also present, often appearing to be distributed on the same pole as the large crateriform structures (Fig. 6 and 9). Budding from cells in rosettes seen by light microscopy is also visible in negatively stained preparations (Fig. 6 and 7). Stalks appear to be absent, although amorphous fibrillar material has been seen on isolated cells of one strain. In thin sections, the absence of an obvious peptidoglycan layer was apparent, the cell wall exhibiting an outer electron-dense layer and an inner layer separated by an electron-transparent layer (Fig. 8). Thin sections also confirmed the prokaryotic nature of these bacteria.

Antibiotic sensitivities of isolates. As seen in Table 2, both isolates have similar antibiotic sensitivity spectra as judged by MICs. Growth endpoints, the highest antibiotic concentrations at which growth still occurred, are also given to illustrate resistance. Of special interest are the resistances shown to antibiotics with targets concerned with cell wall synthesis (vancomycin, cephalothin, phosphomycin, cycloserine, penicillin G, and bacitracin) and the antibacterial antibiotics included in the prawn tissue culture medium in which the original contamination was observed (penicillin G and streptomycin). The strains are significantly sensitive only to tetracycline and polymyxin.

TABLE 1. Carbon substrate utilization for PRPL-1 and PRPL-2 as compared with *Pirellula staleyi* (UQM 2488) and *Pirellula marina* (UQM 3344)

Substrate	Utilization by:			
	PRPL-1	PRPL-2	UQM 2488	UQM 3344
Glucose	+	+	+[a]	+[a]
Lactose	+	+	+[a]	+[a]
Lyxose	+	+	−[a]	+[a]
Mannose	+	+	+[a]	+[a]
Maltose	+	+	+[a]	+[a]
Melezitose	+	+	−[a]	+[a]
Raffinose	+	+	−[a]	+[a]
Rhamnose	+	+	−[a]	+/−[a]
Ribose	−	−	−[a]	+/−[a]
Sucrose	+	+	+[a]	+[a]
Trehalose	+	+	+[a]	+[a]
Xylose	+	+	+[a]	+[a]
Arabinogalactan	+	+	NA[b]	−[c]
Arabinose	−	−	NA	−[c]
Cellobiose	+	+	+[a]	+[a]
Fructose	+	+	+[a]	+[a]
Fucose	+	+	−[a]	−[a]
Galactose	+	+	+[a]	+[a]
Erythritol	−	−	NA	−[c]
N-Acetylglucosamine	+	+	+[a]	+[a]
Acetate	+	+	−[a]	−[a]
Caproate	−	−	−[c]	−[c]
Caprylate	−	−	−[c]	−[c]
Citrate	−	−	−[c]	−[c]
Formate	−	−	−[c]	−[c]
Fumarate	−	−	−[c]	−[c]
Malate	−	−	−[c]	−[c]
Phthalate	−	−	−[c]	−[c]
Pyruvate	+	+	+[c]	+[c]
Succinate	−	−	−[c]	−[c]
Tartrate	−	−	−[c]	−[c]

[a] Results obtained in this study.
[b] NA, not applied.
[c] Compiled from Staley's description of "*Pasteuria ramosa*" (*P. staleyi* ATCC 27377) (33) or from Schlesner's description of *P. marina* (26).

DISCUSSION

The characteristics of the bacterial contaminants occurring in prawn tissue culture medium inoculated with hepatopancreas tissue from *P. monodon* are consistent with their membership in the *Planctomyces-Pirellula* group of bacteria, that is, bacteria of the family *Planctomycetaceae* and the order *Planctomycetales* (29). Criteria consistent with this conclusion are the possession of budding teardrop-shaped cells producing crateriform surface structures and pili, with cell wall structure consistent with the absence of a peptidoglycan layer, and resistance to antibiotics known to target peptidoglycan synthesis (11, 29, 35). In particular, crateriform structures are a defining characteristic of the *Planctomyces-Pirellula* group, formerly referred to as the *Blastocaulis-Planctomyces* group (29, 31, 35).

Within the order *Planctomycetales*, the two isolated strains seem most likely to belong to the genus *Pirellula* (27, 28). This genus was referred to as "*Pirella*" until its reassignment as *Pirellula* due to prior use of the name "*Pirella*" for a genus of fungi (28). Characteristic features of members of this genus include pear- or teardrop-shaped cells, a slightly pointed cell pole, crateriform structures on only one (reproductive) cell pole rather than scattered all over the cell surface, monotrichous flagellation, and the absence of a rigid fibrillar stalk (27). All of these characters are also exhibited

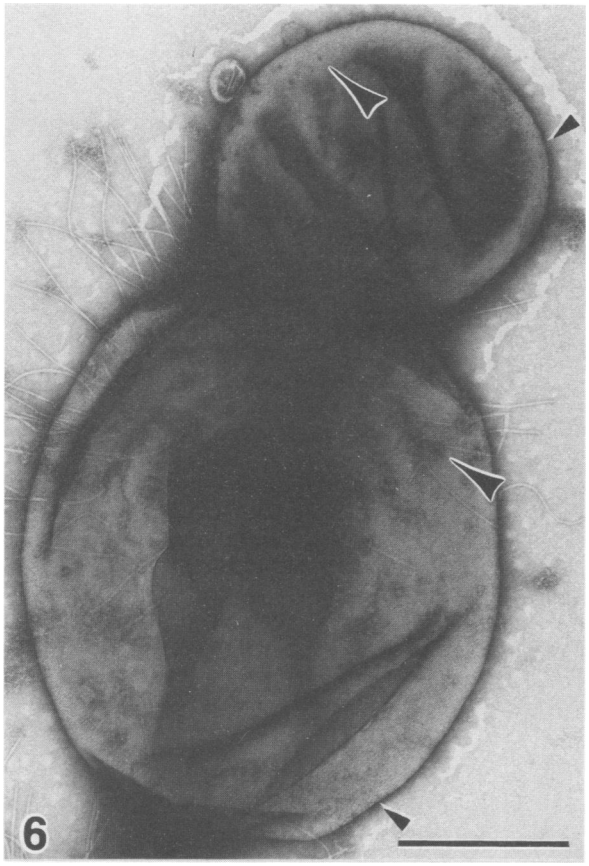


FIG. 6. Electron micrograph of strain PRPL-2 isolated from contaminated prawn tissue culture; negatively stained with 1% uranyl acetate. A mother cell with bud is shown. Large crateriform structures are visible on the reproductive pole of the mother cell and over one-half of the bud (large arrowheads). Clusters of small crateriform structures are seen on both mother cell and bud on poles opposite to the concentrated areas of the large crateriform structures (small arrowheads). Fimbriae arise from the mother cell. Bar, 0.5 μm.

TABLE 2. Growth endpoints and MICs for action of cell wall synthesis target antibiotics on strains PRPL-1 and PRPL-2

Antibiotic	Growth endpoint and MIC (μg ml^{-1}) for:			
	PRPL-1		PRPL-2	
	Endpoint[a]	MIC[b]	Endpoint	MIC
Vancomycin	250	500	250	500
Cephalothin	250	500	250	500
Phosphomycin	1,000[c]		1,000[c]	
Cycloserine	10	25	10	25
Penicillin G	500	1,000	500	1,000
Bacitracin	1,000		1,000	
Tetracycline	1	2	1	2
Chloramphenicol	10	25	10	25
Streptomycin	1,000		1,000	
Polymyxin	1	2	<1[d]	1

[a] Concentration in the last tube in the series showing growth.
[b] Concentration in the first tube in the series not showing growth.
[c] Growth in all tubes.
[d] No growth in any tube.

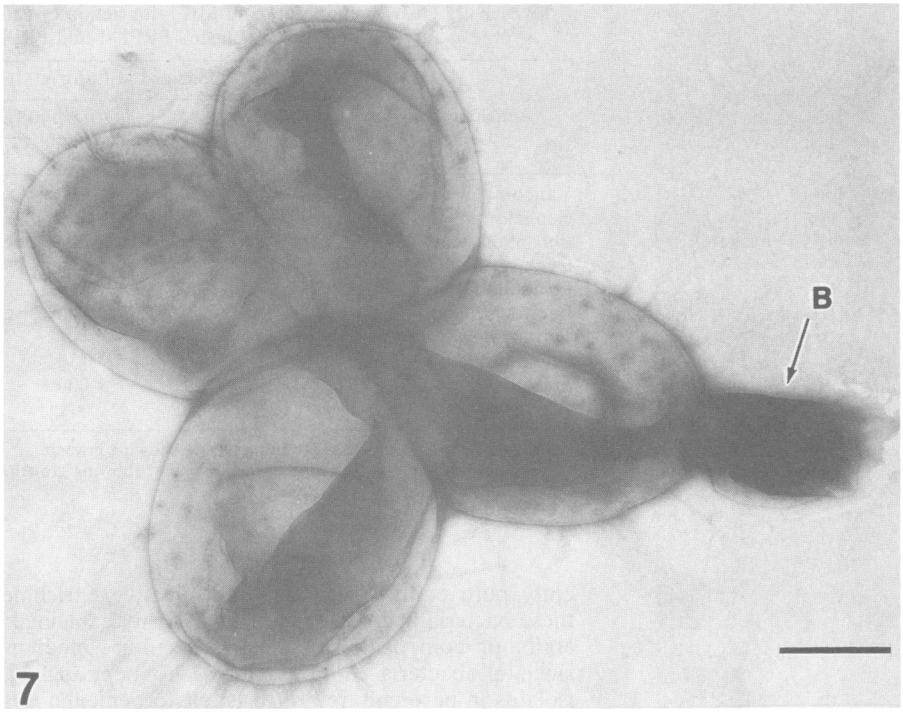
by the prawn tissue isolates. Schlesner and Hirsch (27) specifically emphasize cell shape and crateriform structure distribution in their distinction of *Pirellula* from *Planctomyces*.

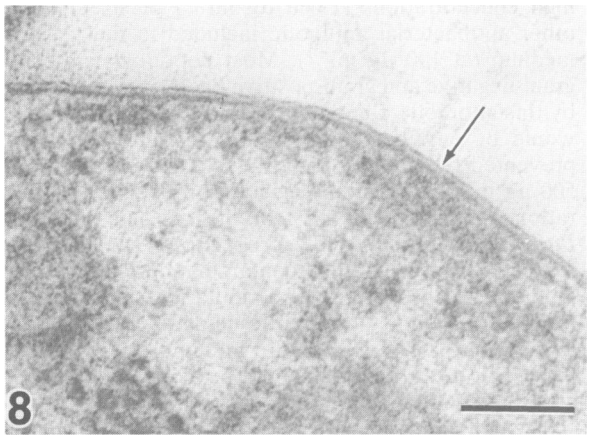
With respect to possible species identity within the genus *Pirellula*, the isolates are most similar to *Pirellula marina*, represented by brackish-water isolates from the Baltic Sea (26), as judged by crateriform structure distribution, salinity tolerance, and carbon substrate utilization pattern. However, the teardrop or pearlike cell shape of our isolates is more similar to *Pirellula staleyi* than to *Pirellula marina*, and Gram stain results for our strains are more similar to those found for *Pirellula staleyi* (33). Further characterization will be needed to resolve the species identity of our strains.

The *Pirellula* bacteria are of special significance in that they complicate attempts at establishing prawn tissue culture, since their formation of sheets of growth on the culture vessel may imitate successful tissue culture establishment. A tendency to attach to the surface of culture vessels was noted in the original description of *Pirellula staleyi* (33).

The appearance of bacteria of the *Planctomyces-Pirellula* group in the original prawn tissue culture medium in apparently pure culture suggests that selective enrichment for these bacteria had occurred. This is easily explained by the antibiotic composition of the tissue culture medium used, since all bacteria in this group lack peptidoglycan and isolates in pure culture are resistant to penicillin G at high concentrations, e.g. 500 to 1,000 μg ml^{-1} (11, 13, 30). In addition, the particular strains isolated are also resistant to high concentrations (1,000 μg ml^{-1}) of streptomycin, the other antibacterial antibiotic included in the tissue culture medium (at 100 μg ml^{-1}). Most peptidoglycan-containing, gram-negative and gram-positive bacteria would be inhibited by this antibiotic combination, and thus the *Pirellula* strains would be enriched. Such contamination was successfully prevented by addition of tetracycline at a concentration of 500 μg ml^{-1} in the washing solution used for tissue preparation and at 50 μg ml^{-1} in the tissue culture medium. *Pirellula* bacteria thus have properties of special significance for prawn tissue culture contamination; they form sheets adhering to the culture dish, imitating tissue culture, and they are resistant to antibiotic combinations included in at least some prawn cell culture medium formulations. Problems with these bacteria should be avoidable with appropriate use of tetracycline.

The contaminant bacteria are assumed to have arisen from prawn tissue, but it is not known whether such bacteria occur normally associated with such tissue. Although there are reports suggesting that the gut of some crustaceans may be sterile (2), other reports indicate that the digestive tracts of *Penaeus* species harbor a rich commensal bacterial flora, including *Vibrio*, *Pseudomonas*, or *Aeromonas* species (6, 9, 38, 40). The possibility of a specific *Vibrio*-like gut flora has also been raised in a study of marine copepods of the species *Acartia tonsa* Dana (32). *Pirellula*-like bacteria might be a normal component of the commensal gut flora of juvenile prawns but, in the presence of a high background of classical gram-negative rods, might be detected only rarely. Such a minor normal component might only be detected by a selective enrichment in the presence of peptidoglycan synthesis-inhibiting antibiotics. Although an infection of the prawn with *Pirellula* species is a possible source, *Pirellula* species are normally isolated as free-living aquatic bacteria and are not known to be pathogenic to invertebrates, vertebrates, or plants. An alternative is that digestive tracts of stressed cultured juvenile prawns with incipient baculovirus





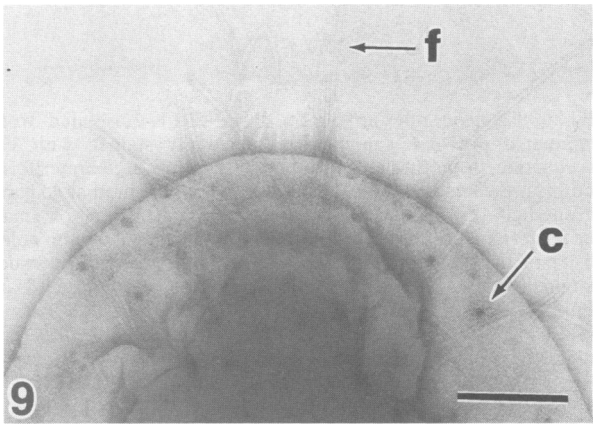


FIG. 7–9. FIG. 7. Electron micrograph of strain PRPL-1 isolated from contaminated prawn tissue culture; negatively stained with 1% uranyl acetate supplemented with 0.4% sucrose. A rosette of ovoid cells is visible; one cell possesses a bud (B). Crateriform structures are present over half of each cell. Fimbriae are concentrated at the cell poles. Bar, 0.5 μm.

FIG. 8. Electron micrograph of thin section of strain PRPL-2 isolated from contaminated prawn tissue culture. Cell wall (arrow) is composed of outer and inner electron-dense layers separated by an electron-transparent layer. The inner electron-dense layer is thicker than the outer layer. Bar, 100 nm.

FIG. 9. Electron micrograph of strain PRPL-1 isolated from contaminated prawn tissue culture; negatively stained with 1% uranyl acetate supplemented with 0.4% sucrose. Details of large crateriform structures (c) and fimbriae (f) are seen on a cell pole. Bar, 0.2 μm.

infection may possess high numbers of *Pirellula*-like bacteria which may "bloom" as commensals under such conditions. The juvenile prawns used here as a source of tissue for primary tissue culture appeared to be healthy, but some individuals in the same batch were later found to be infected with baculovirus as indicated by inclusion bodies. Changes in bacterial microflora with stress caused by salinity changes have been noted in a study of the freshwater shrimp *Palaemon paucidens* (37). The hepatopancreas or midgut gland, however, produces a number of digestive enzymes, including proteases (23), which might be expected to lyse even *Pirellula*-like bacteria with proteinaceous cell walls. A more probable source for such bacteria may be hemolymph itself, since similar contaminants were observed in tissue culture medium inoculated with hemolymph of stressed juvenile *P. monodon*. Bacteria have, of course, been isolated from hemolymph of lobsters (*Homarus americanus*), blue crab (*Callinectes sapidus*), and horseshoe crab (*Limulus polyphemus*) (3, 36, 39). Recent studies attempting primary tissue culture from prawns have encountered contamination

problems, attributed to chytrid protists in the case of a *P. japonicus* cell culture (22) and to hemolymph-derived bacteria with possible *Vibrio* affinities in the case of a *P. semisulcatus* cell culture (24). In the latter case, the hemolymph of *P. semisulcatus* used in the culture medium was found to harbor at least three distinct bacterial strains (24). Together with evidence from the present study, this appears to indicate that planning attempts at prawn tissue culture should take into account the possible inherent contamination of prawn tissue inoculum with a variety of microorganisms, including some, such as *Pirellula*-like bacteria, with inherent resistance to bacterial cell wall synthesis-targeting antibiotics.

The isolation of budding bacteria from contaminated prawn tissue culture most probably related to the order *Planctomycetales* is an interesting complication of the use of antibiotics in tissue culture media, but may also reflect an unrecognized role for such bacteria in crustacean biology. Such a role may prove to be of importance for future aquaculture science and technology. A wider significance of such bacteria for future studies in mariculture microbiology may be indicated by the studies of Austin (1), who found that budding bacteria were commonly isolated from marine fish-rearing units.

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