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# Rickettsial and Mollicute Infections in Hepatopancreatic Cells of Cultured Pacific White Shrimp (*Penaeus vannamei*)

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# Rickettsial and Mollicute Infections in Hepatopancreatic Cells of Cultured Pacific White Shrimp (*Penaeus vannamei*)

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**Abstract**

Infections by multiple species of bacteria occurred in hepatopancreatic epithelial cells of cultured Pacific white shrimp (*Penaeus vannamei*). Grossly, hepatopancreases of moribund shrimp were pale white. Light microscopically, hepatopancreatic tubules appeared atrophied and were associated with granulomas. Examination by scanning and transmission electron microscopy revealed heavy cytoplasmic infections by three forms of microorganisms: (1) a rickettsia-like bacterium, (2) a helical form of a mollicute-like bacterium, and (3) a filamentous mollicute-like bacterium. The rod-shaped rickettsia (900 nm long by 300 nm wide) appeared to be free in the cytoplasm and had both a plasma membrane and a cell wall. Neither form of mollicute possessed a cell wall. The helical mollicute was blunt at its wide end (about 260 nm in diameter) where it contained electron-lucent bodies. Helical turns along its tapered axis resembled those of a spiroplasma (the only helical form of mycoplasma in the class Mollicutes) or a spirochete. The helical bacterium did not possess periplasmic flagella characteristic of spirochetes, which lends support to its being a type of spiroplasma. The filamentous mollicute consisted of masses of short, branched filaments 60 nm wide with intermittent spherical dilations and terminal blebs on the branches. The presumed mollicutes have not been reported previously in crustaceans. Each bacterium, or concurrent infections of the bacteria, are pathogenic to cultured shrimp, could impact culture operations, and thus deserve more study.

**Keywords:** Pacific white shrimp, *Penaeus vannamei*, hepatopancreas, intracellular bacteria rickettsia, mollicute, aquaculture, transmission electron microscopy, scanning electron microscopy

## Introduction

This paper concerns the occurrence of hepatopancreatic bacterial infections in postlarval Pacific white shrimp (*Penaeus vannamei*) that began dying of an unknown cause after introduction into an aquaculture facility. We examined the hepatopancreases of moribund shrimp by light and electron microscopy and found coinfections of three types of unusual bacteria that are tentatively described as a rickettsia and two forms of mollicute, a helical form and a filamentous form.

Many species of bacteria infect and cause mortality in cultured penaeid shrimp (Sindermann and Lightner, 1988). Rickettsia have been reported in penaeid shrimp and other crustaceans, including the hepatopancreatic epithelium of wild-caught juvenile *P. marginatus* in Hawaii, cage-cultured *P. merguiensis* in Singapore, Malaysia, and in experimental infections in *P. stylirostris* (Lightner et al., 1985; Brock et al., 1986; Brock, 1988). Anderson et al. (1987) reported concurrent rickettsia, monodon baculovirus, reo-like virus, and bacterial infections in pond-reared juvenile tiger prawn (*P. monodon*). Reports of rickettsia in other crustaceans include those involving infections in the hepatopancreatic epithelium of the crab *Paralithodes platypus* (Johnson, 1984) and the connective tissues of the crab *Carcinus mediterraneus* (Bonami and Pappalardo, 1980).

The class Mollicutes includes pleomorphic mycoplasmas (Razin and Freundt, 1984). Spiroplasma, the only reported helical form of mycoplasma (Davis et al., 1972), has been found in arthropods and plants (Whitcomb and Tully, 1984). Other mollicutes have been reported from several marine and freshwater organisms; however, few of them have been cultivated and isolated. Harshbarger et al. (1977) reported a mycoplasma in the goblet cells of the American oyster (*Crassostrea virginica*). Mycoplasma-like organisms were associated with the coiling stunt disease of the brown algal sea tangle (*Laminaria japonica*) (in Hackett and Clark, 1989) and were reported from the marine bryozoans *Watersipora cucullata* (Zimmer and Woollacott, 1983) and *W. arcuata* (Boyle et al., 1987). A mycoplasma has also been recovered from a freshwater fish, the European tench (*Tinca tinca*) (Kirchhoff et al., 1987). Here, we describe the morphology and pathogenesis of concurrent infections of these three forms of bacteria in hepatopancreatic cells of *P. vannamei*.

## Materials and Methods

### *Animals*

Postlarval specimens of *P. vannamei* were shipped from Hawaii to Texas. The shrimp showed no evidence of disease when transferred to the Texas aquaculture facility. The shrimp were cultured in tanks equipped with a semiclosed circulating water system. When mortalities begin to occur, representative specimens were sent to Coast Research Laboratory for examination.

### *Light Microscopy*

For light microscopy, 24 shrimp were fixed whole in Davidson's fluid. Gill, nerve cord, and hepatopancreas were removed, processed in a graded series of alcohols, and embedded in paraffin. Sections were cut at 5 μm, stained with Harris' hematoxylin and eosin for

routine examination, and by a variety of special methods that included Ziehl-Neelsen for acid-fast bacteria, Gram stain for bacteria, Grocott's methenamine silver stain for fungus, and Pinkerton's, Giemsa, Ordway, and Warthin Starry stains for rickettsia and spirochetes.

***Electron Microscopy***

Heart, gill, and ventral nerve cord were removed from four shrimp and prepared for transmission electron microscopy (TEM). Hepatopancreases from those shrimp were prepared for both TEM and scanning electron microscopy (SEM). All tissues were fixed in 3% glutaraldehyde in sodium cacodylate buffer, postfixed in 1% osmium tetroxide, and processed in a graded series of alcohols. Specimens for TEM were embedded in epoxy resin, sectioned, stained with uranyl acetate and lead citrate, and examined with a JEOL 100SX transmission electron microscope. For SEM, tissues were processed in a manner similar to the plunge-freezing technique described by Chapman and Staehelin (1986). Tissues were processed to 100% alcohol as described above, wrapped in tubes of Parafilm, and frozen in liquid Freon in a brass cup mounted in a container filled with liquid nitrogen. Frozen tissues were placed on a metal plate submerged in liquid nitrogen and cracked with a single-edged razor blade held with a pair of pliers. The tissues were thawed in 100% ethanol and critical point-dried with carbon dioxide. Dried tissues with the cracked surfaces exposed were mounted on aluminum stubs, sputter-coated with gold-palladium, and examined with a JEOL T330 scanning electron microscope.

## Results

Shrimp began dying about 25 days after introduction into the tank system in Texas and by 40 days, 35% of them had died. On gross examination, the only indication of disease was a pale white coloration of the hepatopancreas in some moribund specimens. Likewise, the hepatopancreas was the only tissue which exhibited evidence of disease at either the light or electron microscopic level. Light microscopically, the cells of the hepatopancreatic epithelium had a normal brush border and a typical rounded cell nucleus with a central nucleolus and dispersed chromatin (Fig. 1). The cytoplasm of cells suspected to be infected appeared grainy. Hepatopancreatic tubule lumens were often collapsed. There was evidence of necrosis and sloughing of cells into the tubule lumen. Hemocytes accumulated in the hemal sinuses associated with the hepatopancreatic tubules. A few granulomas consisted of flattened layers of hemocytes that encapsulated entire. infected hepatopancreas tubules (Figs. 1, 2). Hepatopancreas tissue produced a Gram-positive stain reaction. All other special stains were inconclusive.

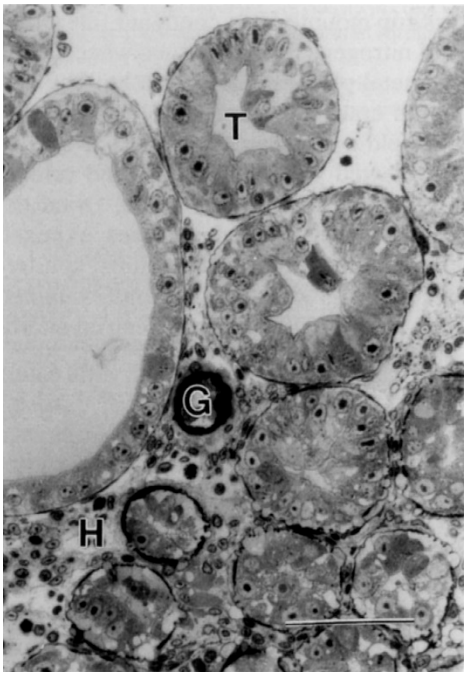


**Figure 1.** Light micrograph of hepatopancreas tubules of *Penaeus vannamei*. Note the typical tubule (T) with hepatopancreas cells surrounding the tubule lumen. The hemal space is filled with hemocytes (H). Granuloma (G) formation occurs as hemocytes surround infected tubules. Bar = 75 μm.

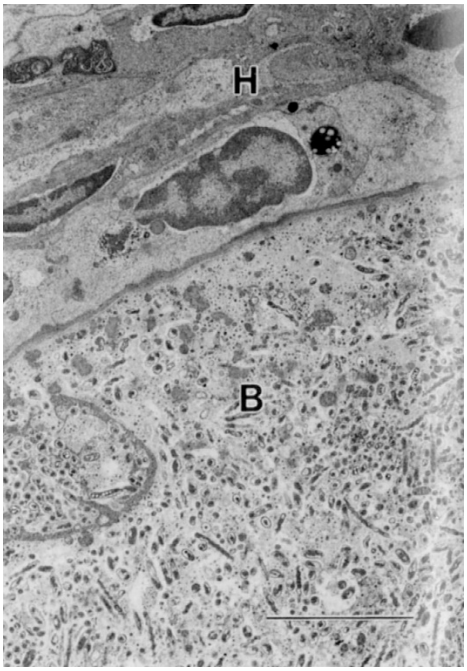


**Figure 2.** Transmission electron micrograph of a granulomatous hepatopancreas tubule composed of a core of multiple bacterial types (B) encapsulated by layers of hemocytes (H). Bar = 10 μm.

Hepatopancreatic epithelial cells of three of the four shrimp examined by TEM were infected multiply by a rickettsia (Figs. 3, 4), a helical mollicute (Figs. 3, 4), and a filamentous mollicute (Figs. 3, 5). The other shrimp was infected only by the rickettsia. The only organelles that appeared normally distributed in the cell cytoplasm were mitochondria and ribosomes (Fig. 3). Because of the absence of other normal cytoplasmic constituents, we could not determine which type of hepatopancreatic cell was infected. In infected shrimp, approximately 80% of the hepatopancreatic cells contained bacteria. There was no evidence of phagocytosis of any type of bacteria within cells. The only host response appeared to be the presence of granulomas in hemal spaces. The core of some granulomas was a hepatopancreatic tubule with no remaining eel and tubular architecture; it contained a mass of multiple bacterial types (Fig. 2). No bacteria were observed free in hemolymph. The rickettsia, a short rod 900 nm long by 300 nm wide, was the predominant bacterium in the hepatopancreatic cells. It possessed a trilaminar cell wall and a trilaminar plasma membrane (Figs. 6, 7). The helical mollicute had no cell wall or envelope and was bounded only by a plasma membrane. The organism was blunt at its wide end, with helical turns about its tapering form. It measured 260 nm across at the wide end which also contained electron-lucent bodies. There was no evidence of axial flagella or other organs of locomotion (Fig. 7). Masses of filaments filled the cytoplasm of some cells (Fig. 5). Like the helical form, the filamentous mollicute also had no cell wall. The filaments were 60 nm wide with terminal blebs, or knobs, on short, branched processes (Figs. 5, 8). Spherical dilations occurred on some of the filaments (Fig. 6).

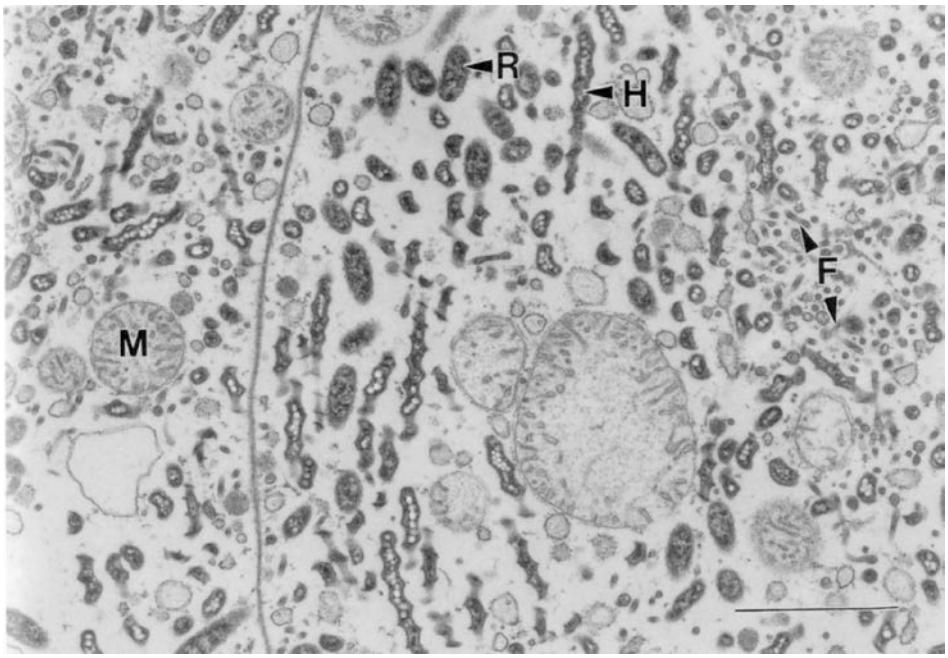


**Figure 3.** Transmission electron micrograph of cytoplasm of hepatopancreatic cells infected with rickettsia (R), a helical mollicute (H), and a filamentous mollicute (F). Note the paucity of cytoplasmic organelles other than mitochondria (M). Bar = 2 μm.

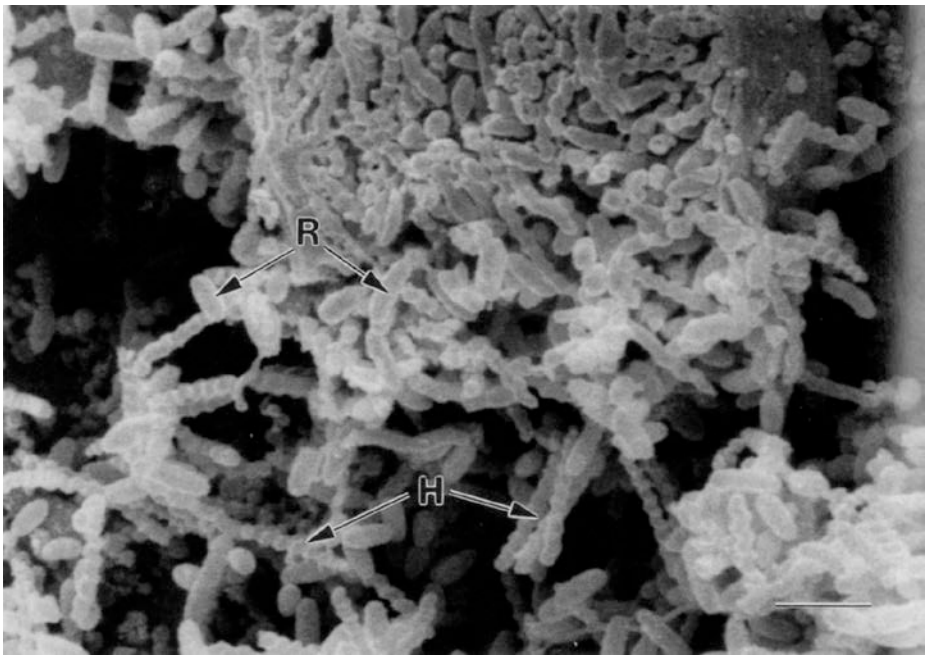


**Figure 4**. Scanning electron micrograph of freeze-cracked hepatopancreas cell containing rickettsia (R) and a helical mollicute (H). Bar = 1 μm.

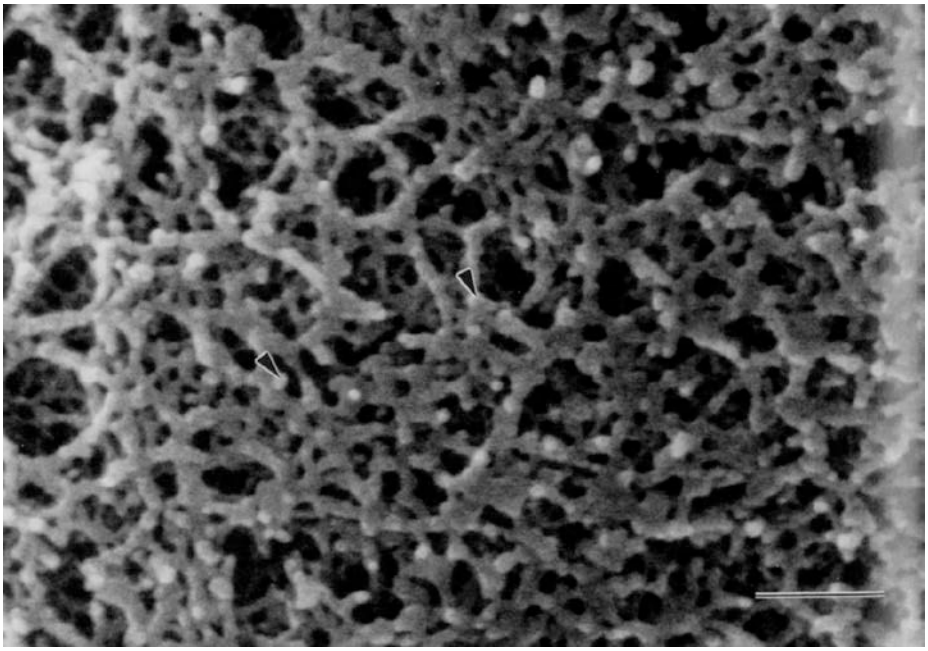

**Figure 5.** Scanning electron micrograph of masses of filamentous mollicute filling hepatopancreas cell cytoplasm. Note the terminal knobs (arrows). Bar = 0.5 μm.

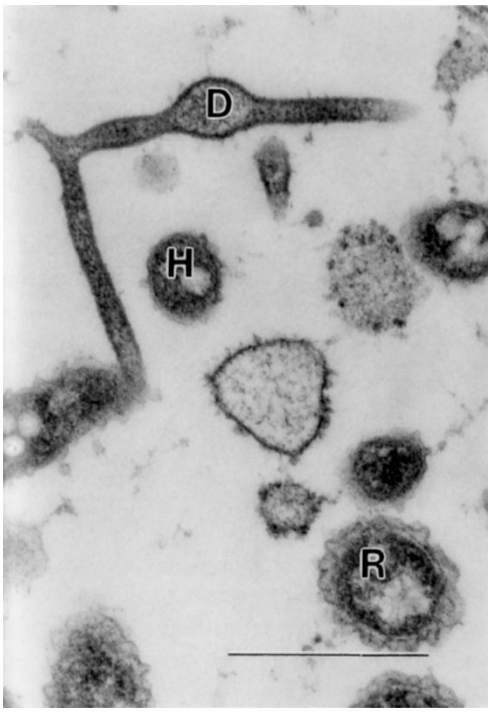


**Figure 6.** Transmission electron micrograph of a branched filamentous mollicute with a spherical dilation (D). Note the rickettsia (R) and helical mollicute (H) in the cell cytoplasm. Bar = 0.5 μm.

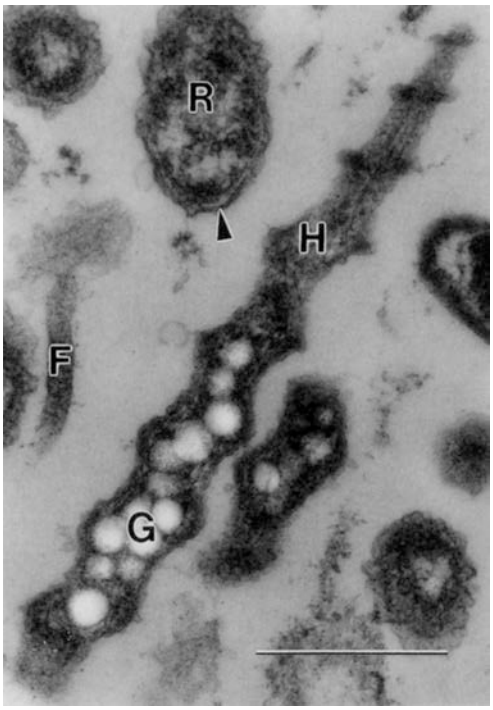


**Figure 7.** Transmission electron micrograph of rickettsia (R) and helical mollicute (H). Note the cell wall (arrow) of the rickettsia, the electron-lucent granules (G) at the blunt end of the helical mollicute, and a portion of filamentous mollicute (F). Bar = 0.5 μm.

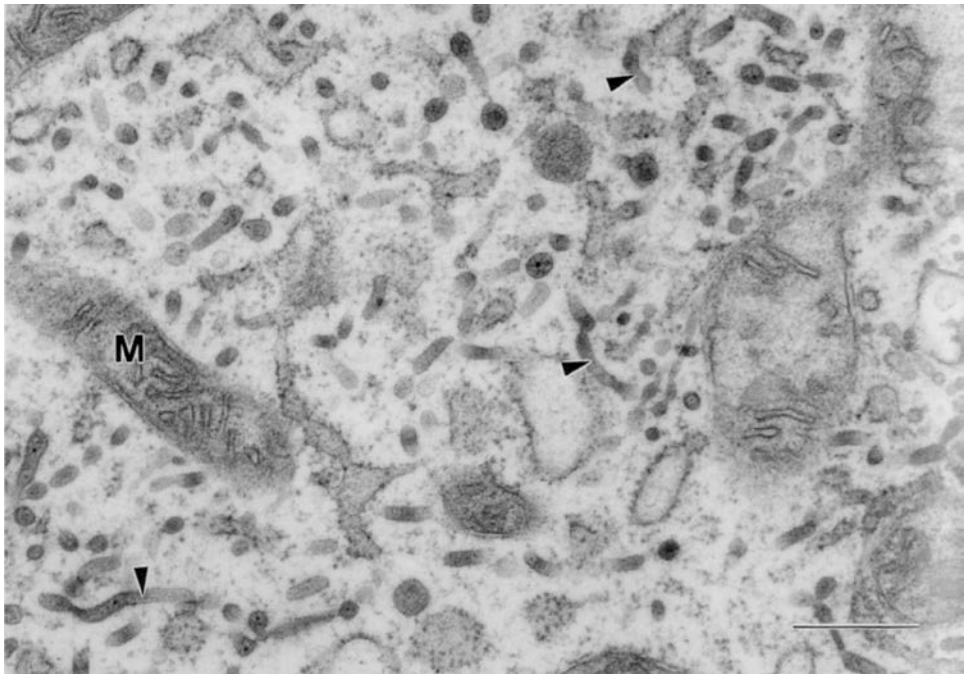


**Figure 8.** Transmission electron micrograph of a filamentous mollicute (arrows) in hepatopancreas cell cytoplasm. Note the mitochondria (M). Bar = 0.5 μm.

## Discussion

Whereas definitive diagnosis must await bacterial culture, tentative identification of the bacteria in *P. vannamei* can be based on morphological, primarily electron microscopical, features. Other studies have reported that rickettsial infections are difficult to detect in the early stages and may not be diagnosed by conventional histological methods until 30 days after infection (Johnson, 1984; Brock et al., 1986). By light microscopy, only the granularity of the hepatopancreatic cell cytoplasm and granulomatous tubules was readily apparent and special stains did not facilitate a definitive diagnosis.

The infections appeared to stimulate a host response in the hepatopancreas. Similarly, hemocytic encapsulation of infected hepatopancreatic tubules was found in rickettsial infections in *P. marginatus* and in *Paralithodes platypus* (Johnson, 1984; Brock et al., 1986). Anderson et al. (1987) reported rickettsial infections in the connective tissues of the hepatopancreas of *P. monodon*. That infection was accompanied by bacterial septicemia. Although hemocytes proliferated in the areas of encapsulation, bacterial septicemia was not diagnosed in the *P. vannamei* in this study or in *P. marginatus* examined by Brock et al. (1986).

The rod-shaped bacterium appeared to be a rickettsia because it had a trilaminar plasma membrane and envelope characteristic for that group of bacteria (Weiss and Moulder, 1984). The rickettsia in *P. vannamei* conformed to the size measurements for those reported in *P. monodon*, *P. marginatus,* and *P. platypus* (Johnson, 1984; Brock et al., 1986; Anderson et al., 1987). Rickettsial infections, although accompanied by mortality, generally elicit few

signs in infected shrimp. Pale white coloration of the hepatopancreas and lethargic behavior have been reported in other shrimp with rickettsial infections (Brock et al., 1986; Anderson et al., 1987; Sindermann and Lightner, 1988). The hepatopancreatic cells of *P. vannamei* did not exhibit the microcolonies usually associated with rickettsial disease in other shrimp or in crabs (Johnson, 1984; Brock et al., 1986; Anderson et al., 1987; Sindermann and Lightner, 1988).

Some agents in the shrimp we examined were Gram-positive whereas most bacteria that infect shrimp are Gram-negative. Several Gram-positive bacteria are found in shrimp, but only two that are infectious and may cause disease have been isolated. Both have been reported in hepatopancreas tissue. These include an acid-fast rod in hemocytic granulomas in a moribund *P. vannamei* and cocci in *P. monodon* with red disease (Lightner, 1988). Most rickettsias and mollicutes are Gram-negative (Razin and Freundt, 1984; Weiss and Moulder, 1984). Spiroplasmas are unusual mollicutes because they are Gram-positive. Classification of mollicutes on the basis of Gram-staining techniques is questionable, however, because the group has no cell wall (Whitcomb, 1980).

Both the helical and filamentous organisms are considered to be mollicutes because of their lack of a cell wall and because of their pleomorphic morphology. Although spiroplasmas resemble spirochetes in that both have a helical conformation, the helical bacterium in *P. vannamei* lacked the outer cell envelope and periplasmic flagella characteristic of spirochetes (Canale-Parola, 1984). Furthermore, this organism was not typical of known spiroplasmas because it exhibited tightly coiled helicity and contained electron-lucent bodies at the blunt end (K. E. Hackett and J. G. Tully, pers. comm.). It has not been reported previously in crustaceans and perhaps is a variant of a spiroplasma seen in other arthropods.

Hemolymph and gut are the two habitats from which spiroplasmas have been found most frequently in insects. More than 16 spiroplasmas have been isolated from the insect gut (Whitcomb and Tully, 1989). Most are found in extracellular locations such as gut lumen, although there are reports of spiroplasmas which penetrate and grow in insect cells (Garnier et al., 1984; Whitcomb and Tully, 1989). Mollicutes that are usually helical have been reported in such intracellular locations as the midgut cells of the Colorado potato beetle (Clark and Whitcomb, 1984). The mollicutes are rarely observed in membrane-bound vacuoles within the cell. Also, only nonphagocytic cells have been observed containing spiroplasmas (Whitcomb and Tully, 1989). These features are consistent with the infection seen in *P. vannamei*.

The wall-less filamentous bacteria with spherical dilations in the hepatopancreatic cells of *P. vannamei* may also be a type of mycoplasma. On the other hand, some spiroplasmas have been reported to lose their helicity in an intracellular environment. Their translational motility is a feature that either relates to their adaptation for an extracellular existence or responds to nutritional factors, growth stage, or environmental parameters such as osmolarity or pH (Williamson et al., 1989).

Most bacterial diseases represent secondary conditions in shrimp, occurring as a result of other primary factors such as other infectious diseases, inadequate nutrition, or stress and wounds (Sindermann and Lightner, 1988). No other evidence of disease was found in the *P. vannamei* that we examined, indicating that the shrimp may have been subjected to extreme stress and nutritional factors in culture conditions. Perhaps the bacteria caused a

chronic infection in these shrimp. The hepatopancreatic cells retained most of their normal architecture except for the loss of some organelles. There was little evidence of cell sloughing or cell necrosis despite the high number of infected cells. Death may occur when a large enough number of these cells become affected; disrupting normal metabolic processes. Further analysis will be necessary to determine the identity of these bacteria, their pathogenesis in cultured shrimp, and any relationships among the various types of bacteria.

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