

Prokaryote infections in the New Zealand scallops *Pecten novaezelandiae* and *Chlamys delicatula*

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ABSTRACT: Four intracellular prokaryotes are reported from the scallops *Pecten novaezelandiae* Reeve, 1853 and *Chlamys delicatula* Hutton, 1873. Elongated (1025 × 110 nm), irregular (390 × 200 nm), or toroidal (410 × 200 nm) mollicute-like organisms (M-LOs) occurred free in the cytoplasm in the digestive diverticular epithelial cells of both scallop species. Those in *P. novaezelandiae* bore osmiophilic blebs that sometimes connected the organisms together, and some had a rod-like protrusion, both of which resemble the blebs and tip structures of pathogenic mycoplasmas. The M-LOs in *C. delicatula* had a slightly denser core than periphery. Round M-LOs, 335 × 170 nm, occurred free in the cytoplasm of agranular haemocytes in *P. novaezelandiae*, without apparent harm to the host cell. In *P. novaezelandiae*, 2 types of highly prevalent (95 to 100%) basophilic inclusions in the branchial epithelium contained Rickettsia-like organisms (R-LOs). Type 1 inclusions occurred in moderately hypertrophied, intensely basophilic cells, 8 to 10 µm in diameter, containing elongate intracellular R-LOs, 2000 × 500 nm. Type 2 inclusions were elongated and moderately basophilic in markedly hypertrophic branchial epithelial cells, 50 × 20 µm in diameter, containing intracellular organisms 500 × 200 nm in diameter. The possible roles of these organisms in pathogenesis is discussed.

KEY WORDS: Scallops · *Pecten* · *Chlamys* · Digestive epithelium · Branchial epithelium · Haemocytes · Rickettsias · Mollicutes · Mycoplasmas · Tip structure · Ultrastructure

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INTRODUCTION

Since the first reports of Rickettsia-like (RL-O), chlamydia-like (CL-O) and mollicute-like (ML-O) organisms from bivalves (Harshbarger et al. 1977), there have been numerous reports of RL-Os, less of CL-Os, and few reports of M-LOs (Harshbarger et al. 1977, Azevedo 1993). Scallops have been reported with R-LOs (Gulka et al. 1983, Morrison & Shum 1983, Gulka & Chang 1984, Leibovitz et al. 1984, Elston 1986, Le Gall et al. 1988), C-LOs (Morrison & Shum 1982, Leibovitz 1989) and intracellular bacteria (Bower & Meyer 1991), sometimes associated with lesions (Gulka & Chang 1984) or mass mortalities (Gulka et al. 1983, Le Gall et al. 1988, Leibovitz 1989).

Annual mortality rates among wild New Zealand scallops *Pecten novaezelandiae* have been estimated at 23 to 39% (Bull 1976), and while a percentage of mortalities may be due to senescence, disease is suspected to also be the cause of some of these events. Light microscope examination of sections through *P. novaezelandiae*, to determine whether these on-going intermittent mortalities may be due to an infectious agent, has shown considerable sloughing of digestive epithelial cells. A subsequent ultrastructural study revealed the presence of virus-like particles (V-LPs) in sloughing cells (Hine & Wesley 1997). As similar V-LPs were previously reported to be associated with mortalities among cultured mussels *Perna canaliculus* (Jones et al. 1996), the V-LPs in scallops may cause excessive sloughing and mortalities. However, subsequent observations on *P. novaezelandiae* showed that many sloughing digestive epithelial cells also contained prokaryotes, in the presence or absence of V-LPs.

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This ultrastructural study was conducted to identify the prokaryotes in the digestive epithelia and to assess their impact on scallop health. During this study, prokaryote infections were also demonstrated in branchial epithelial cells and haemocytes. These infections were also studied, and another commercially important scallop *Chlamys delicatula* was examined for comparison with *Pecten novaezelandiae*.

MATERIALS AND METHODS

Samples of the gill and digestive tract were excised from 220 scallops *Pecten novaezelandiae* from around the North Island and top of the South Island, collected in 1996 to 1998, 388 *P. novaezelandiae* from the north of the North Island collected in 1999, and 60 queen scallops *Chlamys delicatula* from the north of the South Island, collected in 1998. Sections through the gills, mantle and digestive organ of *P. novaezelandiae* and the digestive organ of *C. delicatula* were fixed in Davidson's fixative or 10% formalin in filtered seawater and stained with haematoxylin and eosin.

For transmission electron microscopy (TEM), small (1 mm³) pieces of the gill and digestive gland of *Pecten novaezelandiae* and the digestive gland of *Chlamys delicatula* were excised immediately after opening the valves. They were fixed in 2.5% glutaraldehyde in 0.22 µm filtered seawater (FSW) for 1 h, washed twice in FSW, and post-fixed in 1% OsO₄ for 1 h. They were then dehydrated in ascending (50 to 100%) ethyl alcohol, embedded in Araldite, thick-sectioned and stained with 1% toluidine blue in 1% borax solution. Ultrathin sections were stained with 5% uranyl acetate for 10 min and 5% lead citrate for 5 to 6 min, and examined on a Philips 420ST TEM. Other sections were stained with ruthenium red to compare surface coating of prokaryotes with that of the plasma membrane (Taylor-Robinson et al. 1991).

RESULTS

The gills and digestive tract of *Pecten novaezelandiae* were examined by light microscopy, and TEM, and the digestive tract of *Chlamys delicatula* by light microscopy and TEM, for comparison with *P. novaezelandiae*.

Pecten novaezelandiae

Prokaryotes in digestive epithelial cells

Sections through the digestive diverticulae showed the normal highly vacuolated ghost cells and concentric

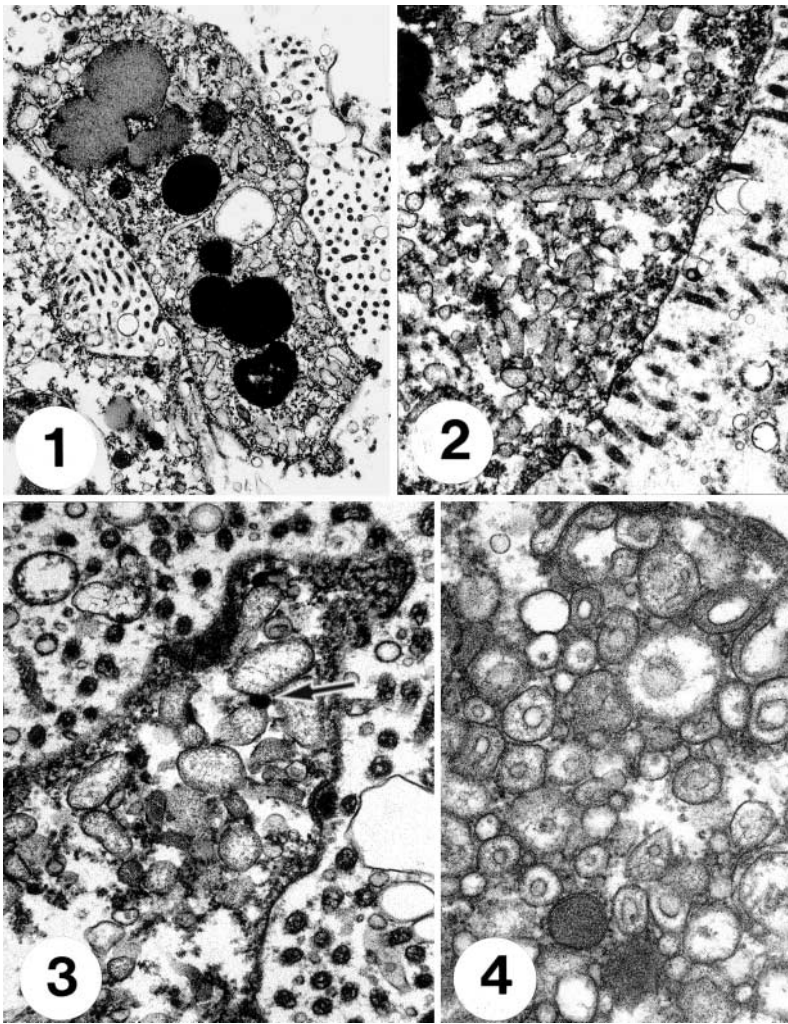
masses of yellowish amorphous matter in the lumen, commonly found in *Pecten* spp. There was no evidence of basophilic inclusions in the remnant basophil and absorptive cells lining the diverticulae, or in sloughing absorptive cells. Prevalence of prokaryotes in sloughing digestive epithelial cells could not therefore be determined by light microscopy. Ultrastructural observations on sloughing digestive epithelial cells of 127 of the 220 *Pecten novaezelandiae* sampled, showed 117 (92%) of scallops to be infected. As only 1 section through the digestive diverticulae could be examined from each scallop, the 10 apparently negative scallops may also have been infected elsewhere in the digestive diverticulae. Within individual oysters, nearly all the absorptive cells were infected.

Ultrastructurally, attached and sloughed digestive epithelial cells contained intracytoplasmic polymorphic prokaryotes, lacking a cell wall and bounded by a single trilaminar membrane, which were dispersed throughout the cells, in direct contact with the cytosol (Figs. 1 to 3). These prokaryotes were vermiform, 1025 × 110 nm (Fig. 2), irregular, 390 × 200 nm (Fig. 3) or toroidal, 410 × 200 nm (Fig. 4) in shape, sometimes with a clear elongated protrusion (Figs. 5 to 7), and internally had a few peripheral ribosomes and central filaments (Figs. 3 to 6). Elongated forms bore a terminal osmiophilic bleb (Figs. 3 & 8) that sometimes appeared striated or laminated (Fig. 9), with striations 4 to 6 nm in periodicity, and these were frequently seen to attach one organism to another (Fig. 10). Identical laminated structures also lay free in the cytoplasm (Fig. 11). Sometimes the prokaryotes lay in intracytoplasmic vacuoles, with the vacuole showing a similar slightly flocculent surface to that of the prokaryote (Fig. 7). Rarely, groups of apparently degenerating bodies with an osmiophilic core, and dense bodies, 230 to 130 nm, bounded by a single trilaminar membrane, were seen among the cellular debris (Fig. 12). Groups of similar bodies had a dense core and clear space between the core and membrane (Fig. 13).

Some sloughing digestive cells were co-infected with V-LPs, which occurred among the prokaryotes. Host-cell Golgi bodies were occasionally seen to be actively producing an osmiophilic substance, and host-cell mitochondria were very dense and surrounded by a double membrane. The cytosol was also composed of membrane-attached V-LPs, ribosomes, membranous debris, glycogen and dense granules bounded by a double membrane.

Prokaryotes in haemocytes

As this study was specifically carried out on digestive epithelial cells and branchial epithelial cells, haemo-



Figs. 1 to 4. *Pecten novaezelandiae*. Infection of digestive epithelial cells. Fig. 1. Sloughing digestive epithelial cell heavily infected with prokaryotes ($\times 11550$). Fig. 2. Cytoplasm of a sloughing cell containing elongated prokaryotes ($\times 19380$). Fig. 3. Detail of sloughing cell showing polymorphic prokaryotes; note that 2 are attached by an osmiophilic bleb (arrow) ($\times 41140$). Fig. 4. Toroidal prokaryotes lying directly in the host cell cytoplasm ($\times 49550$)

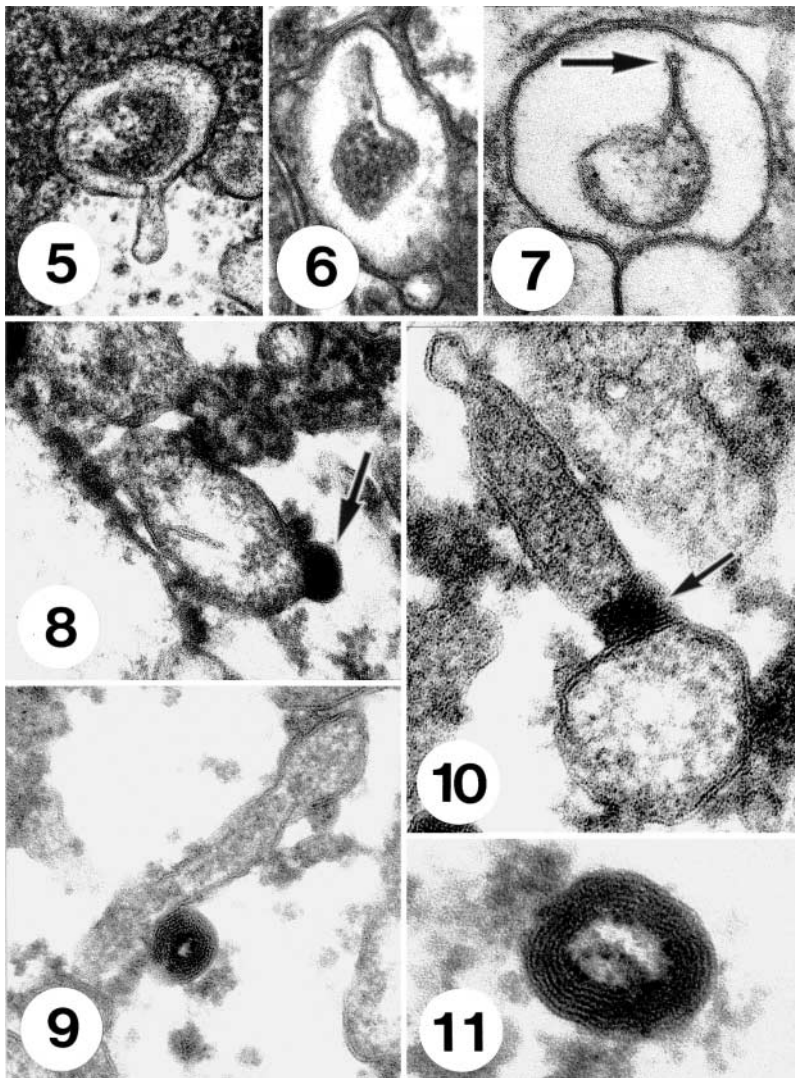
cytes were rarely observed. However, haemocytes were observed in 16 of the 127 *Pecten novaezelandiae* studied by TEM, and prokaryotes were observed in the haemocytes of 14 of the 16 scallops. Intracytoplasmic spherical prokaryotes, 335×170 nm, lacking a cell wall and bounded by a single trilaminar membrane, also occurred in agranular haemocytes (Fig. 14), in direct contact with the cytosol (Fig. 15). They contained ribosomes, but nucleic acid filaments were not apparent. Constriction of some individuals, and attachment of twin organisms suggested that they divided by binary fission (Fig. 16). Although these prokaryotes occupied much of the cytoplasm, the nuclei of the haemocytes appeared to be normal, suggesting they were unaffected by their presence.

Prokaryotes in branchial epithelial cells

Branchial epithelial cells (Fig. 17) of 80 to 100% of the scallops examined were infected by 2 types of basophilic inclusions containing prokaryotes. Type 1 inclusions occurred in moderately hypertrophied, intensely basophilic cells, 8 to 10 μm in diameter, containing elongate intracellular organisms, 2000×500 μm (Fig. 18). Type 2 inclusions were elongated and moderately basophilic in markedly hypertrophic branchial epithelial cells, 50×20 μm in diameter (Fig. 19), containing intracellular organisms 500×200 nm in diameter. Type 1 inclusions were common at all sites, but Type 2 inclusions were variable and intermittent in distribution. Ultrastructurally, the prokaryotes occurred in an inclusion and were rod-shaped, with a periphery of dense ribosomes and a less dense central region containing putative filaments of nucleic acid (Fig. 20). Each organism was surrounded by a wavy membrane (Fig. 20). Large elongated basophilic inclusions also occurred in the epithelium of the mantle in 23 to 100% of scallops from each area (data not shown).

Chlamys delicatula

Of the 60 *Chlamys delicatula* studied, 20 were examined ultrastructurally, and all of these 20 had prokaryotes in the digestive epithelial cells. Histologically, the digestive diverticulae appeared as in *Pecten novaezelandiae*. Ultrastructurally, some attached and many sloughed digestive epithelial cells contained intracytoplasmic polymorphic prokaryotes, 205×115 nm, lacking a cell wall and bounded by a single trilaminar membrane. They were dispersed throughout the cells, in direct contact with the cytosol (Fig. 21). They were circular or irregular in shape and contained many ribosomes but lacked nucleic acid filaments (Figs. 21 & 22), or were circular with peripheral ribosomes and a central filamentous nucleoid (Fig. 23). The circular to irregular forms with many ribosomes appeared to develop central dense nucleoids as they elongated and divided by binary fission to form circular forms with a nucleoid (Fig. 24). Host cells lacked organelles such as mitochondria and Golgi



Figs. 5 to 11. *Pecten novaezelandiae*. Morphology of prokaryotes in digestive epithelial cells. Fig. 5. Toroidal prokaryote showing protrusion into a space containing glycogen granules ($\times 70650$). Fig. 6. Prokaryote packed with ribosomes showing rod-like protrusion ($\times 117200$). Fig. 7. Prokaryote lying in a vacuole, showing protrusion of a tip-structure (arrow) and a slightly flocculent surface similar to that lining the vacuole; ruthenium red ($\times 135050$). Fig. 8. Intracytoplasmic prokaryote bearing an osmiophilic bleb (arrow) ($\times 116200$). Fig. 9. Striated or laminated structure lying on a prokaryote ($\times 135000$). Fig. 10. Two prokaryotes joined by a striated bleb (arrow) ($\times 193700$). Fig. 11. Striated structure lying free in the cytosol ($\times 240300$)

bodies, and the cytosol mainly comprised ribosomes and membranous debris.

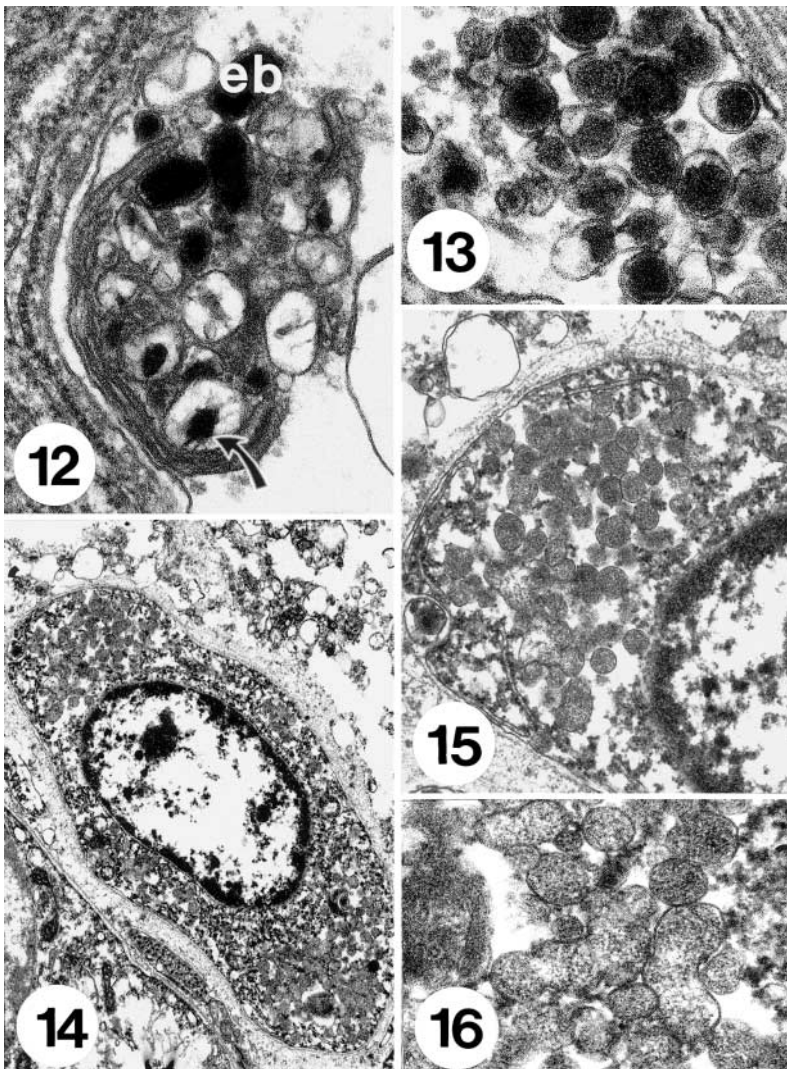
DISCUSSION

With the exception of a recent study identifying an intracellular prokaryote causing withering syndrome in abalone as a member of the Rickettsiales (Friedman

et al. 2000), identification of intracellular prokaryotes of molluscs has relied on ultrastructural studies. While such studies do not permit specific or generic identification, they can be used to identify the group to which the prokaryotes belong. However, it is difficult to determine the prevalence and intensity of infection, because infection may be localized and not encountered in the few ultra-thin sections examined, and intensity may differ in different parts of an infected organ. The branchial basophilic prokaryotic inclusions in *Pecten novaezelandiae* resemble R-LOs reported from *P. maximus* both in the 2 types of inclusions and in the ultrastructure of the R-LOs within the inclusions (Le Gall et al. 1988).

The other prokaryotes encountered are less easy to identify. The organisms in epithelial cells of the digestive diverticulae of *Pecten novaezelandiae* and *Chlamys delicatula*, and in the haemocytes of *P. novaezelandiae* possessed a trilaminar plasma membrane but lacked cell walls, thus resembling mollicutes. However, the presence or absence of cell walls must be treated with caution as both rickettsial and chlamydial prokaryotes may produce cell wall defective variants (Kordová 1978, Hase 1985). Normal R-LOs and C-LOs have walls similar to Gram-negative bacteria, although rickettsial walls may vary between species even in the same genus (Silverman 1991), and chlamydial walls lack a peptidoglycan layer (Moulder 1991). The absence of an inclusion membrane is taxonomically important, as R-LOs (Leibovitz et al. 1984, Elston 1986, Le Gall et al. 1988) and C-LOs (Morrison & Shum 1982, Leibovitz 1989, Johnson & Le Pennec 1995) occur in intracellular inclusions, visible as basophilic inclu-

sions under the light microscope. The chlamydial inclusion membrane is particularly important as it mediates contact with host cell organelles (Moulder 1991, Hackstadt et al. 1997, Hackstadt 2000). Mollicutes occur either in deep invaginations of the plasma membrane or free in the cytoplasm (Taylor-Robinson et al. 1991, Baseman et al. 1995, Razin et al. 1998), and are not visualized by light microscopy. Based on the lack of cell wall and the absence of an inclusion, the



Figs. 12 & 13. *Pecten novaezelandiae*. Possible stages of a prokaryote in digestive cells. Fig. 12. group of possible prokaryotes showing a possible nucleoid (arrow) and elementary bodies (eb) ($\times 69\,230$); Fig. 13. group of bodies resembling elementary bodies of a prokaryote ($\times 96\,260$). Figs. 14 to 16. Prokaryotes in haemocytes. Fig. 14. Agranular haemocyte containing intracytoplasmic prokaryotes ($\times 122\,210$); Fig. 15. greater detail of the haemocyte in Fig. 14 showing prokaryotes free in the cytosol ($\times 30\,010$); Fig. 16. wall-less prokaryotes in the haemocyte cytoplasm ($\times 54\,830$)

digestive cell and haemocyte organisms are considered mollicutes.

Two notable features of the prokaryotes in the digestive cells of *Pecten novaezelandiae* were the common occurrence of osmiophilic bleb-like protrusions (Figs. 3 & 8), and an elongated rod-like structure (Figs. 5 to 7). Mollicutes lack features, but some pathogenic mycoplasmas show polarity and have apical bleb-like protrusions (Archer 1981) or nap (Tully et al. 1983), which may be utilized in attachment to host cells or to each other (Jensen et al. 1994), and which

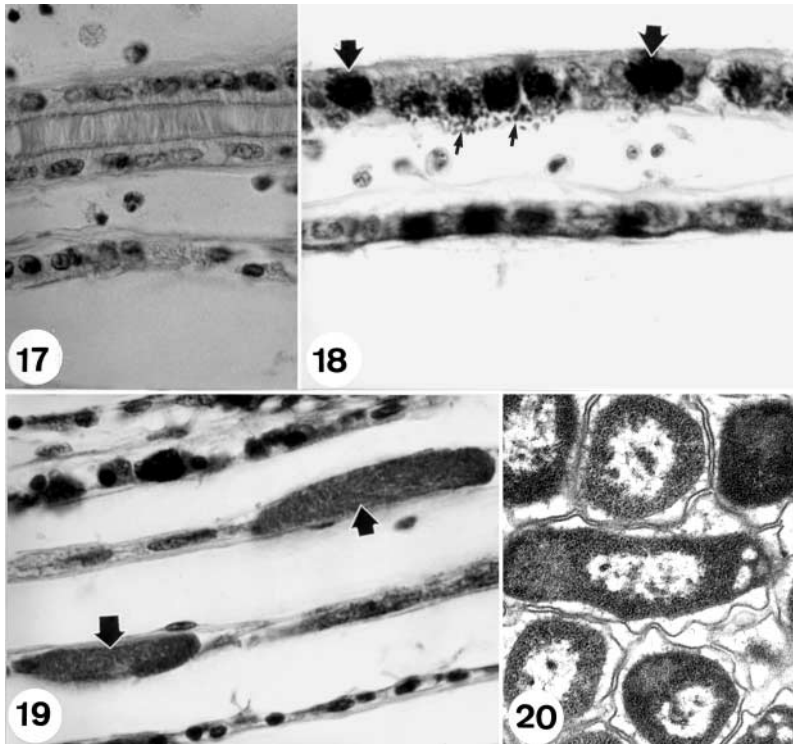
may be striated (Tully et al. 1983; see present Figs. 9 to 11). They contain enzymes and resemble the nap-like protrusions observed here. Vesicular inclusions in the periphery of mollicutes infecting red claw crayfish *Cherax quadricarinatus* have also been identified as blebs (Jiménez et al. 1998), but they were not osmiophilic and did not protrude from the surface of the mollicute, as observed in mammalian mycoplasmas and in this study.

Also, the long rod-like extension shown in Figs. 6 & 7 resembles the tip structure used by pathogenic mycoplasmas to attach to and penetrate host epithelial cells (Lo et al. 1993, Jensen et al. 1994, Abdul-Wahab et al. 1996, Trachtenberg 1998, Romero-Arroyo et al. 1999). Tip structures utilize cytoadhesins for attachment, but attachment and penetration can also occur in the absence of tip structures (Salih & Rosenbusch 1988, Jensen et al. 1994). Mycoplasmas of higher animals utilize nap or tip structures when actively invading epithelial cells, but they are unnecessary when mycoplasmas are endocytosed by host cells (Razin et al. 1998). The presence of apical nap and apparent tip structures in the mollicutes infecting the digestive epithelial cells of *P. novaezelandiae* suggests that the cells may be actively invaded by the mollicute. However, mollicutes bearing nap were only observed in direct contact with the cytoplasm (Figs. 3 & 5), and those with tip structures in vacuoles (Figs. 6 & 7). The function of these structures in relation to cytoadhesion and penetration needs to be clarified before their identity can be verified.

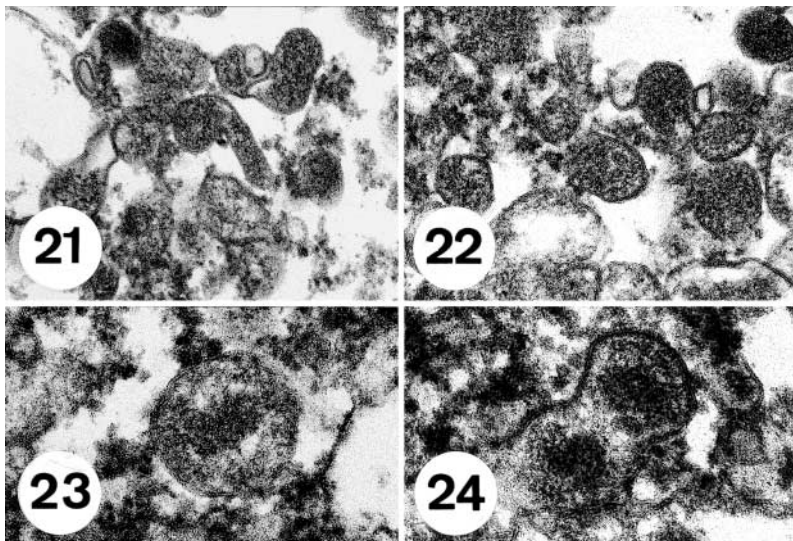
The dense bodies rarely observed in the necrotic debris of degenerating

Pecten novaezelandiae digestive cells (Figs. 12 & 13) may or may not be developmental stages of a prokaryote. The dense bodies do resemble the elementary bodies of chlamydias (Morrison & Shum 1982, Leibovitz 1989, Miyashita et al. 1993), and even rickettsias may form similar bodies (Popov et al. 1995). They may be stages of an underlying chlamydial infection, or simply secretory granules. Further research is needed to determine their identity.

Few serious, or potentially serious, infective organisms have been reported from scallops (Moyer et al.



Figs. 17 to 20. *Pecten novaezelandiae*. R-LOs in branchial epithelial cells. Fig. 17. Normal branchial epithelium ($\times 1375$). Fig. 18. Branchial epithelium with highly basophilic Type 1 R-LO inclusions (large arrows) and elongate intracellular organisms (small arrows) ($\times 1375$). Fig. 19. Two elongated, moderately basophilic Type 2 R-LO inclusions (arrows) ($\times 1375$). Fig. 20. TEM of rod-shaped intracellular R-LOs from the branchial epithelium showing wavy outer membrane, periphery of dense ribosomes and less dense central region ($\times 49000$)



Figs. 21 to 24. *Chlamys delicatula*. Prokaryotes in digestive epithelial cells. Fig. 21. Polymorphic wall-less prokaryotes free in the cytosol of a digestive epithelial cell ($\times 72860$). Fig. 22. Rounded prokaryotes free in the cytosol of a digestive epithelial cell, showing the organisms packed with ribosomes ($\times 90630$). Fig. 23. Prokaryote showing a dense nucleoid-like centre ($\times 118400$). Fig. 24. Division of forms with a dense nucleoid-like centre ($\times 127920$)

1993, Whyte et al. 1994, Bower et al. 1998), and yet mass mortalities are common in wild and cultured scallop stocks in many countries (Leibovitz et al. 1984). The presence of intracellular prokaryotes in scallops raises the question of their possible role in pathogenesis. Mortality rates of 23 to 39% reported among *Pecten novaezelandiae* by Bull (1976) are similar to the 30% mortalities in rickettsial epizootics in *P. maxima* (Le Gall et al. 1991), and in both cases the R-LOs are highly prevalent (90 to 100%). Branchial R-LO infections may also occur in *Chlamys delicatula*, similar to those reported from *C. varia* and *C. operculata* in France (Le Gall et al. 1991). Branchial R-LO infections may adversely affect the host directly by release of enzymes such as acid phosphatases and catalase (Le Gall & Mialhe 1992), or indirectly by destroying the respiratory function of branchial epithelial cells, causing respiratory impairment and, when many cells are infected, death. The mollicutes in haemocytes may be similar to those reported from the haemocytes of Japanese scallops *Patinopecten yessoensis*, which were associated with failure to thrive, lesions in the adductor muscle, widespread connective tissue lesions, or death, depending on the level of infection (Bower & Meyer 1991, 1995).

The possible role in pathogenesis of the digestive cell mollicute in *Pecten novaezelandiae* is more difficult to assess because of the co-occurrence of V-LPs in many sloughing cells. Detached cells contained V-LPs only, mollicutes only, or both, but proportions varied greatly between tubules and between scallops. M-LOs may reduce food absorption by the scallops, and secretion of hydrogen peroxide and superoxide radicals directly into the cytoplasm is probably mildly toxic to host cells (Razin et al. 1998). Nap and rod-like tip structures are characteristics of pathogenic *Mycoplasma* spp. (Tully et al. 1983, Abdul-Wahab et al. 1996), and may indicate pathogenicity of the mollicute in *P. novaezelandiae* digestive cells. Considered separately,

the V-LP and prokaryote infections of scallops may not appear harmful to the host, but considered together at moderate to high intensities, and in the apparent absence of other possible pathogens, they may physiologically stress the host, resulting in mass mortalities.

There are 2 possible reasons why scallops are particularly prone to prokaryote infections. Firstly, scallops appear to lack granulocytes which, in other bivalves, phagocytose bacteria (Hine 1999). Secondly, R-LOs (Buchanan 1978, Wen et al. 1993, Comps & Tigé 1999) and C-LOs (Harshbarger et al. 1977, Johnson & Le Pennec 1995) of bivalves are often infected with phages, which appear to control populations of these prokaryotes in bivalves. Phages have not been reported from scallop non-eubacterial prokaryotes, and this has possibly permitted them to spread unchecked. Experimental infection studies are needed to determine the pathogenicity of these organisms in scallops.

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