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# *Vibrio parahaemolyticus* and *V. harveyi* cause detachment of the epithelium from the midgut trunk of the penaeid shrimp *Sicyonia ingentis*

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ABSTRACT: Shrimp *Sicyonia ingentis* were either injected with *Vibrio parahaemolyticus* ($10^4$ CFU) or *V. harveyi* ($10^6$ CFU) or immersed in ASW containing either species at $10^5$ CFU $ml^{-1}$. These densities were shown in preliminary experiments to kill approximately half the population by 7 d. On Day 7, surviving shrimp were classified as either diseased or apparently healthy, and their midgut trunks (MGT) were examined by light and electron microscopy. All shrimp immersed in ASW containing either species of *Vibrio* showed detachment of the epithelium in the MGT. In shrimp injected with either species of *Vibrio*, epithelial detachment was common in diseased shrimp but not in apparently healthy animals. Experiments with live shrimp were supported by *in vitro* experiments where MGTs were removed, tied off at both ends, and injected with either pathogenic bacteria (*V. parahaemolyticus* or *V. harveyi*), non-pathogenic bacteria (*Bacillus subtilis* or *Escherichia coli*), or ASW. After 2 h incubations in ASW at 15°C, the MGTs were processed and examined. The epithelium consistently detached from isolated MGTs injected with either species of *Vibrio*, but not from MGTs injected with non-pathogenic bacteria or ASW. Because the MGT epithelium secretes the peritrophic membrane, loss of the epithelium eliminates 2 layers that may restrict penetration of ingested pathogens into the shrimp body and may disrupt the osmoregulatory function of the MGT. A second finding was that fixed, large-granule hemocytes associated with the basal lamina degranulated in the presence of the 2 species of *Vibrio*, but not with the non-pathogenic bacteria or ASW. These blood cells may help fight specific bacteria penetrating the MGT.

KEY WORDS: Shrimp · Vibriosis · Midgut trunk · Hemocytes



## INTRODUCTION

Vibriosis is a bacterial disease responsible for mortality of cultured shrimp worldwide (Lightner & Lewis 1975, Adams 1991, Lavilla-Pitogo et al. 1998, Chen et al. 2000). Outbreaks may occur when environmental factors trigger the rapid multiplication of bacteria already tolerated at low levels within shrimp blood (Sizemore & Davis 1985), or by bacterial penetration of host barriers. The exoskeleton provides an effective physical barrier to pathogens trying to penetrate the external surface of crustaceans, as well as the foregut and hindgut. However, *Vibrio* spp. are among the chitinoclastic bacteria associated with shell disease (Cook & Lofton 1973) and may enter through wounds in the exoskeleton or pores (Jiravanichpaisal & Miyazaki 1994, Alday-Sanz et al. 2002). The gills may appear susceptible to bacterial penetration because they are covered by a thin exoskeleton (Taylor & Taylor 1992), but their surfaces are cleaned by the setobranchs (Bauer 1998). The midgut, composed of the digestive gland (DG) and the midgut trunk (MGT, often referred to as the intestine, see Lovett & Felder 1990), is not lined by an exoskeleton and therefore seems to be a likely site for penetration of pathogens carried in the water, food, and sediment (Ruby et al. 1980, Jayabalan et al. 1982).

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Vibriosis has been experimentally induced in penaeid shrimp by either immersing shrimp in water containing bacteria (Egusa et al. 1988, Chen et al. 1992, Sung et al. 1994, Hameed 1995, Esteve & Herrera 2000) or injecting bacteria into their musculature or hemolymph (Lightner & Lewis 1975, Aruma 1989, de la Peña et al. 1993, Jiravanichpaisal & Miyazaki 1994, Lee et al. 1996). These studies show that regardless of the mode of infection, heavily infected shrimp typically become lethargic, the blood loses its ability to clot, and the gills, lymphoid organ, and in particular the DG show degenerative changes. Specific morphological changes to the DG include tissue necrosis, loss of the epithelium, and infiltration of hemocytes.

This paper describes the detachment of the epithelium lining the MGT in shrimp immersed in seawater containing *Vibrio parahaemolyticus* and *V. harveyi*. Detachment of the MGT epithelium was also observed in *in vitro* experiments where isolated MGTs were exposed to either species of *Vibrio*, but not when artificial seawater (ASW) or non-pathogenic bacteria were injected into isolated MGTs. Evidence is also presented that fixed granulocytes associated with the basal lamina of the MGT contribute to the defense response by degranulating in the presence of these 2 species of *Vibrio* but not the non-pathogenic bacteria.

## MATERIALS AND METHODS

**Organisms.** Penaeid shrimp *Sicyonia ingentis* were collected by otter trawls in 100 m of water off the Palos Verdes peninsula, California, USA, and maintained in aquaria with seawater at 33 ppt and 15°C for at least 7 d prior to use. Only intermolt shrimp that had normal reddish color, vigorously swam away when prodded, and which had eaten were used in subsequent experiments. *Vibrio parahaemolyticus* was purchased from American Type Culture Collection (ATCC 27519), and originally came from a food poisoning incident in Lousiana, USA, involving shrimp. *V. harveyi* was provided by Dr. M. O. Martin at Occidental College, and *Bacillus subtilis* and *Escherichia coli* were purchased from Ward's Natural Science, New York (85W0228 and 85W0400). The latter bacteria were grown in liquid broth (LB) containing tryptone (Difco) 10 g $l^{-1}$, yeast extract (Difco) 5 g $l^{-1}$ and NaCl 10 g $l^{-1}$. Both species of *Vibrio* were streaked onto Tryptic Soy Agar (TSA) with 2.5% NaCl and grown overnight at room temperature. Aliquots (0.5 ml) of the bacteria in freezing media (Tryptic Soy Broth with 2.5% NaCl and 20% glycerol) were frozen (–70°C). For experiment with either species of *Vibrio*, a frozen sample was thawed, added to 10 ml liquid broth with saline (LBS, prepared the same as LB with extra NaCl 20 g $l^{-1}$), and grown overnight at room temperature. The density of *Vibrio* was determined by optical density (OD) at 540 nm. An OD value of 0.1 was shown to be equivalent to $1.0 \times 10^8$ colony-forming units $ml^{-1}$ (CFU $ml^{-1}$) by counting colonies on LBS agar (LBS with 15 g agar $l^{-1}$), and agrees with previous studies (Mikulski et al. 2000). Bacteria were pelleted by centrifugation at $1000 \times g$ for 10 min at 4°C and resuspended in 0.2 µm filter-sterilized ASW (Instant Ocean, Aquarium Systems) for use.

**Preliminary studies.** In preliminary experiments, shrimp were immersed in individual tubs with ASW containing *Vibrio parahaemolyticus* or *V. harveyi* at the following concentrations: $10^9$, $10^7$, $10^5$, $10^3$, and $10^1$ CFU $ml^{-1}$. Twenty shrimp were tested at each concentration and 20 control shrimp were isolated in individual tubs containing ASW. Likewise 20 shrimp were injected with 0.1 ml of ASW containing *V. parahaemolyticus* or *V. harveyi* at each of the following concentrations: $10^9$, $10^7$, $10^5$, $10^3$, and $10^1$ CFU, and then returned to individual tubs containing ASW. Control shrimp (20) received a 0.1 ml injection of ASW. Mortality was tabulated after 7 d. These preliminary experiments showed that the following dosages resulted in killing approximately half of the population in 7 d: injections of $10^4$ and $10^6$ CFU *V. parahaemolyticus* and *V. harveyi*, respectively, and immersion in ASW containing $10^5$ CFU $ml^{-1}$ of either species. These dosages were selected for use in the present study and are also similar to published lethal dose ($LD_{50}$) values on shrimp, although different species, different lengths of exposure, and different stages in shrimp life cycles were used (see Arume 1989, Chen et al. 1992, Jiravanichpaisal & Miyazaki 1994, Hameed 1995, Lee et al. 1996, Goarant et al. 1998, Vandenberghe et al. 1999, Mikulski et al. 2000).

***In vivo* infections.** Individual shrimp (mean carapace length of females = 57 mm; males = 49 mm) were maintained in plastic tubs containing 3 l of ASW at 15°C in constant dark to simulate conditions at the collection site and checked daily. For injection experiments, 60 control shrimp were injected into the hemolymph via the 2nd pleopod with 0.1 ml of filter-sterilized (0.2 µm) ASW. Experimental shrimp (60) received an injection of 0.1 ml of ASW containing $10^4$ CFU *Vibrio parahaemolyticus*, and a second set of 60 shrimp received an injection of $10^6$ CFU *V. harveyi*. For immersion experiments, control shrimp (60) were maintained in tubs containing ASW, and 2 additional sets of 60 shrimp were placed in individual tubs with 3 l of ASW containing $10^5$ CFU $ml^{-1}$ of either bacterium.

The pH of ASW in each tub was measured daily and levels of dissolved oxygen (DO), total ammonia, and nitrites were determined using test kits from Ward's Natural Science (#21W9073, 21W9067, 21W9077). Tubs were not allowed to develop biological filters

with nitrifying bacteria before experiments began so that shrimp were exposed primarily to the 2 species of *Vibrio* being tested. Tubs were not aerated to prevent aerosolization of pathogenic *V. parahaemolyticus* (Howard et al. 1985, Hally et al. 1995). On Day 7, surviving shrimp were categorized as either diseased or apparently healthy. Diseased shrimp typically lay on their sides and did not respond when probed, although movement of appendages, the schphognathites, and/or heart were observed. Shrimp regarded as apparently healthy rested on their legs and moved or showed a startle response with rapid flapping of the abdomen when prodded. On Day 7, MGTs from 7 shrimp from each preparation were processed for light microscopy (LM) and transmission electron microscopy (TEM; Zeiss EM 109).

**Experiments on isolated MGTs.** To further assess the effects of bacteria on the MGT, 3 cm long sections of MGT were removed, tied off at both ends and injected with 0.1 ml of ASW containing 1 of the following bacteria at $10^5$ CFU $ml^{-1}$: *Vibrio parahaemolyticus*, *V. harveyi*, *Bacillus subtilis*, and *Escherichia coli*. Control preparations were injected with ASW. After a 2 h incubation in ASW at 15°C, 10 MGTs from each test were fixed and processed for LM and TEM as described below. In addition to the condition of the epithelium, the layer of fixed (non-circulating), large-granule hemocytes associated with the basal lamina was examined by TEM. Specifically, the extent of degranulation in a minimum of 100 cells from each treatment was recorded as either normal (filled with electron-dense cytoplasmic granules), moderately degranulated (mixture of granules and exocytotic vesicles), or extensively degranulated (few if any granules remaining).

**Tissue processing.** Tissues were fixed at room temperature for 3 h in 3% glutaraldehyde in 0.1 M sodium cacodylate pH 8.0 containing 3% sucrose. Following a brief rinse in buffer, tissues were post-fixed 1 h in 1% $OsO_4$ in 0.1 M sodium cacodylate, stained en bloc in 3% uranyl acetate in 0.1 M sodium acetate buffer for 1 h, dehydrated through a graded series of ethanol, and infiltrated and embedded in Spurr's (1969) low viscosity plastic. Thick (0.5 µm) sections were stained with methylene blue, thin (90 nm) sections with lead citrate, and viewed by LM and TEM, respectively.

## RESULTS

### Mortality

Mortality was low in control shrimp; 5 of the 60 shrimp injected with ASW died by Day 7 as did 4 of the 60 shrimp immersed in ASW. Analysis of the ASW in tubs containing shrimp showed that DO typically dropped from 8 to 4 ppm and pH decreased from 8.0 to 7.4 by Day 7, while total ammonia-N and nitrite-N levels increased from 0 to 2.5 and 0.2 ppm, respectively.

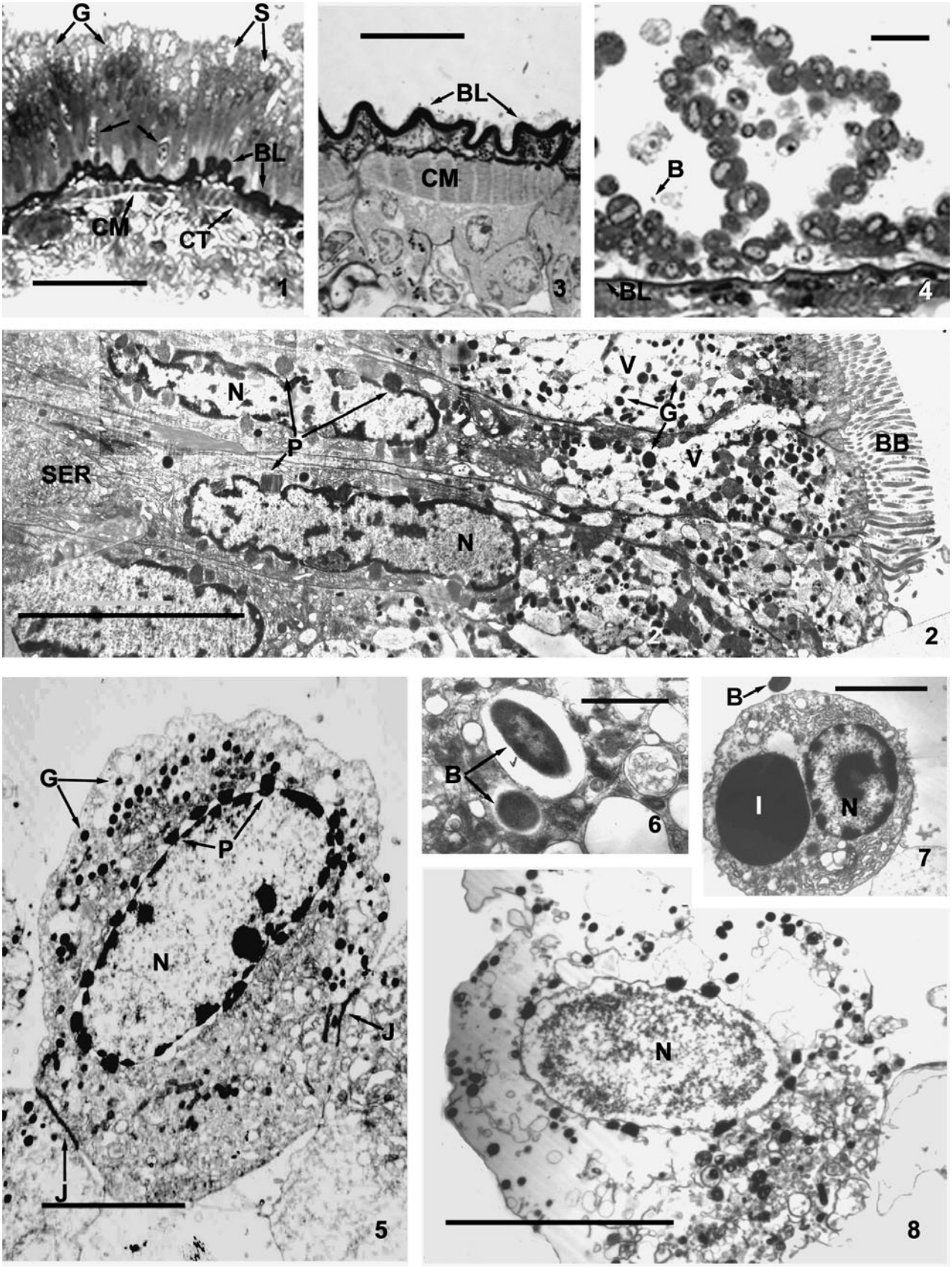
Sixty shrimp were injected with 0.1 ml of ASW containing $10^4$ CFU *Vibrio parahaemolyticus*. Thirty-six shrimp (60%) died by Day 4 and 4 more died by Day 7. Of the 20 shrimp alive on Day 7, 8 were diseased and 12 appeared healthy. Sixty shrimp were injected with 0.1 ml of ASW containing $10^6$ CFU *V. harveyi*. Thirty-three shrimp (55%) died by Day 4 and no more died by Day 7. Of the 27 shrimp alive on Day 7, 7 were considered diseased and 20 were apparently healthy.

Sixty shrimp were immersed in ASW containing $10^5$ CFU $ml^{-1}$ of *Vibrio parahaemolyticus*. Thirty-three (55%) died by Day 4 and 5 more died by Day 7. Of the 22 shrimp alive on Day 7, 12 were diseased and 10 appeared healthy. Sixty shrimp were immersed in $10^5$ CFU $ml^{-1}$ of *V. harveyi*. Six (10%) shrimp were dead by Day 4 and 23 more (48%) died by Day 7. Of the surviving shrimp on Day 7, 8 were diseased and 23 appeared healthy.

### Morphology of the MGT

Seven days following the injection of either strain of *Vibrio* into shrimp hemolymph, the MGT in apparently healthy shrimp appeared normal (Fig. 1); the same as in control animals. The simple, columnar epithelium was bound to a thick (0.8 µm) and wavy basal lamina and was composed of 2 types of cells as previously described (Martin & Chiu 2003). The most numerous type of cell was elongate (65 µm × 4 µm) with a brush border (5 µm long), and the apical cytoplasm was filled with granules and empty-appearing vesicles suggestive of exocytosis (Fig. 2). Ovoid nuclei with patches of heterochromatin around the perimeter of the nuclear envelope occupied the middle of each cell. The basal part of each cell was filled with smooth endoplasmic reticulum. A second type of epithelial cell was ovoid, lay along the basal lamina, and contained small to large inclusions (see Figs. 1 & 7). Outside of the basal lamina was a layer of loose connective tissue containing circular and longitudinal muscle, hemal spaces, and an outer acellular intimal layer. In diseased shrimp 7 d after being injected with either species of *Vibrio*, the MGT epithelium appeared normal in 80% of the shrimp, while in the other 20%, parts or all of the epithelium was missing (see Fig. 3). Remaining epithelial cells in the latter shrimp were typically spherical (see Fig. 4).

After 7 d of immersion in ASW containing either species of *Vibrio*, the epithelium was missing in 85% of apparently healthy and 100% of the diseased shrimp (Fig. 3). Remaining cells still attached or separated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from the basal lamina were spherical and had lost their microvilli (Figs. 4 & 5). A few spherical cells contained phagocytosed bacteria (Fig. 6), and the second type of cell containing an inclusion was occasionally seen (Fig. 7). Detached cells found further into the lumen showed signs of degeneration, with pycnotic nuclei, abundant vesicles and lysed plasma membranes (Fig. 8). No changes were observed in the basal lamina, connective tissue, and muscle layers.

### Isolated MGT experiments

The morphology of the MGT appeared normal with an intact simple columnar epithelium when MGTs were removed from shrimp, tied off at both ends, injected with ASW, and incubated for 2 h in ASW (see Figs. 1 & 2). Likewise, injections of ASW containing non-pathogenic bacteria (*Bacillus subtilis*, *Escherichia coli*) had no effect on the morphology of the MGT epithelium. However, when ASW containing either *Vibrio parahaemolyticus* or *V. harveyi* (at $10^5$ CFU $ml^{-1}$) was injected into the lumen of the isolated MGTs, the epithelium was missing at the end of the 2 h incubations in 64% of the preparations (see Fig. 3). When epithelial cells were observed, none were elongate. Instead, they were either spherical, lacking microvilli, and often attached by junctions (see Figs. 4 & 5), or necrotic (see Fig 8).

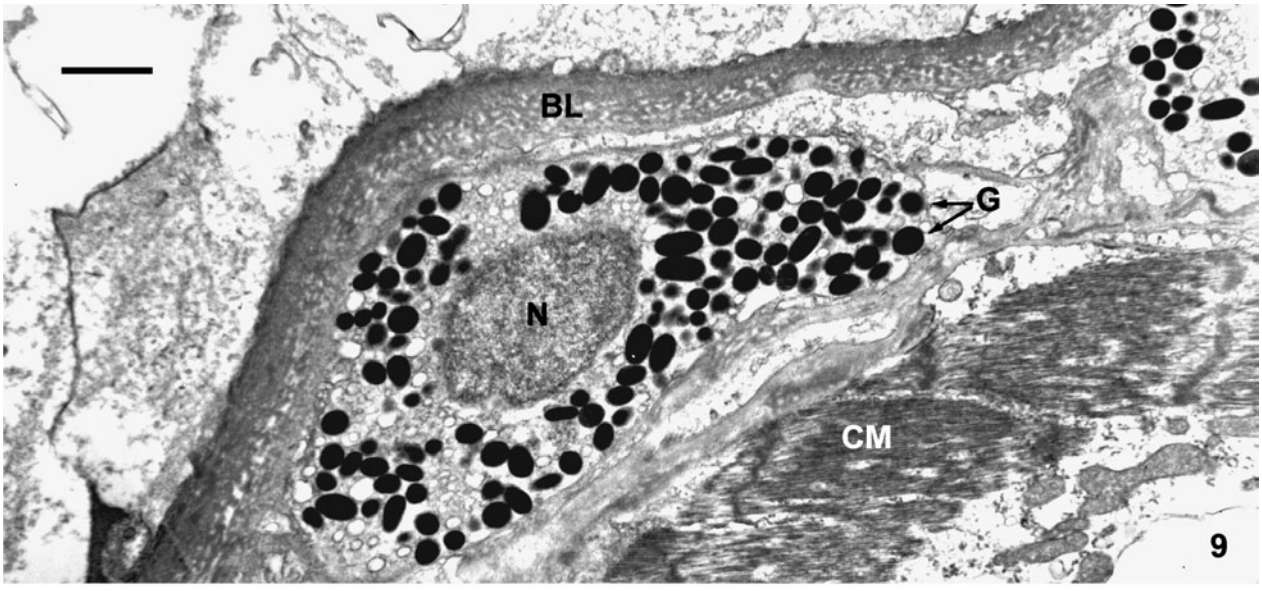
In control preparations where isolated MGTs were injected with sterile ASW, fixed hemocytes associated with the basal lamina were filled with numerous electron-dense ovoid (1.0 × 0.5 µm) granules (Fig. 9) and a smaller number of electron-lucent vesicles (0.4 µm). After 2 h of incubation with *Vibrio parahaemolyticus* or *V. harveyi*, only 18 and 29% of the fixed hemocytes, respectively, retained this morphology. Remaining hemocytes showed signs of moderate (Fig. 10; 31% *V. parahaemolyticus*, 43% *V. harveyi*) or extensive (Fig. 11; 51% *V. parahaemolyticus*, 24% *V. harveyi*) degranulation as well as clumping of heterochromatin along the nuclear envelope. The majority of granulocytes in isolated MGTs injected with either *Bacillus subtilis* or *Escherichia coli* retained the normal morphology (82 and 75% respectively) with no cells showing extensive degranulation.

## CONCLUSIONS

Immersion of the penaeid shrimp *Sicyonia ingentis* in ASW containing either *Vibrio parahaemolyticus* or *V. harveyi* induced the rounding up and detachment of epithelial cells from the basal lamina of the MGT. Seven days after being injected with either species of *Vibrio*, epithelial cell detachment was seen in diseased but not in apparently healthy shrimp. These results suggest that *Vibrio* in the MGT lumen initiate epithelium detachment, whereas *Vibrio* injected into the hemolymph do not affect the MGT unless the infection is not controlled, and septicemia ensues. Detachment of epithelial cells was also observed in isolated pieces of MGT 2 h after these bacteria were injected into their lumens. Epithelial cell detachment was not seen when non-pathogenic bacteria (*Escherichia coli*, *Bacillus subtilis*) or ASW were injected into isolated MGTs for 2 h incubations.

Although there have been numerous studies describing morphological changes to tissues in shrimp infected with *Vibrio* (Lightner & Lewis 1975, Egusa et al. 1988, Aruma 1989, Chen et al. 1992, de la Peña et al. 1993, Jiravanichpaisal & Miyazaki 1994, Sung et al. 1994, Hameed 1995, Lee et al. 1996, Esteve & Herrera 2000, Alday-Sanz et al. 2002), this is the first to demon-

Figs. 1 to 8. *Vibrio parahemolyticus* and *V. harveyi* infecting *Sicyonia ingentis*. Fig. 1. Light microscopy (LM) of the midgut trunk (MGT) wall of a healthy-appearing shrimp 7 d after receiving an injection of *V. parahaemolyticus* into the hemolymph. Wall looked the same as in control shrimp with an epithelium separated from a connective tissue (CT) layer by a wavy basal lamina (BL). Note that apical cytoplasm in the elongate epithelial cells contains granules (G) and spaces (S). A second type of epithelial cells (arrow) was ovoid, lay near the BL, and in some cases contained inclusions. Connective tissue layer contains circular (CM) and longitudinal muscle. Scale bar = 20 µm. Fig. 2. Transmission electron miscroscopy (TEM) of a normal epithelial cell from the MGT. Note the brush border (BB), G and vacuoles (V) in the apical cytoplasm, and abundant smooth endoplasmic reticulum (SER) filling the cytoplasm beneath the nucleus (N). P: nuclear pore particles. Scale bar = 10 µm. Fig. 3. LM of MGT from a healthy-appearing shrimp 7 d after being immersed in ASW containing *V. harveyi*. Note absence of the epithelial layer. Scale bar = 20 µm. Fig. 4. LM of MGT from a healthy-appearing shrimp 7 d after being immersed in ASW containing *V. parahaemolyticus*. Note: epithelium has detached in places from the BL and the cells have rounded up. B: bacteria. Scale bar = 10 µm. Fig. 5. TEM of an epithelial cell from a healthy-appearing shrimp 7 d after being immersed in ASW containing *V. parahaemolyticus*. Note: cell has a circular outline and is still attached to adjacent cells by junctions (J). Although the cells lack microvilli, G and P are still present. Scale bar = 5 µm. Fig. 6. TEM of part of a spherical epithelial cell from a healthy-appearing shrimp 7 d after being immersed in ASW containing *V. parahaemolyticus*. Note: phagocytic vacuole containing a bacterium (B). Scale bar = 1.5 µm. Fig. 7. TEM of a basal cell free in the MGT lumen of a shrimp exposed to *V. harveyi* in ASW for 7 d. Note characteristic large, electron-dense inclusion (I). Scale bar = 5 µm. Fig. 8. TEM of a necrotic epithelial cell from the MGT of a healthy-appearing shrimp exposed to ASW containing *V. harveyi*. Scale bar = 10 µm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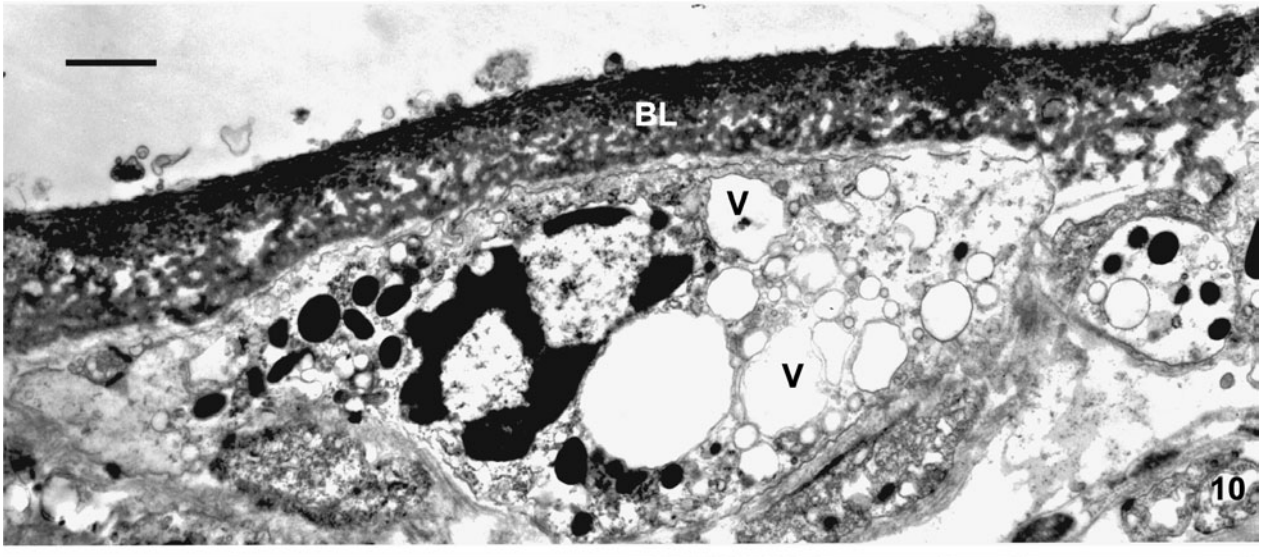
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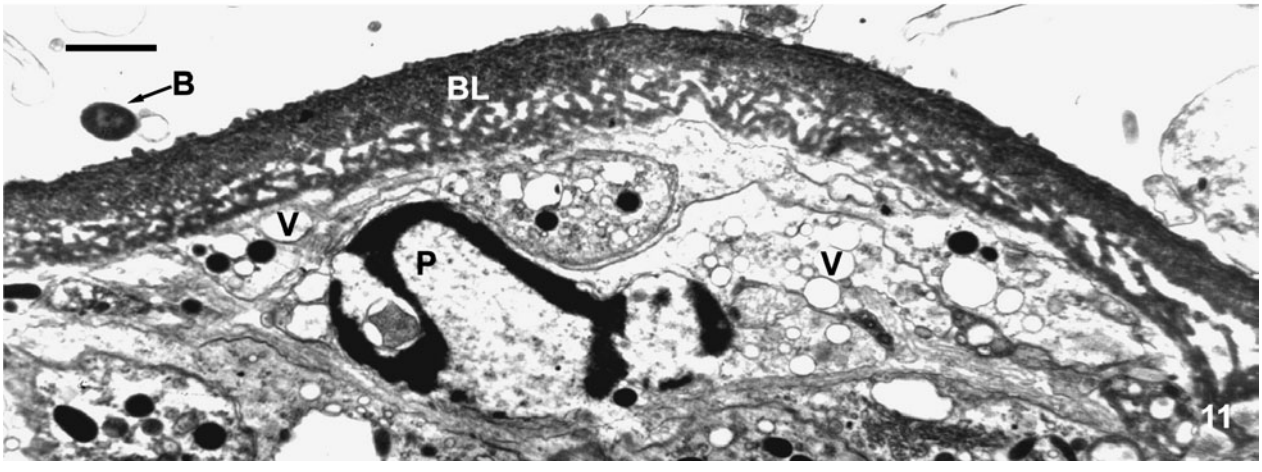
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strate the detachment of the MGT epithelium. In the previous studies, only Chen et al. (1992) described changes to the MGT, i.e. an influx of hemocytes into the MGT of *Penaeus monodon* following exposure to *V. harveyi*. It will be important to determine if the results described in this paper are common in most penaeid shrimp or are specific to *Sicyonia ingentis*. Some researchers, such as Miyawaki et al. (1985), have described the separation of the epithelium (with the cells remaining elongate) as a possible fixation artifact and this may explain the apparent absence of an epithelial layer in the anterior MGT of *P. setiferus* (Lovett & Felder 1990). We suggest that with artifactual detachment of the epithelium, the cells remain elongate and polarized, whereas when detachment is caused by toxins, the cells round up.

Could detachment of the MGT epithelium be due to poor water quality rather than the pathogenic bacteria? In our experiments, shrimp were housed in small (3 l) tubs without aeration, and during the 7 d experiments, the DO levels and pH declined as total ammonia and nitrites rose. Although there is little information on the effect of these changes on shrimp health for cold water species, like *Siyonia ingentis*, water quality in our experiments is within guidelines for warm water species (see Chen & Lei 1990, Boyd 2001a,b), and mortality of our control shrimp was less than 7.5%. It therefore seems probable that the elevated mortality rates in shrimp exposed to *Vibrio* sp. (which are well known to be pathogenic to cultured shrimp) and the detachment of the epithelium from the MGT in surviving shrimp were caused by exposure to these bacteria. Finally, our *in vitro* experiments also demonstrated detachment of the epithelium from the MGT only after exposure to 2 species of *Vibrio*.

The observation that *Vibrio parahaemolyticus* and *V. harveyi* affect the epithelium of the MGT in *Sicyonia ingentis* is not surprising considering the movement of ingested *Vibrio* spp. through the gut tract and previous studies on the effects of their toxins on crustaceans. *V. proteolyticus* releases a toxin that causes disassembly of zonula adherens between adjacent gut cells in the brine shrimp *Artemia*, the subsequent degeneration of these cells, and the penetration of the bacterium deeper into the body (Verschuere et al. 2000). Work on related species of *Vibrio* include identification of toxins in *V. cholerae* that cause cell rounding by affecting the cytoskeleton (Fullner & Mekalanos 2000), a zonula occludens toxin (Wu 1997), a lethal serine protease secreted by *V. alginolyticus* (Chen et al. 2000), and factors that affect hemostasis (Lee et al. 1999). Under natural conditions, shrimp are exposed to multiple microbes, and bacteriophages may affect the toxicity of *V. harveyi* (Ruangpan et al. 1999), and *Bacillus subtilis* may work as a probiotic treatment against pathogenic *Vibrio* (Rengpipat et al. 2003, Vaseeharan & Ramasamy 2003). These interactions could be tested further in the *in vitro* system described in this paper or in recently described primary cell culture systems (Goarant et al. 2000).

A second important finding of this study is that fixed granulocytes associated with the basal lamina in *Sicyonia ingentis* (Martin & Chiu, 2003) degranulated in response to the presence of *Vibrio parahaemolyticus* and *V. harveyi* in the lumen of the MGT. This was seen in the shrimp exposed to either species of *Vibrio* by injection or immersion, and quantified in the isolated MGT experiments. Previously, crustacean hemocytes had been shown to degranulate *in vitro* when exposed to bacteria (Söderhäll et al. 1986), and granules in hemocytes from a variety of decapod crustaceans are known to contain lysosomal enzymes, prophenoloxidase, and antibacterial compounds (Söderhäll & Smith 1983, Martin & Hose 1993, Khoo et al. 1999, Destoumieux et al. 2000, Bartlett et al. 2002). The hemocytes associated with the basal lamina of *S. ingentis* are well placed to fight pathogens passing into the body through the MGT. Longer-term studies are needed to determine if fixed hemocytes and other immune responses (Alday-Sanz et al. 2002, van de Braak et al. 2002) can defend the MGT until the intact epithelial layer can be restored or if death of a shrimp lacking the epithelium is inevitable. Ingested pathogens like *Vibrio* spp., which cause detachment of the epithelium in the MGT, can affect high mortality in *S. ingentis* by eliminating 2 layers that protect the shrimp from infections: the epithelium and the peritrophic membrane it secretes. In addition, loss of the epithelium may affect the regulation of water and ion uptake into the body (Mykles 1977, Neufeld & Cameron 1994).

Figs. 9 to 11. *Sicyonia ingentis*. TEM showing the basal lamina (BL) and associated fixed granulocyte. Fig. 9. BL from an isolated midgut trunk (MGT) injected with artificial seawater (ASW)and incubated in ASW for 2 h. Lumen of the MGT is to the top of the page. Note: granulocyte contains numerous granules (G) and very few smaller electron-lucent vesicles. CM: circular muscle; N: nucleus. Scale bar = 2 µm. Figs. 10 & 11. BL in isolated MGT 2 h after receiving an injection of *Vibrio parahaemolyticus*. Note moderate (Fig. 10) and extensive (Fig. 11) degranulation of the cell, the abundance of exocytotic vesicles (V), and the pycnotic (P) nuclei. B: bacteria. Scale bars = 2 µm

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