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ULTRASTRUCTURE OF LIGHT ORGANS OF LOLIGINID SQUIDS AND THEIR BACTERIAL SYMBIONTS: A NOVEL MODEL SYSTEM FOR THE STUDY OF MARINE SYMBIOSES

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Abstract

The class Cephalopoda (Phylum Mollusca), encompassing squids and octopuses, contains multiple species that are characterized by the presence of specialized organs known to emit light. These complex organs have a variety of morphological characteristics ranging from groups of simple, light-producing cells, to highly specialized organs (light organs) with cells surrounded by reflectors, lenses, light guides, color filters, and muscles. Bacteriogenic light organs have been well characterized in sepiolid squids, but a number of species in the family Loliginidae are also known to contain bacteriogenic light organs. Interest in loliginid light organ structure has recently arisen because of their potential as ecological niches for *Vibrio harveyi*, a pathogenic marine bacterium. This also implies the importance of loliginid light organs as reservoirs for *V. harveyi* persistence in the ocean. The present study utilized transmission and scanning electron microscopy to characterize the morphology of loliginid light organs and determined the location of bacterial symbiont cells within the tissue. It was determined that the rod-shaped loliginid symbionts lack flagella, as similarly observed in other light organ-associated bacteria. Also, the interaction of individual cells to light organ tissue is not as defined as reported for other squid-*Vibrio* systems. In addition, SEM observations show the presence of two pores leading to the bacterial chamber. Data presented here offer support for the hypothesis of environmental transfer of bacterial symbionts in loliginid squids.

Keywords

CEPHALOPODA; LIGHT ORGAN; VIBRIO; SYMBIOSIS; *UROTEUTHIS*; LOLIGINIDAE

INTRODUCTION

Several families within the class Cephalopoda (Mollusca) are characterized by the presence of light-producing organs, also known as photophores (Young 1972). Cephalopod photophores have a wide range of morphological features ranging from simple groups of photogenic cells, to organs with photogenic cells surrounded by reflectors, lenses, light guides, color filters, and muscles. These luminescent organs vary from very small, simple photophores (less than 0.2 mm in diameter) such as those of the mesopelagic squid *Abralia trigonura* (Young & Arnold 1982), to larger, more complex organs from squid of the family Sepiolidae (McFall-Ngai & Montgomery 1990). Complex photophores, also referred to as light organs, are found in the widely studied sepiolid squid *Euprymna scolopes* (McFall-

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Ngai & Montgomery 1990, Nishiguchi *et al.* 2004), as well as a number of other species in the families Sepiolidae and Loliginidae (Naef 1912a). Light organs found in sepiolids are oftentimes comparable in complexity to the compound eyes of many animals, including cephalopods themselves.

Light organs are able to readily adjust color, intensity, and angular distribution of light produced from within (Ferguson & Messenger 1991). In oceanic cephalopods, photophores emit intrinsic luminescence (autogenic) with light emanating from their own photocytes (Pringgenies & Jorgensen 1994). These cells are also able to emit different spectra of light (Herring *et al.* 1992). In contrast, photophores of most neritic cephalopods have extrinsic luminescence (bacteriogenic), with light produced by bacteria housed in a specialized light organ complex within the mantle cavity of their host (Naef 1912b, McFall-Ngai & Montgomery 1990, McFall-Ngai & Ruby 1991). Studies observing *E. scolopes* demonstrate that down-welling light intensity can be matched by the squids luminescence in a behavior termed counterillumination (Jones & Nishiguchi 2004). This confirms the hypothesis proposed by Young & Roper (1977) that production of light ventral to the squid mantle cavity is used for counter-shading down-welling light from the moon. Light organs of similar morphology are also found in oceanic teuthid squids (epipelagic and mesopelagic), such as *Chiroteuthis* spp., *Chiropsis* spp. and *Taningia* spp. However, according to Herring (1977), these anal light organs never house luminous bacteria.

Within the family Loliginidae, a number of species are known to possess bacteriogenic light organs (Alexeyev 1992). Loliginids are neritic, muscular squids, ranging from 3 to 100 cm in total body length. In contrast to sepiolids, loliginids have a gladius that extends along the mantle. They are also characterized by the existence of a branchial canal in the gill (Vecchione 2008). Light organs in these squids are bilobed and situated on the ventral side of the ink sac near the anus. They are known to harbor luminous bacteria from the family Vibrionaceae, which have been characterized both by molecular methods (Guerrero-Ferreira & Nishiguchi 2009, 2007) and biochemical and morphological assays (Guerrero-Ferreira *et al.* in review). These studies have provided evidence confirming that the pathogenic marine bacterium *V. harveyi* colonizes light organs in loliginid squid, and posed questions regarding the role of loliginid light organs as a reservoir for *V. harveyi* persistence in the ocean. However, the morphology of loliginid light organs has not been well described in the scientific literature, most likely because their potential as *V. harveyi* reservoir was not entirely understood. Thus, the objective of this study was to describe the anatomy and ultrastructure of loliginid light organs using transmission electron microscopy and scanning electron microscopy techniques. In addition, the presence of symbiotic bacteria in the light organ would clarify whether loliginid light organs resembled those of sepiolid squids. The results of this study describe in detail the morphology of light organs in loliginid squids from the genus *Uroteuthis*, as well as the location of symbiotic bacteria existing inside the light organ complex.

MATERIALS AND METHODS

Sample collection and fixation

Specimens (~10 of each species) of the loliginid squids *Uroteuthis chinensis* and *Uroteuthis etheridge* were collected off the coasts of Cairns and Townsville (Australia) respectively, by trawl netting at daytime. Light organs were dissected from the mantle cavity and immediately fixed in 4 % paraformaldehyde and 0.5 % glutaraldehyde (Electron Microscopy Sciences, Hartfield, PA, USA) in 0.1 M marine phosphate buffer (MPB, 0.66 M NaCl, 0.2 M phosphate buffer, pH 7.2). After fixation, light organs were kept at 4°C until processed for light microscopy and transmission electron microscopy. Tissue samples were simultaneously collected for confirmation of squid species identity by cytochrome c oxidase

gene (COI) sequencing using universal primers (Folmer 1994) under the following amplification conditions: initial denaturation at 94°C followed by 25 cycles of 94°C for 50 s, 50°C for 75 s, and 72°C for 90 s. Sequences were compared with the National Center for Biotechnology Information (NCBI) database using BLAST 2.2.11 (Basic Local Alignment Search Tool, NCBI, NLM, NIH, Bethesda, MD) to confirm species identity.

Sample preparation for scanning and transmission electron and light microscopy

Scanning electron micrographs were directly taken using a Hitachi TM-1000 tabletop microscope, without further sample preparation. For preparation of light organs for transmission electron microscopy and light microscopy, samples were rinsed with 0.1 M MPB and post-fixed with 0.5 % osmium tetroxide in 0.1 M MPB for 1 hour, stained with % tannic acid (in dH₂O₂) for 1.5 hours, rinsed with water and subsequently dehydrated using a 50–100 % ethyl alcohol gradient. Light organs were incubated for 1 hour in each ethanol solution and finally incubated in 100 % ethyl alcohol overnight. Samples were then infiltrated with 3:1 ethanol: Spurr's resin for 1 hour (Electron Microscopy Sciences, Hatfield, PA, USA), and then transferred to a 1:1 ethanol: Spurr's solution overnight. A 1:3 ethanol: Spurr's media was used to infiltrate the samples on the second day overnight, and then finally transferred to 100 % Spurr's resin overnight for the last infiltration. All infiltration steps were completed on a rotating table at room temperature (~22°C). Tissue was embedded in Spurr's resin in plastic molds. Resin was polymerized at 68°C overnight.

Thick Sectioning and imaging

Polymerized blocks were trimmed and sectioned using a Leica UC6 Ultramicrotome (Leica, Bannockburn, IL, USA). Thick sections (2 μm) were collected onto droplets of double distilled water on ethanol-washed glass slides. Slides were dried on a heat block for approximately five minutes to guarantee complete attachment of the sections to the slide. Subsequently, sections were broadly covered with 1 % toluidine blue epoxy stain (EMS, Hatfield, PA) for 1 minute. Sections were then extensively washed with double distilled water to ensure that all excess stain was removed from the slides. Samples were then viewed with a Nikon E800 upright epifluorescence microscope (Nikon Inc., Melville, NY).

Thin sectioning and imaging

Thin sections were collected onto a 300 mesh (squared) copper grid (Electron Microscopy Sciences, Hatfield, PA, USA) previously washed with a mild solution of hydrochloric acid. Subsequently, sections were positively stained with 2 % uranyl acetate (EMS, Hatfield, PA) for 10 min. and then with 1 % lead citrate (Polysciences Inc., Warrington, PA) for 10 min. Sections were washed with distilled water for one minute between stains. Stained samples were viewed through a Hitachi H7650 Transmission Electron Microscope (Hitachi, Schaumburg, IL, USA) at an accelerating voltage of 80kV.

RESULTS

Specimens were identified through sequencing of the cytochrome *c* oxidase subunit one gene (COI) and when compared with the NCBI database using BLAST 2.2.11, were confirmed as two species of the family Loliginidae, *Uroteuthis chinensis* and *Uroteuthis etheridge*.

Light organs in these species of squid are located on the ventral face of the ink sac. With respect to the size of *U. chinensis* and *U. etheridge* specimens (approximately 30 cm of dorsal mantle length), the light organs are considerably smaller when compared to the homologous structures in members of the family Sepioidae, such as the Hawaiian bobtail squid *Euprymna scolopes* (McFall-Ngai & Montgomery 1990). Ventral dissection of the

squid *U. etheridge* reveals the relatively small, bilobed light organ situated within the mantle cavity (Fig. 1).

A histological cross-section of one lobe of the light organ reveals the existence of labyrinths of crypts where bacteria are housed (Fig. 2). Light microscopy also illustrates the location of the reflector tissue (RT) with respect to the light organ crypts within the bacterial chamber (BC). These observations confirmed results made by Pringgenies & Jorgensen (1994), which illustrated a layer of tissue representing a reflector that was immediately in contact with the bacterial chamber (Fig. 2). Their results were also similar to earlier descriptions of light organ morphology in sepiolid squids (summarized in Nyholm & Nishiguchi 2008), where bacterial crypts are also surrounded by reflector tissue (dorsal) as well as a lens (ventral).

By completing transmission electron microscopy of the reflector tissue area, it was possible to confirm the presence of flat, structural platelets or lamellae organized in alternate layers (Fig. 3). Secreted ink granules from the ink sac are also clearly visible in the micrograph. The lamellae have a constant width of approximately 100 nm, but their length varies considerably. These structures have been studied in *E. scolopes* and have been found to contain proteins called reflectins (Crookes *et al.* 2004) which makes them an exception to most aquatic animals that contain reflectors made of crystals of purinic nucleotides, specially guanine and hypoxanthine (Denton & Land 1971).

Uroteuthis etheridge light organs (Fig. 1) are known to house luminescent bacteria of the family Vibrionaceae (Guerrero-Ferreira & Nishiguchi 2007). Because recent studies in our lab have demonstrated that at least one member of the bacterial symbiotic population within these organs is the marine pathogen *Vibrio harveyi*, it was of great interest to structurally describe the state of the interaction between the two partners. A particular goal of this study was to determine where in the light organ crypts these bacterial symbionts reside, as well as to gather information regarding the symbiotic morphology of the bacteria, particularly the presence of bacterial cell appendages which might facilitate adhesion to squid tissues.

Figure 4 portrays the location of the bacterial cells when associated with *U. chinensis* squid tissue. The most noticeable feature of the symbionts is the absence of flagella. Even though bacteria from the family Vibrionaceae are known to possess either monotrichous or peritrichous flagella (Baumann 1984), bacterial cells in *U. chinensis* light organ lack flagella. Previous research studying the association between *E. scolopes* and *V. fischeri* has also shown that loss of flagella is a characteristic process that occurs upon establishment of the association (Ruby & Asato 1993).

The morphology of the bacteria colonizing light organs was the characteristic rod shape of members in the family Vibrionaceae. Figure 5 illustrates one of these symbionts and clearly defines the cell wall (CW) and cytoplasm (CY) of the bacterium. In general, transmission electron microscopy of the bacteria reveals they have the ovoid or oblong shape, characteristic of vibrios. Their sizes vary, with smaller bacteria measuring approximately 0.5 μm in length, whereas the largest having dimensions of up to 3.0 μm in length. Symbiotic bacteria residing in the light organ exhibit a dense cytoplasm, with the presence of vacuoles approximately 100 nm in diameter (Fig. 6). In addition, cell appendage pili are present that might allow for bacterial attachment to squid tissue, as it is known to occur in *V. fischeri* bacteria colonizing *E. scolopes* (Nair and Nishiguchi 2009, McFall-Ngai & Ruby 1991).

Scanning electron microscopy was also used to determine the presence of pores that are necessary for initial colonization by symbionts infecting the crypt region of the light organ. In the *E. scolopes* light organ complex, three pores exist in the juvenile squid after hatching. Upon successful colonization by their symbiotic partner *V. fischeri*, developmental changes occur leading to the formation of a single pore that allows for the dial cycle of bacterial

venting that occurs throughout the life of the mollusc (Foster *et al.* 2002). Interestingly, SEM illustrates that *U. chinensis* has a light organ with two pores leading into the bacterial chamber in the adult squid (Fig. 7). The size of these pores is approximately 5 μm , which is similar to the size reported by Montgomery and McFall-Ngai in sepiolid squids (1993). Previous results have shown that pore number varies among sepiolid squids; the genus *Sepiolo* has 4 pores on each light organ lobe prior to infection by their *Vibrio* bacteria (Foster *et al.* 2002). The functional advantage for the occurrence of two pores in adult *U. chinensis* is still unclear, but their presence may be required for similar venting behaviors to those found occurring in adult sepiolids.

DISCUSSION

This study provides evidence that physical interactions between bacterial cells and loliginid squid light organs is somewhat sporadic. Electron micrographs illustrate an infrequent distribution of bacterial cells with respect to each other (*i.e.*, no aggregation) and with the squid host light organ tissue. This is in agreement to the description given by Pringgenies and Jorgensen (1994). According to their study, scarcity of bacterial cells was due to the crypts in the light organ being used exclusively for expulsion of excretion products from both symbiont and host. However, it is currently understood that the symbiont community in sepiolid light organs has a diurnal fluctuation (McFall-Ngai & Ruby 1991, Ruby & McFall-Ngai 1992), which may account for the low density of bacteria observed in the micrographs presented here. In addition, absence of a strong physical connection between host cells and symbionts may account for the lack of species-specificity found in loliginid light organ symbiosis and may explain the diversity of bacterial genotypes found in their crypts. Recent studies have established that more than one species of Vibrionaceae bacteria colonize light organs of squids in the family Loliginidae (Guerrero-Ferreira & Nishiguchi 2007). Further studies would need to address whether light organ venting occurs with the onset of dawn in loliginid squids, and if the lack of pili corresponds to a less specific interaction on the cellular level.

This is also true in the case of light organs in the leiognathid fish *Nuchequula nuchalis*, where *Vibrio harveyi* was recently found as a member of the bacterial community (Dunlap *et al.* 2008). In this species, more than two distinct ecological variants of *Photobacterium leiognathi* were also found colonizing the light organ tissue. Transmission electron micrographs of *N. nuchalis* show a similar scattered distribution of symbionts within the light organ tubules, which supports the aforementioned idea of a relationship between lack of specificity and limited physical attachment to host tissue.

In addition, vacuole-like, refractive granules were observed within the symbionts in quantities varying from one to five. These granules have been previously proposed to be poly- β -hydroxybutyrate (PHB) (Dunlap *et al.* 2008), a compound that is produced in *V. harveyi* cells at high density in the presence of endogenous *lux* autoinducer, *n*-(3-D-hydroxybutanoyl) homoserine lactone (Sun *et al.* 1994). This restricts the production of PHB to *V. harveyi* strains living under confined conditions (such as the light organ of loliginid squids) which might account for the classification of *V. harveyi* cells as PHB negative (Baumann 1981). Because of this, it is possible that PHB containing bacteria living in conditions such as those of fish and squid light organs were misclassified as species different than *V. harveyi*, as observed by Miyamoto *et al.* (1998).

Tissue organization in loliginid light organs observed through TEM resembles the structure of sepiolid light organs with reflector and lens tissue surrounding the chambers where the bacterial symbionts are located (McFall-Ngai & Montgomery 1990). In sepiolid squids, the arrangement of these various tissue types allows the animal to utilize light produced by the

bacteria in a behavior known as counterillumination (Jones & Nishiguchi 2004). Our results present morphological evidence suggesting that *Uroteuthis* spp. use their association with Vibrionaceae bacteria for the same purpose. Further behavioral studies investigating light production under controlled down welling light intensity will contribute to our understanding of the evolutionary significance of this association for loliginids. Additionally, temporal studies with light organs from squids collected at several times of the day would help determine whether low bacterial densities within light organ crypts are due to diurnal variation in bacterial populations in loliginid squids.

Moreover, we can argue that association of loliginid squids with their bacterial symbionts is currently in an ancestral state. Nishiguchi *et al.* (2004) presented a hypothesis for the origin of bacteriogenic light organs from the accessory nidamental gland (ANG) complex of sepiolid squids. This organ complex has more than 20 strains of bacteria present that are acquired from the surrounding seawater environment (Kaufman *et al.* 1998). If ANG in loliginid squids contributes to the symbiont population in loliginid light organs, this may explain the diversity of bacteria found in these complexes and the lack of a strong physical association between partners (Guerrero-Ferreira & Nishiguchi 2007). Strong selection by the host for particular strains or species of *Vibrio* bacteria will need to occur in order to accommodate strain specificity and cospeciation (Nishiguchi *et al.* 1998, Nishiguchi 2002, Nyholm & Nishiguchi 2008). Likewise, these environmentally transmitted symbionts that colonize loliginid light organs will need to be successful in their free-living stages in order to out-compete other bacteria for their niche in this mutualism. Trade-offs between these two life history strategies appear to be the ultimate driving forces for the evolution of this dynamic and evolutionary significant symbiosis; whether multiple or single strains can coexist needs to be taken into careful consideration when contemplating the origin of bacteriogenic light organs (Nishiguchi *et al.* 2008, Nyholm & Nishiguchi 2008).

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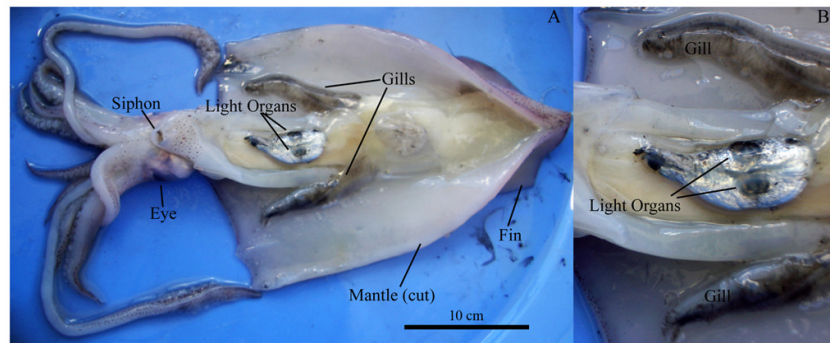


Fig. 1. Ventral view of the mantle cavity of an adult *Uroteuthis etheridge*. A: Whole specimen B: closeup of the light organs. Dorsal mantle length: 30 cm.

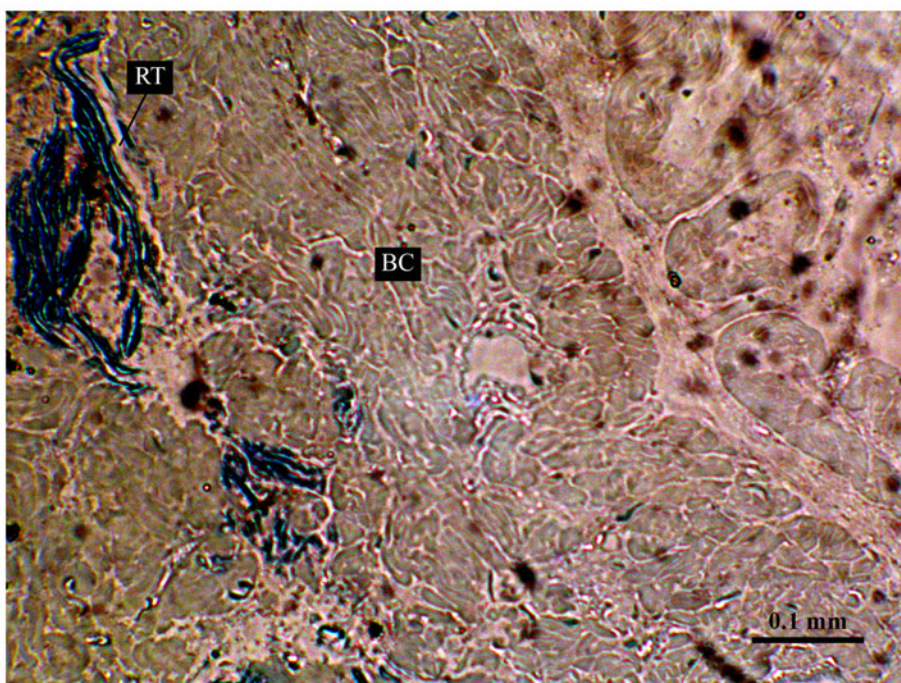


Fig. 2. Light micrograph of a section showing the bacterial chamber (BC) of the loliginid squid *Uroteuthis etheridge*. Highly electron dense material corresponds to the reflector tissue (RT). Scale bar: 0.1 mm

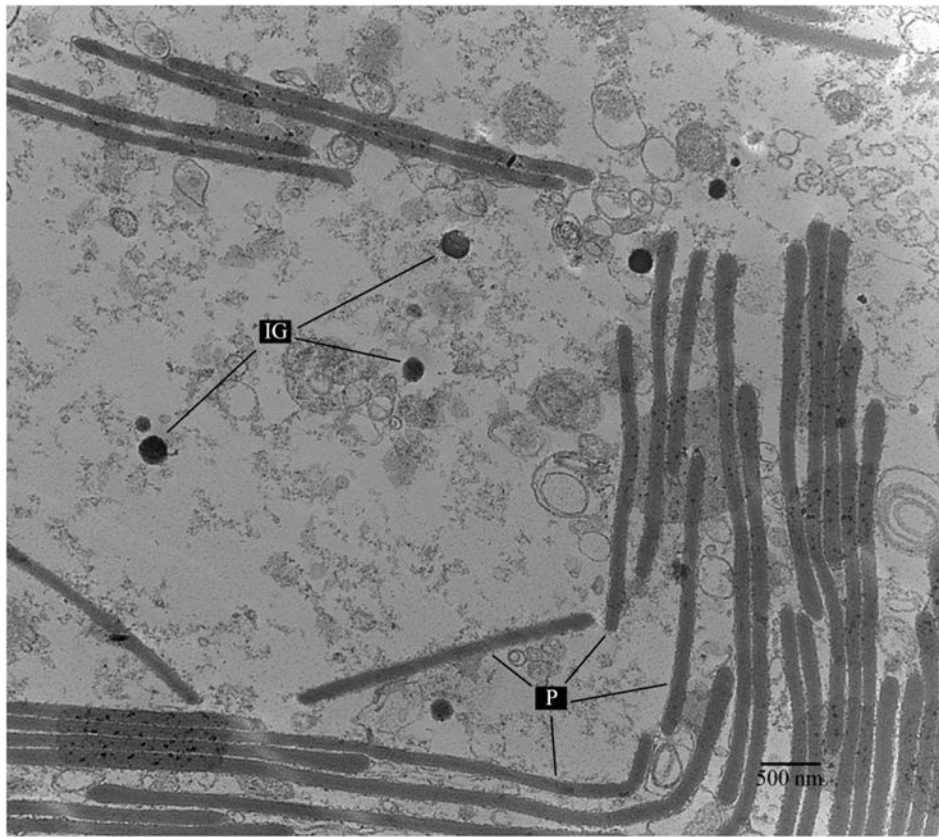


Fig. 3. TEM image of a region of *U. etheridge* light organs. Ink granules (IG) and platelets (P) are shown in the field. Scale bar: 500 nm.

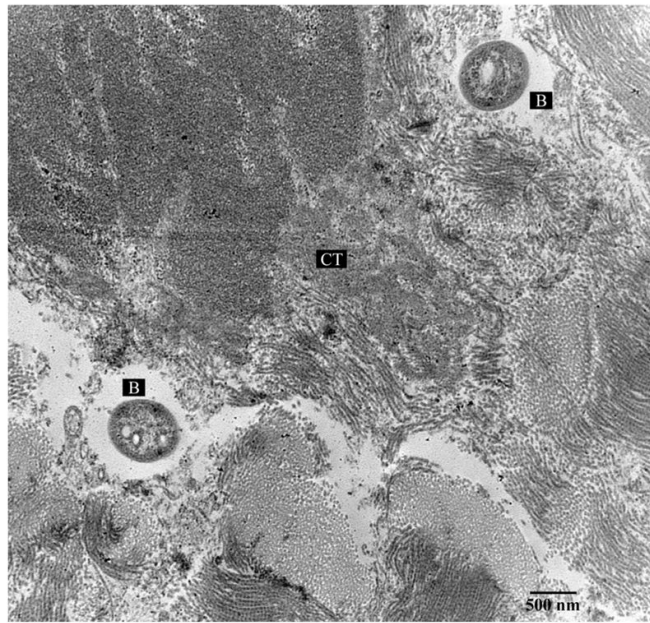


Fig. 4. *Vibrio* bacteria (B) associated to *Uroteuthis chinensis* squid tissue (CT for connective tissue). Cells are located inside crypt spaces. Scale bar: 500 nm.

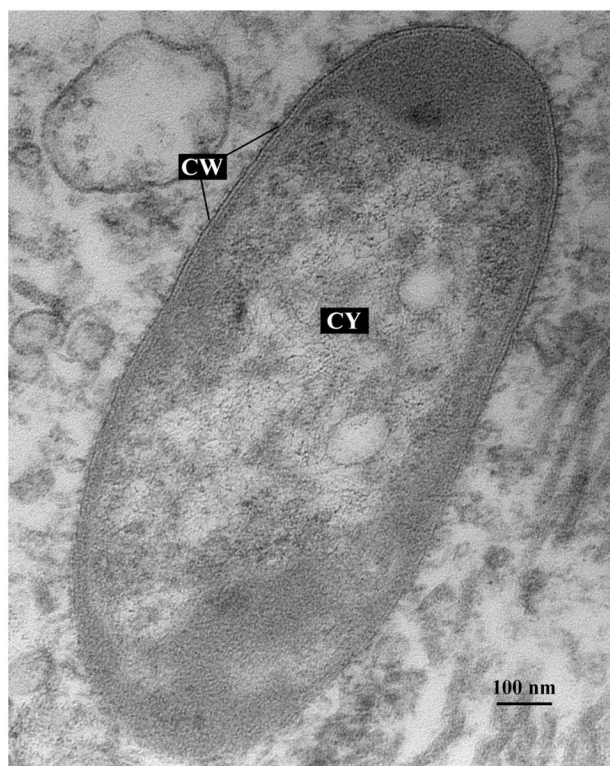


Fig. 5. Bacterial symbiont within the crypt region of *Uroteuthis etheridge* light organ. CW: Cell wall. CY: Cytoplasm. Scale bar: 100 nm.

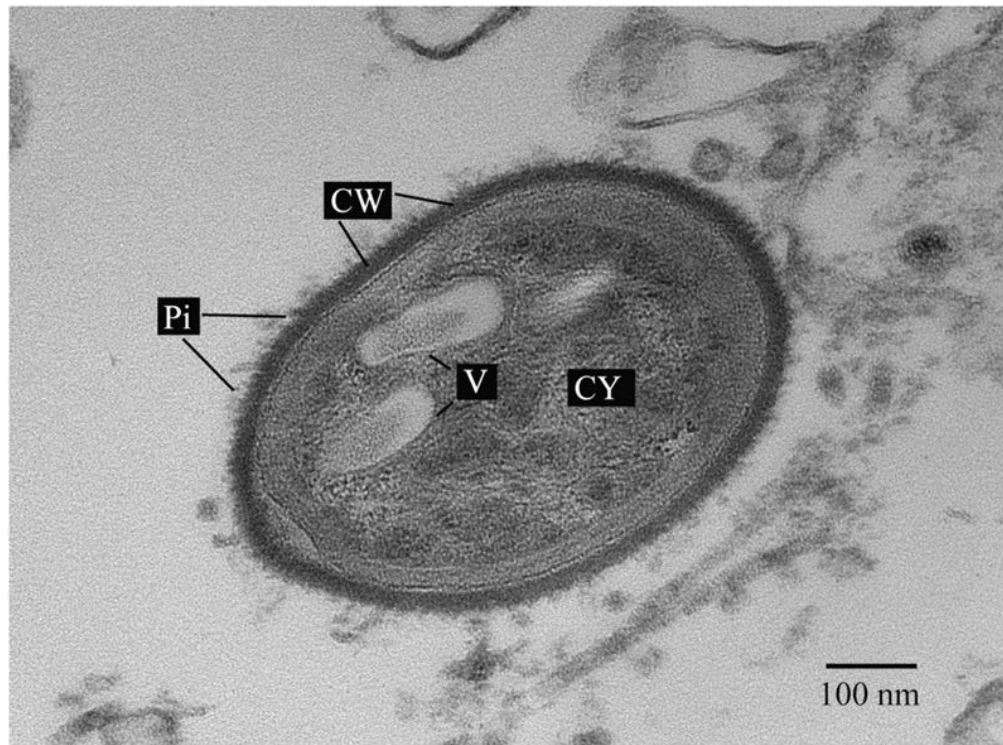


Fig. 6. Bacterial cell associated to the squid *Uroteuthis etheridge* with pili (P) and vacuoles (V) present. Scale bar: 100 nm.

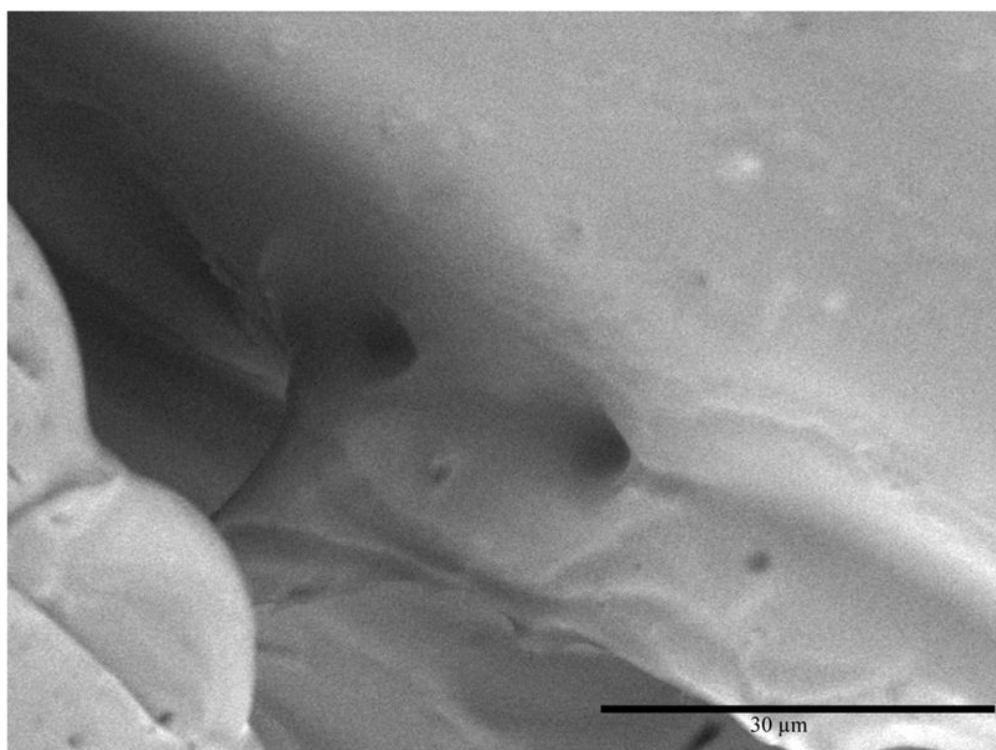


Fig. 7. Scanning electron micrograph of *Uroteuthis chinensis* light organ showing two pores leading into the bacterial chamber. Scale bar: 30 μm.