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BACTERIAL SYMBIOSIS IN *SYSSITOMYA POURTALESIANA* OLIVER, 2012 (GALEOMMATOIDEA: MONTACUTIDAE), A BIVALVE COMMENSAL WITH THE DEEP-SEA ECHINOID *POURTALESIA*

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ABSTRACT

For the first time, bacterial symbiosis is recognized in the bivalve family Montacutidae of the superfamily Galeommatoidea. The ctenidial filaments of *Syssitomya pourtalesiana* Oliver, 2012 are extended abfrontally and a dense layer of bacteriocyte cells cover the entire surface behind a narrow ciliated frontal zone. The bacteria are extracellular and held within a matrix of epithelial extensions and microvilli. There is no cuticular layer (glycocalyx) covering the bacteria as in many thyasirid symbioses. The bacteriocytes hold more than one morphotype of bacteria, but bacilli, 1–3 μm in length, dominate. Scanning electron microscopy observations show a surface mat of filamentous bacteria over the extreme abfrontal surfaces. Filter feeding was confirmed by the presence of food particles in the stomach and the bivalve is presumed to be mixotrophic. *Syssitomya* is commensal and lives attached to the anal spines of the deep-sea echinoid *Pourtalesia*. In this position, echinoid feeding currents and echinoid faecal material may supply the bacteria with a variety of nutrient materials including dissolved organic matter.

INTRODUCTION

Within the Bivalvia, bacterial chemosymbiosis has now been recognized in six families: Solemyidae, Mytilidae, Lucinidae, Thyasiridae, Vesicomyidae (Taylor & Glover, 2010) and most recently the Nucinellidae (Oliver & Taylor, 2012). In these families, the bacteria are housed in the ctenidia and are mostly thiotrophic and/or methanotrophic (Fisher, 1990; Distel, 1998; Cavanaugh *et al.*, 2006; Dubilier, Bergin & Lott, 2008; Taylor & Glover, 2010). In the Solemyidae, Lucinidae, Vesicomyidae and Nucinellidae, where all studied species possess symbionts, the relationship is probably obligate for the bivalve (Stewart & Cavanaugh, 2006; Taylor & Glover, 2006; Krylova & Sahling, 2010; Oliver & Taylor, 2012). For the Mytilidae, only the Bathymodiolinae clade (*Bathymodiolus*, *Tamu*, *Gigantidas*, *Idas* and *Adipicola*) has been shown to possess symbionts (DeChaine & Cavanaugh, 2005; Southward, 2008; Duperron, 2010), and in the Thyasiridae some species have abundant symbionts, while others have few or no bacteria (Southward, 1986; Dufour, 2005).

In most chemosymbiotic taxa, the ctenidial filaments are typically thickened, fleshy in appearance and opaque white, cream, pink, red or brown-purple in colour, as seen in many of the thyasirids reported upon by Southward (1986). In 1987, one of us (ECS) observed such a ctenidium in small bivalves attached to the irregular echinoid *Pourtalesia miranda* collected from depths of 1500 to 2000 m in the Bay of Biscay, and tentatively identified as '*Lepton*', a genus of the Galeommatoidea. Following a taxonomic review by Oliver (2012), this associate of *Pourtalesia miranda* Agassiz, 1869 and *P. jeffreysi* Wyville Thomson, 1873 was given the name *Syssitomya pourtalesiana* Oliver, 2012 and attributed to the galeommatoidean family Montacutidae.

This paper describes in detail the functional morphology of *Syssitomya*, comparing it with other bivalves that harbour symbiotic bacteria. Attempts to acquire specimens suitable for molecular analysis have so far been unsuccessful, but we hope that this discovery of an unexpected and novel relationship will invite fresh investigations.

MATERIAL AND METHODS

Collection data

The specimens from the southern Bay of Biscay, including the individual bivalve used for transmission electron microscopy (TEM), came from RV *Frederick Russell* cruise 87/13A, Station 45 (43°46′N 03°47′W, 1 June 1987, depth 1786 m, coll. E.C. &

A.J. Southward). The specimens used for gross morphology and the SEM study came from the Norwegian Sea (Ormen Lange gas field, off Sør-Trøndelag, 63°47′N 03°35′E, 815–925 m, 2009, Swedish Museum of Natural History). A further eight specimens from RRS *Challenger* station ES137 in the Rockall Trough (54°40′N 12°19′W, 2900 m, 22 February 1978, National Museum of Wales) were examined and these were found to be gravid. These specimens are likely to be part of those recorded from the Rockall Trough by Gage *et al.* (1985).

Shells

No shells remain from the 1987 specimen studied; all descriptions are based on the Norwegian material. Images were made using a computer-aided digital photographic system powered by AutoMontage™ and by scanning electron microscopy (SEM) with a JEOL Neoscope scanning electron microscope.

Gross anatomy

The Norwegian specimens had previously been fixed in formaldehyde, without opening the shells, before being stained with rose Bengal and stored in 70% ethanol. The shell was removed in dilute hydrochloric acid and the anatomy was viewed and dissected from the left side. Images were made using a computer-aided digital photographic system powered by AutoMontage™.

Critical-point drying and SEM examination

Specimens for SEM examination were decalcified in dilute hydrochloric acid and the ctenidia excised and sliced transversely with a razor blade into several pieces. The tissue pieces were then dehydrated in 100% ethanol and critical-point dried. The specimens were then mounted on stubs, sputter-coated with gold and examined using a JEOL Neoscope scanning electron microscope. The whole stomach was dissected from the body, critical-point dried and then cracked open to reveal the stomach contents before coating with gold for examination.

TEM examination

A specimen of *Syssitomya pourtalesiana* taken from a Biscay *Pourtalesia miranda* was prepared as follows. The two ctenidia were removed from the fresh specimen and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, with 14% sucrose. They were post-fixed in 1% OsO_4 in phosphate buffer, dehydrated in an ethanol series and embedded in Spurr's resin. One ctenidium was sectioned transversely, the other longitudinally. Thin sections were mounted on grids and stained with uranyl acetate and Reynolds' lead citrate for electron microscopy. They were examined in a Phillips 300 electron microscope.

Deep-sea photographs

Sea-floor photographs and benthic samples were taken by A.J. and E.C. Southward in 1974, 1975 and 1976, in the Biscay habitat of *Pourtalesia miranda* (43°45′N, 3°45′W), on cruises of RV *Sarsia*. The suspended camera system (Southward *et al.*, 1976) employed two cameras in stereoscopic mode. Comparative benthos samples were taken by dredge and Agassiz trawl.

RESULTS

Gross morphology

Shell (Fig. 1A, B): The shell was fully illustrated by Oliver (2012) from the material used here and by Bouchet & Warén (1979) from samples collected in the Norwegian Sea at a depth of 2930–2960 m. The maximum recorded length is 4.2 mm. The shell is thin and semi-transparent, its shape tumid, equivalve and inequilateral, with beaks slightly behind the midline. The outline is obliquely subovate, the anterior distinctly more expanded than posterior and the lunule area is depressed. Sculpture is weak, consisting of fine commarginal ridges. The hinge plate is weak; the right valve has a single cardinal peg, anterior to which is a shallow excavation; the left valve is without cardinal teeth and the lunule margin has a projecting flange. The ligament is deeply sunken on a shallow resilifer entirely behind the beaks. Internally the muscle scars are weak and the pallial line entire. The larval shell is small, prodissoconch I measuring 148–150 μm in breadth and prodissoconch II 390–407 μm ($n = 3$).

Anatomy (Fig. 1C–G): The mantle edge is fused from approximately the midline to the posterior adductor, except for a small posterior exhalent opening (exa). Anteriorly there is a large pedal gape (pg) (with inhalant aperture, ia) with its anterior part bordered by a prominent frill along the inner mantle edge [me(f)]. The gill axis is fused to the mantle edge (ct/me) to form a small suprabranchial chamber. The anterior and posterior adductor muscles are of equal size (aa and pa) and roughly oval in outline. There is a pair of anterior pedis retractor muscles (apr) inserted dorsal to the anterior adductor; the posterior retractor muscles (ppr) are inserted dorsal to the posterior adductor muscle. The foot (f) has a large rounded 'toe' and a smaller but prominent 'heel'. A byssus (by), consisting of a basal strap that subdivides into numerous threads, emanates from the byssus gland opening medially on the sole of the foot; it can be seen attached to an echinoid spine in Figure 1C. The ctenidium is fleshy (Fig. 1D) and consists of a single demibranch, partially reflected, with filaments of a laminar form. Labial palps (lp) are present, short but deep, and have the appearance of protruding lips with few sorting ridges. The alimentary system is complete: the stomach (s) is large, situated anteriorly, close to the dorsal surface of the visceral mass; the style sac (ss) is separate, extending from the right side of the stomach; the midgut exits posteriorly and is contiguous with the hind gut (hg), not looped before passing through the pericardium and over the posterior adductor muscle to the anus. Digestive diverticula (dg) are confined to the surrounds of the stomach. Gonads (gd) with developing eggs take up much of the visceral mass and extend into the foot. A pair of dome-shaped structures (dss) lies on the ventral face of the posterior adductor over the visceral gangli; their function is unknown, but they lie in the position of the seminal receptacles seen in other montacutids (Jespersen, Lützen & Oliver, 2007).

Specimens collected from the Rockall Trough were gravid, with released larvae held between the descending and ascending parts of the gill filaments (Fig. 1F) and in the suprabranchial chamber. The larvae were numerous and averaged 112 μm in diameter (Fig. 1G).

SEM study of ctenidium

The terminology associated with modified gills varies slightly between authors; here we follow Dufour (2005), while noting that her bacteriocyte zone corresponds to the lateral zone of Passos, Meserani & Gros (2007).

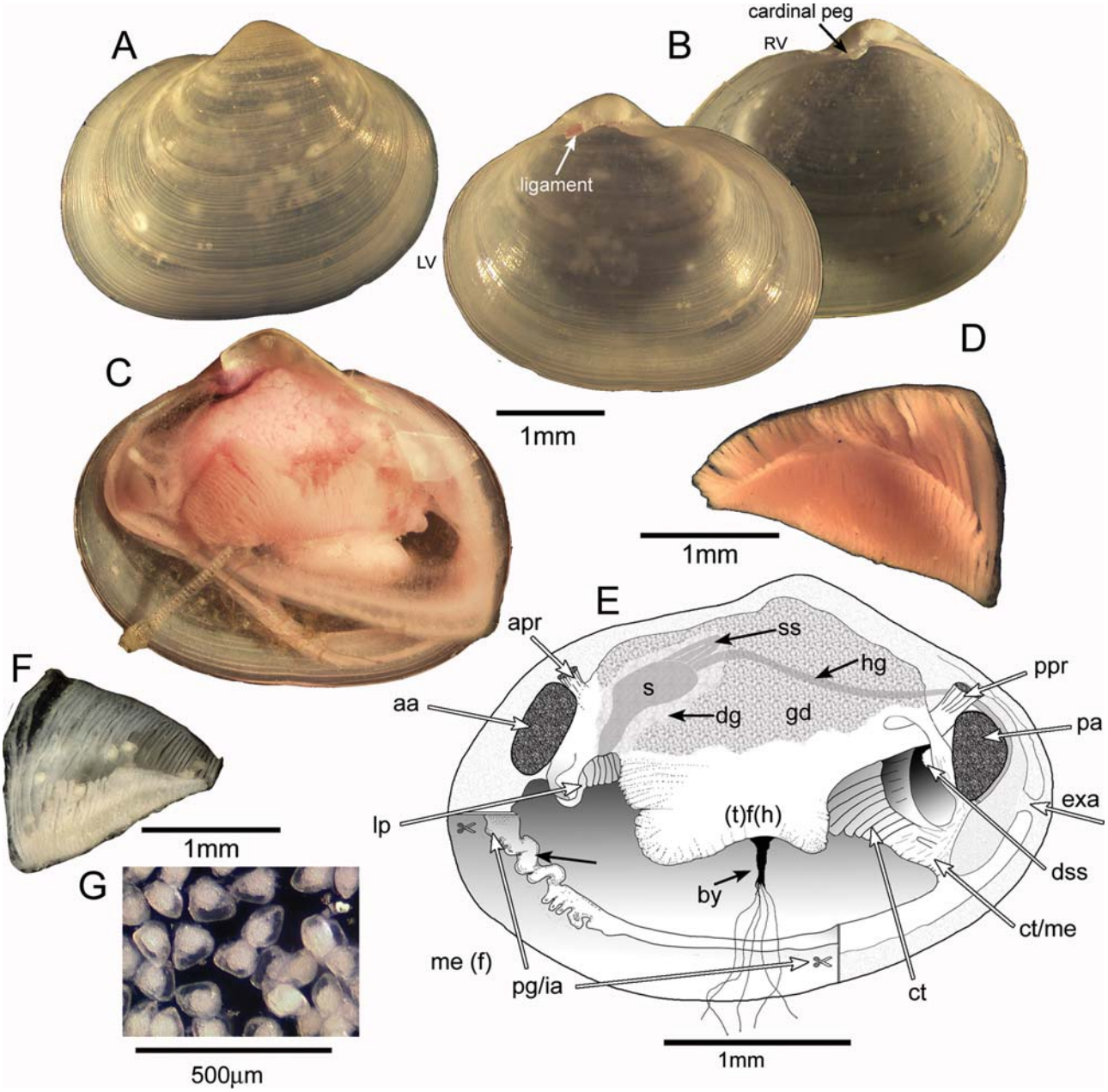

Figure 1. Gross morphology of *Syssitomya pourtalesiana* Oliver, 2012. **A–E.** Specimens from Norwegian Sea. **F–G.** Specimens from Rockall Trough. **A.** External view of left valve. **B.** Internal views of right (RV) and left (LV) valves. **C.** Right valve removed, byssus attached to an echinoid spine. **D.** Excised ctenidium. **E.** Diagram of anatomy after dissection from the right side. **F.** Excised ctenidium with larvae between ascending and descending arms of filaments. **G.** Larvae removed from suprabranchial chamber. aa, anterior adductor muscle; apr, anterior pedal retractor muscle; by, byssus: ct, ctenidium; ct/me, ctenidial/mantle edge junction; dg, digestive gland; dss, domed structures; exa, exhalant aperture; gd, gonad; hg, hind gut; lp, labial palps; me(f), mantle edge folds; pa, posterior adductor muscle; pg/ia, pedal gape and anterior inhalant aperture; ppr, posterior pedal retractor muscle; s, stomach; ss, style sac; (t)f(h) toe and heel of foot.

Each ctenidium consists of a single demibranch with about 30 filaments, which are partially reflected to just over half of their length. Each filament is extended abfrontally and laminar in form, up to 250 µm deep and 20–25 µm wide (Fig. 2A). Two distinct zones can be identified (Fig. 2C), a narrow frontal ciliated zone and an expanded lateral or bacteriocyte zone. The frontal area, as in many suspension-feeding bivalves, is densely ciliated with frontal cilia, laterofrontal cirri and lateral cilia (Fig. 3A). The laterofrontal cirri are very prominent and arise from a double row of attachments set on raised cuticular ridges. A food-acceptance tract is present on the lower edge of the ctenidium (Fig. 3B). The frontal zone is very narrow, with bacteriocytes lying immediately below the ciliated surface of the frontal zone. Interfilament connections appear to be restricted to ciliated junctions between the frontal zones, but there is evidence from the TEM study of very fine tissue junctions immediately behind the frontal zone. The abfrontal, bacteriocyte zone is greatly extended (Fig. 2C) and lined on either side by a layer of bacteriocytes (Fig. 2D, E), roughly polygonal in surface outline. There is no microvillar cuticle or glycocalyx covering the bacteriocytes. Under the SEM, the surface of the bacteriocyte zone appears as a mosaic

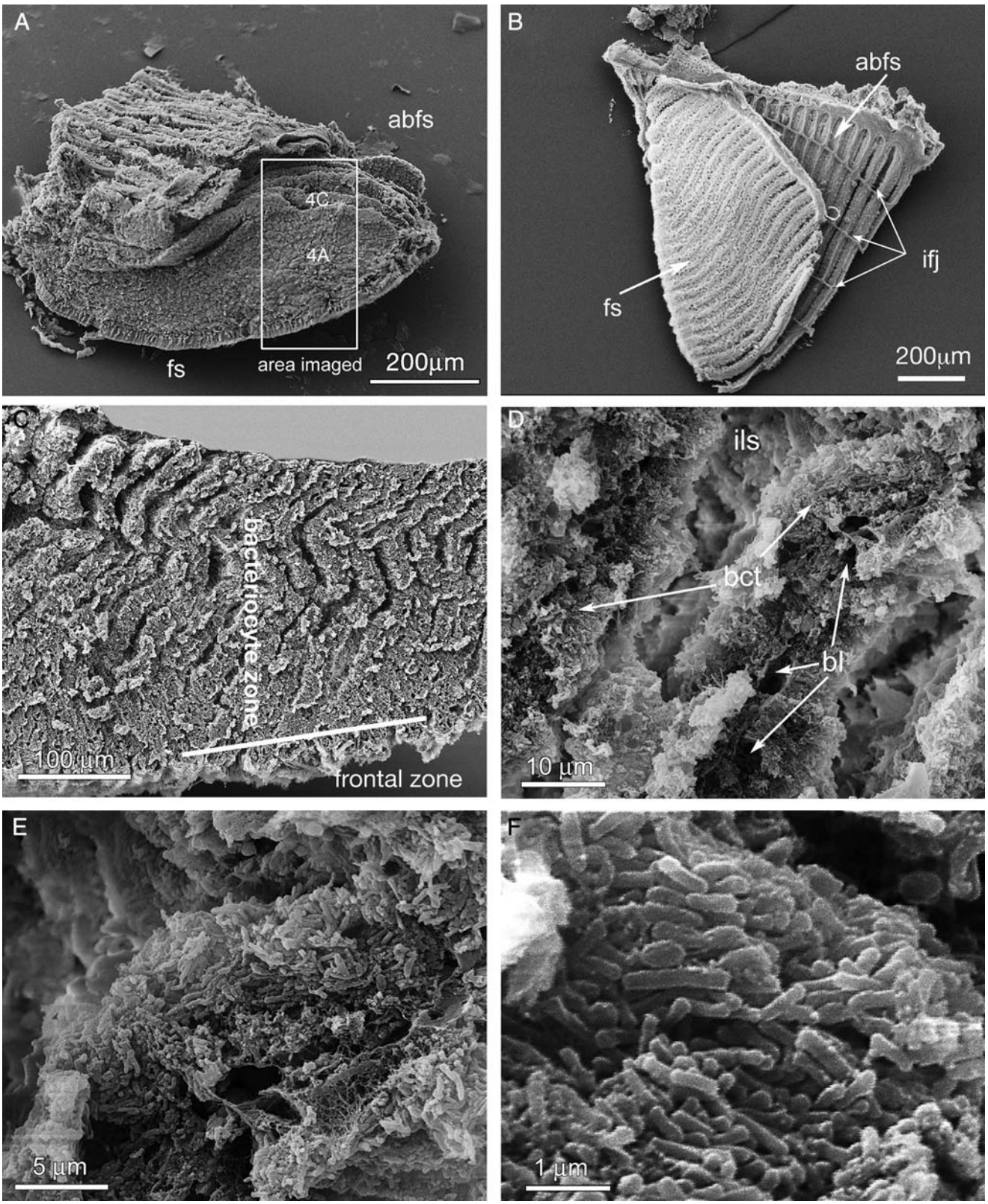

Figure 2. A, C–F. SEMs of the ctenidium of *Syssitomya pourtalesiana*. B. SEM of ctenidium of *Montacuta substriata*. **A.** Part of excised ctenidium showing laminar form of filaments, abfrontal edges (abfs) and frontal edge (fs). **B**. Whole ctenidium showing narrow abfrontal zone and inter-filament junctions (ifj). **C.** Transverse section through a series of filaments showing extended abfrontal/ bacteriocyte zone. **D.** Transverse section of two adjacent filaments showing bacteriocytes (bct) lining surfaces, with blood lacunae (bl) within filament and interlamellar filament space (ils). **E.** Single bacteriocyte packed with rod-shaped bacteria. **F.** Bacteria within bacteriocyte; two morphotypes visible, short rods and ovals.

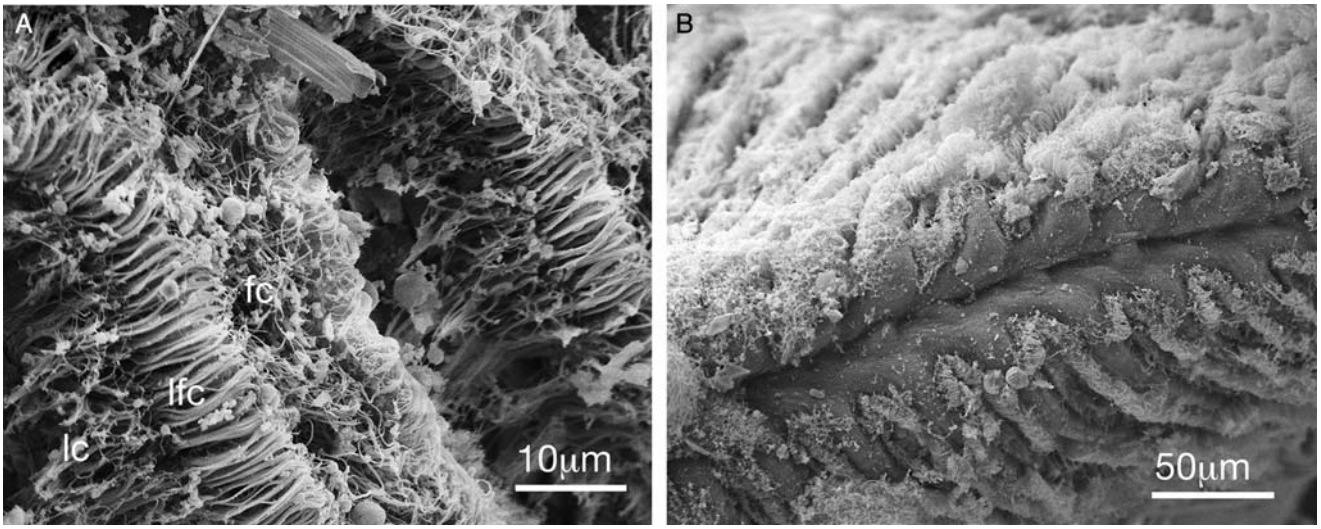

Figure 3. SEMs of the frontal region of the ctenidium of *Syssitomya pourtalesiana*. **A**. Frontal face with frontal cilia (fc), lateral frontal cirri (lfc) and lateral cilia (lc). **B**. Ventral food-acceptance tract.

of polygonal bacteriocytes (Fig. 4A) with the bacteria exposed (Fig. 4B, C, E). In places the bacteria have thread-like projections (pili) connecting them to each other (Fig. 4E). In transverse section, the boundaries of the bacteriocytes are difficult to discern, but a median blood lumen and tubules are visible along the midline (Fig. 2D). The bacteriocytes are densely packed with bacteria (Fig. 2E); most are small bacilli 0.7–1.2 μm in length, but some are coccoid, *c*. 0.15 μm in length (Fig. 2F).

Interspersed between the bacteriocytes and on top of the main bacterial layer are numerous irregular cells, in the size range 2–7 μm (Fig. 4A–C), which are interpreted as phagocytic and referred to as haemocytes. The more regular haemocytes are concentrated between the bacteriocytes, suggesting that they are migrating to or from the surface. Haemocytes on top of the layer of bacilli stand proud of the surface, have a dense appearance and a prostrate, cushion-shaped form and many have pseudopodial and filipodial extensions (Fig. 4C). The density of haemocytes in Figure 4A is 2×104 mm^2.

Where the faces of the abfrontal zone are exposed, particularly on the abfrontal edges, the surfaces are obscured by a tangled mat of filamentous bacteria (Fig. 4D), from 4 to 8 μm in length and 0.12 to 0.15 μm in width. There are relatively few filamentous bacteria close to the frontal edge and over the median area of each filament. These bacteria are on the surface of the ctenidium, mainly on top of the layer of bacilli, often with swollen terminations visible. Some of the latter are simple swellings while others are much larger and in the form of a flattened, ovate blade or paddle (Fig. 4F). These bacteria appear not to be embedded in the cell walls or within the layer of bacilli; remains of these filamentous bacteria cannot be seen within the bacteriocytes.

TEM study of ctenidium

The 1987 TEM images of the Biscay specimen provide a survey of a transverse section of the frontal parts of two ctenidial filaments and adjacent bacteriocytes, plus some details at higher magnification of these bacteriocytes (Fig. 5). In addition, there are four images showing bacteriocytes in a small part of a longitudinal section of a filament. The arrangement of frontal cilia, laterofrontal cirri and lateral cilia in the sections is as seen in the SEMs. The bacteriocytes (bct) are separated from the lateral ciliated cells (cc) by a single row of very small intermediate cells (Fig. 5C, arrowed). The bacteriocytes are large cells lining both faces of the abfrontal zone and the interfilament junctions. They have large nuclei (Fig. 5B; n) and large, irregularly shaped pseudopodia-like extensions (Fig. 5A, B; pl) surrounding superficial pockets. Slender microvilli (Fig. 5E; arrow) extend from the cell surface and branch to form a mesh-like matrix around the extracellular bacteria (ba) in the pockets (Fig. 5A–C, F). The distal tips of the microvilli do not adhere to form a glycocalyx (cuticle) over the bacterial layer (Fig. 5A; ext). The bacteria are numerous, taking a variety of forms: short thick rods ($0.4 \times c.$ 1.0 μm) and longer, narrower rods ($0.2 \times c.$ 3.0 μm) (Fig. 5H); wider elongate-oval shapes about 0.5 μm in diameter are also common (Fig. 5F, G). Phagocytes are present underlying the bacteriocyte layer (Fig. 5D; phagocyte nucleus, n). Large phagosomes (ps) are present within vacuoles in these phagocytes. Smaller vacuoles holding bacteria (Fig. 5D; arrow) are also visible. Changes in the appearance of the bacteria in these vacuoles indicate that digestion is taking place.

SEM study of stomach

The gross anatomy and SEM study of the ctenidium suggest that *Syssitomya pourtalesiana* is capable of suspension feeding.

Given the very small size of the stomach, the cracking process was rather destructive, but the anterior cavity remained intact (Fig. 6A). Here, some stomach contents could be seen; among amorphous debris, pieces of tests of diatoms were recognizable (Fig. 6A, B). Filamentous structures, the shape and size of the filamentous bacteria seen on the ctenidia were also recognizable (Fig. 6C; arrowed), as was a haemocyte (Fig. 6C; hm).

Habitat and behaviour of Pourtalesia

Colour *in situ* photographs showed *Pourtalesia miranda* on a fairly flat brownish-grey mud surface, often criss-crossed by narrow trails, some clearly made by the urchins themselves. Branching sandy Foraminifera (*Rhabdammina* sp.) were common on the sediment surface and tended to accumulate in depressions, often with dark flocculent debris, sometimes with

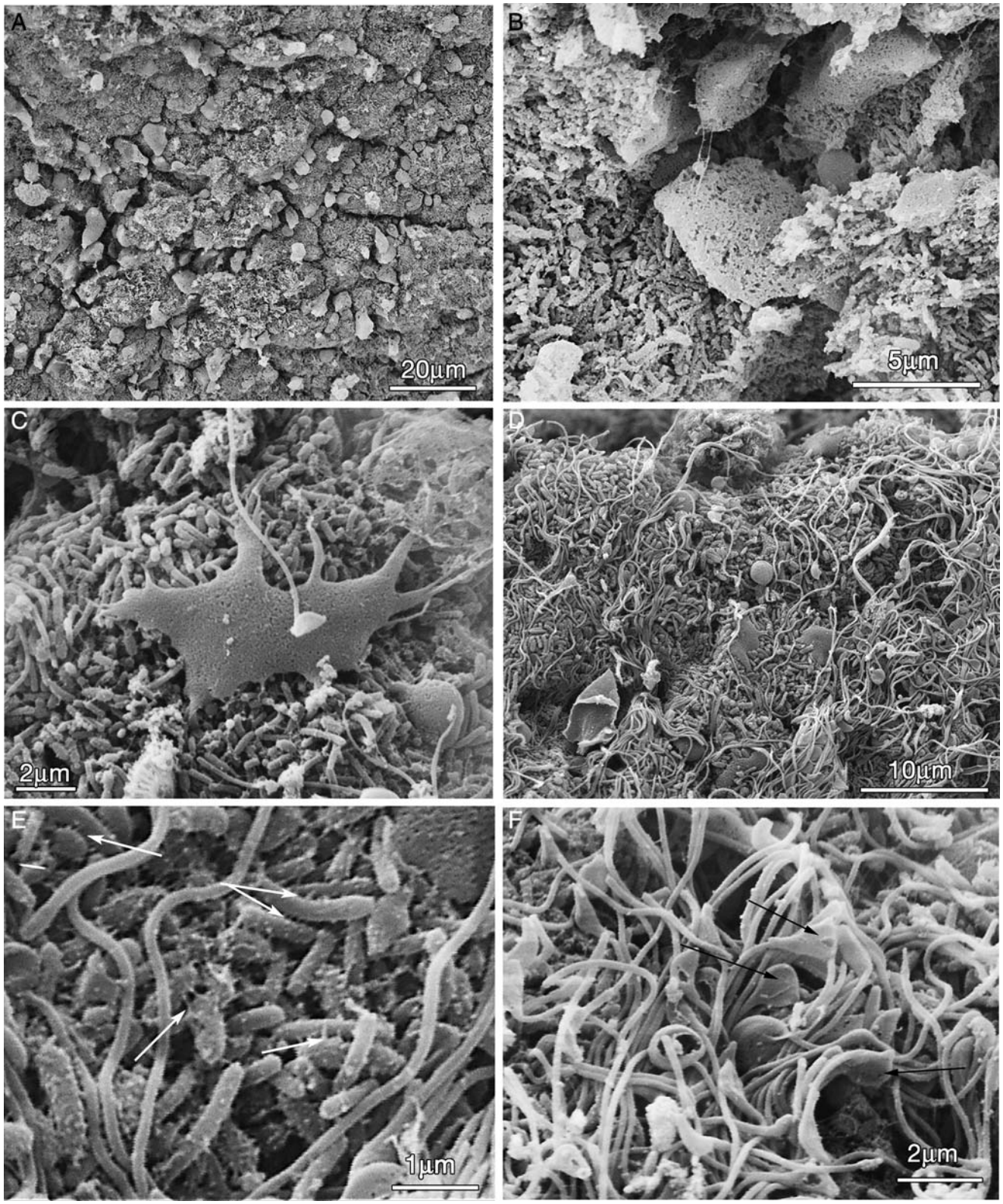

Figure 4. SEMs of the surface of ctenidium of *Syssitomya pourtalesiana*. **A.** At low magnification, showing numerous phagocytic cells emanating from spaces between bacteriocytes. **B.** Spongiform phagocytic cells. **C.** Phagocytes with pseudopodial extensions sitting on surface of bacteriocytes along with sparse filamentous bacteria. **D.** Leading edge of abfrontal zone with dense aggregation of filamentous bacteria. **E.** Rod-shaped bacteria within bacteriocyte; pili indicated with arrows; a few filamentous bacteria are also visible. **F.** Hyphomicrobial cells with paddle-like mother cells (arrowed).

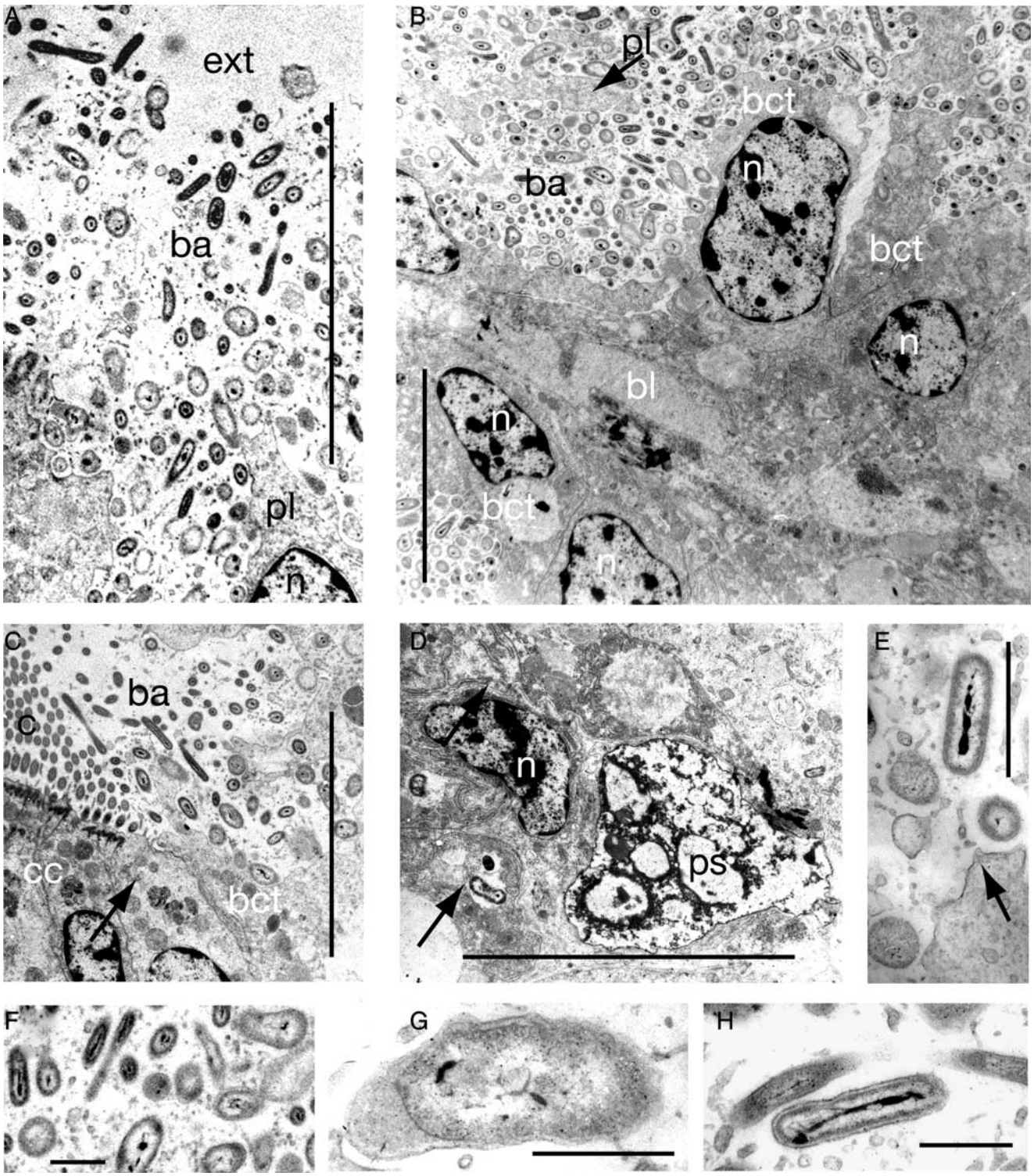

Figure 5. TEMs of bacteriocyte region of ctenidium of Biscay specimen of *Syssitomya pourtalesiana*. **A**. Outer margin of bacteriocyte, showing absence of exterior glycocalyx (ext), a variety of bacteria (ba), microvilli among them and a small part of a pseudopodial lobe (pl), with nucleus (n). **B.** Transverse section of filament, central tissue, with blood lacuna (bl) and bases of several bacteriocytes (bct), with their nuclei (n); a cell inside the blood lacuna may be a haemocyte with nucleus. The bacteria are extracellular, in pockets between pseudopodia-like folds or lobes (pl) of the cell. **C.** Junction of bacteriocyte region with inner edge of lateral ciliated band (cc); a small part of an intermediate cell (arrowed) without bacteria lies in front of a lobed bacteriocyte (bct) with associated bacteria. **D.** Several phagocytes underlying or between bacteriocytes, one large phagosome vacuole (ps) and a small vacuole (arrowed) containing two bacteria apparently undergoing digestion, lower left in another cell. **E.** Bacteria and microvilli with origin of microvillus (arrowed) from cell lobe in lower right corner. **F.** Variety of bacteria among microvilli. **G.** Oval bacterium in longitudinal section. **H.** Two rod-shaped bacteria, a long slender form above a short stout one. Scale bars: **A**–**D** = 10 μm; **E**, **F** = 1 μm; **G**, **H** = 0.5 μm.

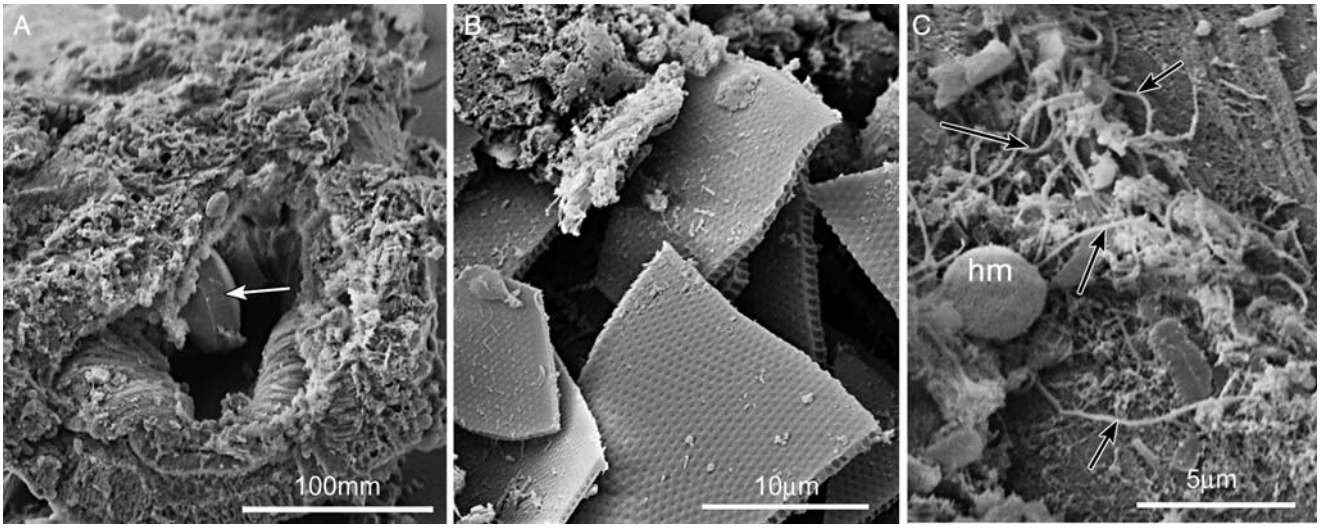

Figure 6. SEMs of stomach and stomach contents of *Syssitomya pourtalesiana*. **A.** Large ingested particles (perhaps diatoms) in anterior chamber. **B.** Large crushed diatom test. **C.** Filamentous bacteria (arrowed) in stomach contents.

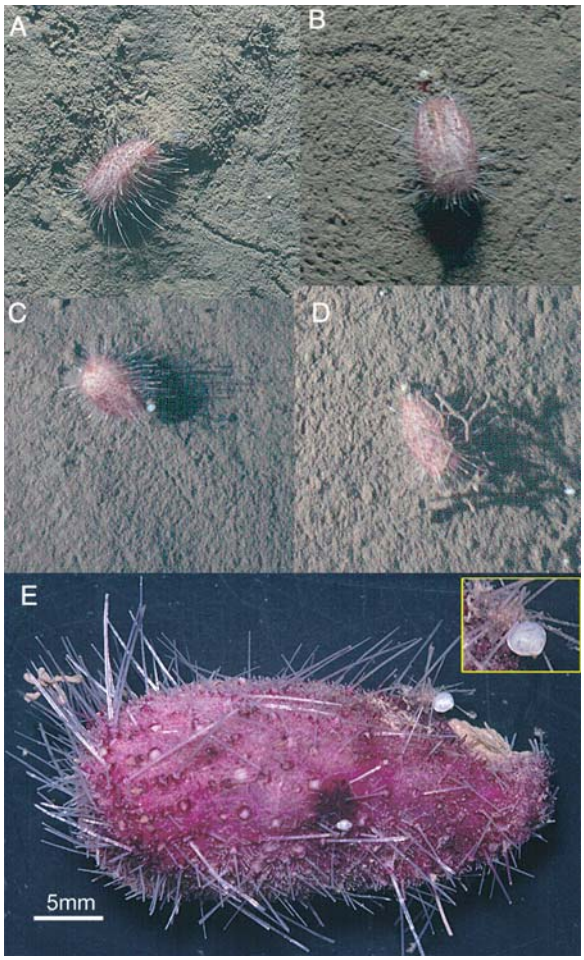

Figure 7. A–D. Towed-camera photograph of *Pourtalesia miranda*, Santander Canyon, *c.* 2000 m, (photo A.J. Southward). **E.** *Pourtalesia miranda* with *Syssitomya pourtalesiana* attached, Santander Canyon, Bay of Biscay (photo A.J. Southward).

dead tests of *Pourtalesia*. Small galatheids (*Munidopsis curvirostra*) were often present on the surface. Dredge samples of the mud were rather stiff or clayey, under a softer surface layer. They contained a much greater diversity of animals than was visible in the photographs. *Pourtalesia miranda* was obtained in samples from depths of about 1550–2900 m and was visible in photographs taken between 1970 and 2330 m depth. The average density in the series of photographs was about 2 m^{-2} The mud temperature was about 4.5°C in box-core samples taken in 1987 (Dando *et al.*, 2008). Collections contained *P. miranda* specimens up to 27 mm long. A specimen 20 mm long, with two bivalves attached, is shown in Figure 7E. The mouth is in a deep funnel-shaped depression at the front end (left), while the anus is in a shallow depression on the upper side near the hind end. The attached bivalves are usually on spines around the anal region, but some may be attached elsewhere, like the small one on a lateral spine.

In situ close-up photographs (Fig. 7A–D) showed four *P. miranda* active on the sediment surface with single *Syssitomya* visible on three of them (Fig. 7B–D). The trails left by *Pourtalesia* on the sediment indicate a variety of behaviours: (1) ejecting rather loose faecal material from the anus, leaving a wide, low ridge of mud behind as they move forward (Fig. 7B); (2) ploughing forward through the surface layer and leaving a groove (Fig. 7C); (3) moving forward on their spines over the surface without feeding or defaecating, leaving only faint traces of the spines (Fig. 7D). They can also burrow forward and downward to become partly buried. It was not possible to determine from these still photographs whether they can bury themselves completely. However, *Pourtalesia* species lack the shovel-shaped spines found in typical deep-burrowing echinoids such as *Echinocardium* and *Brissopsis*.

DISCUSSION

Gill filament and bacteriocyte structure

The ctenidium of *Syssitomya pourtalesiana* is reduced to a single demibranch with partially reflected laminar filaments. The laminar form is similar to that seen in many thyasirids, but

most thyasirids hosting symbiotic bacteria in the gills retain both demibranchs. The reduction in demibranchs is seen in many montacutids and is probably a reflection of the small size of most species. A typical montacutid ctenidium, showing no abfrontal extension, is that of *Montacuta substriata* (a commensal with the echinoid *Spatangus purpureus*; Fig. 2B).

The gill bacteria of *S. pourtalesiana* are extracellular, located in pockets on the surface of well-defined bacteriocytes lining the greatly elongated abfrontal region of the ctenidial filaments. This arrangement is similar to that seen in thyasirids (Southward, 1986) and specifically to the Type 3 described by Dufour (2005). *Syssitomya pourtalesiana* differs in that there are numerous cytoplasmic extensions of the bacteriocytes, with surface microvilli, but no cuticle or glycocalyx formed by the adhesion of the distal ends of the microvilli. This condition is more specialized than the Type 2 thyasirid form (Dufour, 2005) in which the bacteria are present between the microvilli of undifferentiated epithelial cells. Species of Mytilidae on wood-falls also harbour extracellular symbiotic bacteria (Duperron *et al.*, 2008), but the associated cell structure is less defined, with the bacteria held between the microvilli of epithelial cells. On the bacteriocytes of *S. pourtalesiana* the bacteria are enmeshed by the pseusopodia-like extensions and microvilli; their stability is apparently further aided by the self-adhesion of the bacteria using surface pili (Fig. 4E). In the Thyasiridae, uptake of nutrients from the bacteria takes place by endocytosis and phagocytic vacuoles have been frequently observed (Southward, 1986; Dufour, 2005). Similar phagocytic vacuoles are seen in *S. pourtalesiana* (Fig. 5D).

The haemocytes observed on the bacteriocytes of *S. pourtalesiana* were particularly abundant. This might suggest a response to a pathogen (Canesi *et al.*, 2002; Paillard, Le Roux & Borrego, 2004), although the appearance of the gill and attached bacilli and cocci do not resemble any described bivalve pathology. A similar array of haemocytes was also observed on the ctenidia of a specimen taken from the Rockall Trough, thus dispelling conjecture that they are artefacts of preservation or a consequence of disease. An alternative interpretation of the more rounded cells observed between the bacteriocytes might be that they are equivalent to the intercalary cells recorded consistently from lucinid gills (Frenkeil, Gros & Mouëza, 1996: Fig. 13) and to a lesser extent from thyasirids (Southward, 1986). Such intercalary cells are part of the epithelium and have apical nuclei and surface microvilli. They do not form pseudopodial extensions.

Haemocytes form pseudopodia when exposed to foreign cells (Donaghy *et al.*, 2009) and this explains the different morphologies observed in the SEM studies of the bacteriocyte surface, haemocytes between the bacteriocytes having a more rounded outline than those on the surface of the bacteria. The haemocytes do not concentrate around the filamentous bacteria, but their visible protoplasmic projections are seen to be directed towards the bacilli.

In *Ostrea edulis* wandering haemocytes are abundant in the mantle cavity, including on the gill surface, as well as in stomach and gut, and have an important role in phagocytosing large particles (Yonge, 1925). Yonge suggested that the digested products were transferred by the haemocytes to the epithelial cells or the vesicular connective tissue cells. Although haemocytes have been observed in other bivalves with symbiotic bacteria, they are scarce on the external surface of the gill (Bettencourt *et al.*, 2008; 2009). *Syssitomya pourtalesiana* is unusual in having no glycocalyx or microvilli covering the bacteria on the surface of the bacteriocytes; it is possible that the abundant haemocytes on the bacteriocytes are used by this bivalve to supplement the phagocytosis of the bacteria by the bacteriocyte cells, as well as removing foreign particles from the mantle cavity and gills. Ingestion of the bacteria by surface haemocytes may represent an early stage in the evolution of symbiosis with bacteria. The clustering of the haemocytes in the spaces between the bacteriocytes suggests a two-way migration between the bacteriocyte surface and the lumen, analogous to their behaviour in *Ostrea* (Yonge, 1925). Further studies are required to determine the ingested material and the roles of the haemocytes in *S. pourtalesiana*.

The bacteria

The SEM study of the bacteriocytes recognized two morphotypes of bacteria, bacilli and cocci, but the TEM study revealed that the bacilli were of three forms. However, the bivalves used for the SEM and TEM studies came from different geographical areas and different species of *Pourtalesia*. The large surface-dwelling filamentous bacteria are discussed below.

Without molecular data, we cannot be sure what types of bacteria are present. In the majority of chemosymbiotic bivalves, the chemoautotrophic bacteria rely on oxidation of reduced sulphur compounds (Distel, 1998; Stewart, Newton & Cavanaugh, 2005; Dubilier *et al.*, 2008). Circumstantial factors suggest that the gill bacteria in *S. pourtalesiana* are not sulphur oxidizers. There are no sulphur storage vacuoles in the bacteria, a feature of the bacterial symbionts in species of thyasirids and lucinids with thiotrophic capacity.

Stewart *et al.* (2005), in discussing the evolution of chemosynthetic endosymbiosis, stress the predominance of the symbiosis at chemoclines across oxic–anoxic interfaces. Given the observations on the echinoid, it does not live at an oxic–anoxic interface and its predominantly surface-dwelling habit precludes it from entering the reducing layer found below the surface of these continental slope and upper abyssal environments (Dando *et al.*, 2008). The bivalves are most often found close to the anal region of the urchin (Fig. 7B) and in such a position can be in contact with defaecated material. If sulphides are present in the faeces (*cf.* Thorsen *et al.*, 2003; Gomes da Silva *et al.*, 2006), they would be diluted and oxidized rapidly on contact with oxidized water. Other fermentation products produced during digestion and excreted with the faeces would include hydrogen, ammonia and dissolved organic matter (DOM) and these may provide the nutritional source for the epibiotic bacteria in *Syssitomya*. Assimilation of host organic waste products (acetate, propionate, succinate and malate) by a consortium of epidermal symbiotic bacteria has recently been shown for the gutless oligochaete, *Olavius algarvensis*. This consortium also has the potential to use hydrogen as an energy source (Kleiner *et al.*, 2012).

Surface-dwelling filamentous bacteria

The morphology of the filamentous bacteria is most reminiscent of 'hyphomicrobial cells', probably belonging to the genus *Hyphomonas*, since the morphologically similar sister clade, *Hyphobacterium*, is not marine (Poindexter, 2006). The paddle-shaped end is the mother cell and the simple swollen end is the budding daughter cell joined to the mother by the hypha. However, *Hyphomonas* has rarely been isolated from living animals, only once on gills of Turbot (Mudarris & Austin, 1988). *Hyphomonas*-like bacteria have been recognized as a primary food source for the hydrothermal vent limpet *Lepetodrilus schrolli*, which is an active grazer on bacterial films (Beck, 1993). *Hyphomonas* are considered to utilize DOM and require amino acids (Poindexter, 2006).

In *S. pourtalesiana* the position of the filamentous bacteria on the ctenidum indicates that they are unlikely to be utilized as food.

Mixotrophy

Syssitomya pourtalesiana has been shown to harbour bacteria on bacteriocytes and to be capable of digesting these through a process of endocytosis, with the possible additional involvement of wandering haemocytes. The bivalve is, however, capable of filter-feeding as indicated by the fully ciliated frontal gill region and the presence of food particles in the stomach. Mixotrophy in chemosymbiotic species is not unusual and has been demonstrated in *Bathymodilous* (Page *et al*., 1991; Martins *et al*., 2008; Riou *et al*., 2010) and *Idas* (Southward, 2008). The metabolites available to the bacteria are likely to originate in the echinoid faeces, but the bivalve is not always attached to the anal region of the echinoid. Given the likely unreliability of nutrients for the bacteria, it would be advantageous for *S. pourtalesiana* to obtain nutrition from a combination of filter feeding, from ingestion of symbiotic bacteria and possibly from direct epidermal uptake of DOM.

Systematic position of Syssitomya

The shell morphology of *Syssitomya* is similar to that found in the Montacutidae and to *Montacuta* in particular (Oliver, 2012). More detailed examination of the anatomy supports this family placement, together with comparisons with the numerous species studied recently by Lützen and Jespersen and co-workers (Jespersen & Lützen, 2001; Jespersen, Lützen & Nielsen, 2004; Jespersen, Lützen & Oliver, 2007; Rotvit *et al*., 2007; Lützen, Hong & Yamashita, 2009) as well as with earlier studies such as those by Morton (1980) and Ockelmann & Muus (1978).

Like other montacutids, *S. pourtalesiana* is small and is commensal with other invertebrates. The shell is expanded anteriorly, with the ligament set wholly or partly internally on a resilifer behind the beaks. The hinge teeth are small, cardinals peglike and with or without laterals. The adductor muscles are roughly equal in size and oval in section. The mantle edge is partly fused, with a large anterior pedal aperture that also functions as the inhalant aperture; the exhalant aperture is small. The gill axis is fused with the mantle edge and the gill is reduced to a single demibranch. The byssus is functional. The style sac is separate from the midgut. The presence of dimorphic sperm was recorded by Ockelmann & Muus (1978) and is strongly characteristic of the Montacutidae (Ockelmann, 1965). The insertion of the protractor pedis muscle in the anterior adductor was not observed here.

It can therefore be concluded that *S. pourtalesiana* is the first montacutid and galeommatoid bivalve shown to have a symbiotic relationship with bacteria. The bacteria are associated with specialized gill cells, and this is the first commensal bivalve known to exhibit this morphology.

Evolution of bacterial symbiosis in the Galeommatoidea

Syssitomya pourtalesiana is just one of the many galeommatids associated with echinoids, but it is the only species yet discovered to have symbiotic gill bacteria. Oliver (2012) listed 16 species of galeommatid known to be commensal with echinoids, but could find no evidence that any of these harboured gill bacteria. *Montacuta substriata* lives attached to the echinoid *Spatangus purpureus*, a shallow-water analogue of *Syssitomya*, but has a typical filter-feeding ctenidial structure (Fig. 2B). The association with echinoids would appear to have no relevance to the adoption of bacterial symbiosis in *Syssitomya*.

One of the driving forces for the evolution of chemosymbiosis is a response to nutrient-poor, oligothrophic conditions such as those found widely in the deep sea (Kleiner *et al*., 2012). Most galeommatoideans inhabit shallow, relatively nutrient-rich waters. Allen (2008) records only 14 galeommatoideans from the deep Atlantic and, of these, two show unique feeding adaptations—*Mysella tumidula* is suctorial and *Draculamya porobranchiata* is fluid-feeding (Oliver & Lützen, 2011). Such observations support the proposition that oligotrophic conditions drive trophic adaptations in the deep sea. Of the other families exhibiting bacterial symbiosis in the gills, only in the Thyasiridae has there been an adaptive radiation of non-symbiotic and symbiotic species (Southward, 1986; Dufour, 2005; Taylor, Williams & Glover, 2007). In the deep sea, this has resulted in a high species diversity with Allen (2008) listing 79 taxa from the Atlantic. Notably, thyasirid species associated with sulphide-rich environments host single bacterial species whereas those in the deep sea, where sulphide levels are low, host up to three bacterial types (Southward, 1986). Having a diverse microbial community in the symbiosis may imply a wide range of trophic pathways (Kleiner *et al*, 2012), giving support to our suggestion that in *S. pourtalesiana* the symbiotic bacteria utilize DOM and/or oxidize ammonia or other reduced compounds derived from echinoid faeces. Sulphide uptake may not be relevant to *S. pourtalesiana*.

Evolution of the association of bacteria with gill cells may be postulated to begin with a tolerance of specific bacteria on the cell surface followed by the harbouring of these bacteria by specialized bacteriocytic cells (Dufour, 2005). For the development of symbiosis, the bivalve must both accept the bacteria and be able to supply them with nutrients. In taxa that do not live in sulphide- or methane-rich environments (e.g. those at cold seeps), other adaptations have evolved to allow them to source such nutrients. Lucinids and many thyasirids burrow deeply into reducing layers of sediments and extract pore water rich in sulphides, as well as being able to 'mine' insoluble sulphides as an energy source (Dando, Ridgway & Spiro, 1994). Most galeommatoideans live in shallow, oxygenated environments without access to sulphides and methane and lack the specialized morphology to 'mine' the former. However, many galeommatoideans are crevice dwelling and live in association with burrowing invertebrates where they may be exposed to concentrations of faecal-derived DOM. Alexandr Mironov (personal communication) has found bivalves living as internal commensals in the intestine, close to the peristome, of the echinoid *Carnarechinus clypeatus*. *Tellimya ferruginosa* develops a metaliferous bacterial biofilm associated with the iron- and sulphide-rich microhabitat found in the anal flow of the echinoid *Echinocardium* (Gillan & DeRidder, 1997). Many other commensal bivalves associated with invertebrate burrows develop a similar film, e.g. *Kurtiella bidentata* and *Epilepton clarkiae* (personal observation) and inhabit, albeit very restricted, reducing environments. In such associations the bivalves are exposed to bacteria and this may be a route for the evolution of the bacterial symbiosis in the Galeommatoidea. *Syssitomya pourtalesiana* may represent an intermediate stage of the development of a symbiotic association with bacteria on the gill, in which the bacteriocyte does not enclose the bacteria and phagocytosis is instead achieved by roaming haemocytes.

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