RESEARCH ARTICLE

Bacterial communities associated with the wood-feeding gastropod *Pectinodonta* sp. (Patellogastropoda, Mollusca)

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Abstract

Even though their occurrence was reported a long time ago, sunken wood ecosystems at the deep-sea floor have only recently received specific attention. Accumulations of wood fragments in the deep sea create niches for a diverse fauna, but the significance of the wood itself as a food source remains to be evaluated. *Pectinodonta* sp. is a patellogastropod that exclusively occurs on woody substrates, where individuals excavate deep depressions, and is thus a potential candidate for a wood-eating lifestyle. Several approaches were used on *Pectinodonta* sampled close to Tongoa island (Vanuatu) to investigate its dietary habits. Host carbon is most likely derived from the wood material based on stable isotopes analyses, and high cellulase activity was measured in the digestive mass. Electron microscopy and FISH revealed the occurrence of two distinct and dense bacterial communities, in the digestive gland and on the gill. Gland-associated 16S rRNA gene bacterial phylotypes, confirmed by *in situ* hybridization, included members of three divisions (*Alpha-* and *Gammaproteobacteria*, *Bacteroidetes*), and were moderately related (90–96% sequence identity) to polymer-degrading and denitrifying bacteria. Gill-associated phylotypes included representatives of the *Delta-* and *Epsilonproteobacteria*. The possible involvement of these two bacterial communities in wood utilization by *Pectinodonta* sp. is discussed.

Introduction

Although their abundance on the sea floor had been established since the end of the 19th century (Agassiz, 1892; Murray, 1895; Bruun, 1959), it is only one century later that large organic falls such as whale falls or sunken drift woods have received specific attention (Turner, 1973, 1977; Smith, 1992; Smith & Baco, 2003). Plant debris of various sizes (leaves, nuts, wood branches or trunks) can reach the oceans through rivers and sink to abyssal depths. Locally, these terrestrial inputs occur in large amounts and attract dense micro- and macrofauna exploiting this resource (Wolff, 1979; Palacios *et al.*, 2006, 2009). Wood-feeders and metazoans grazing bacterial mats on wood surface use these organic matters as a food source. These organisms are then themselves a food source for predators or scavengers (Turner, 1977). Chemoautotrophy is also probably significant. Indeed, sulfate reduction, mediated by heterotrophic bacteria deriving their carbon from the wood, produces sulfide (Leschine, 1995; Laurent *et al.*, 2009; Gaudron *et al.*, 2010). Sulfide then sustains chemoautotrophic bacteria occurring free-living or as symbionts for example in *Idas* and *Adipicola* mytilids (Duperron *et al.*, 2008). Wood remains thus allow the development of long-lasting ecosystems, relying on both wood degradation and chemosynthesis. Their role in deep-sea ecology, although not yet understood, could be significant (Cayré & Richer de Forges, 2002). Fauna colonizing wood deposits at the deep-sea floor is diverse. It comprises gastropod, polyplacophoran and bivalve molluscs, decapod and peracarid crustaceans, polychaetes and echinoderms

(Turner, 1973, 1977; Wolff, 1979; Cayré & Richer de Forges, 2002). Macrofaunal species documented to degrade wood in marine environments include wood-boring molluscs (bivalve families *Teredinidae* and *Xylophagainae*) and crustaceans (isopod families *Limnoridae* and *Sphaeromatidae*) (Gareth Jones *et al.*, 2001).

Cellulose, the main component of the wood, is hydrolyzed into glucose by the action of a group of enzymes known as cellulases. These enzymes have been shown to exist since a long time in plant, fungi and bacteria. In the last few years, recent evidence has revealed the occurrence of endogenous cellulases in a wide range of metazoans, such as arthropods (termites and other wood-boring insects, Watanabe & Tokuda, 2010) and crustaceans (Crawford *et al.*, 2004), nematods (Smant *et al.*, 1998), various molluscs (Watanabe & Tokuda, 2001), echinoderms (sea urchins, Nishida *et al.*, 2007) and sea squirts (Lo *et al.*, 2003). However, the symbiotic cellulase production is still the favored explanation for cellulose digestion in most metazoans (Watanabe & Tokuda, 2001). The best-documented examples of xylophagy are terrestrial and typically involve complex microbial symbiotic communities. For example, lower termites harbor numerous flagellates within their enlarged hindgut paunch, and flagellates themselves harbor both extra- and intracellular symbioses involving multiple *Bacteria* and *Archaea* (Brune & Stingl, 2005). In marine environments, the most documented examples are the wood-boring bivalves of the family *Teredinidae* that benefit from cellulolytic and nitrogen-fixing gammaproteobacterial symbionts (Waterbury *et al.*, 1983; Distel *et al.*, 2002; Luyten *et al.*, 2006).

During several cruises dedicated to the sampling of naturally sunken woods around Vanuatu islands (*BOA0* 2004, *BOA1* 2005, *SantoBOA* 2006), we collected other mollusc species, in particular chitons and limpets, obviously able to degrade wood. Animals were indeed attached or clinging to the surface of dredged pieces of wood, where they sometimes had excavated deep depressions. The most abundant were true limpets (Patellogastropoda) of the genus *Pectinodonta* (family *Acmaeidae*). In this study, we address whether *Pectinodonta* specimens, collected during the *SANTOBOA* 2006 cruise at two different stations of similar depths, are real wood-feeders. A multidisciplinary approach was conducted to investigate potential bacterial partners of *Pectinodonta*, including morphological and ultrastructural observations, localization and molecular characterization of associated bacteria, cellulase activity assays and stable isotope analyses.

Materials and methods

Samples

Pectinodonta sp. specimens were recovered during the *SANTOBOA* 2006 cruise aboard the *RV Alis*, in the Vanuatu archipelago. Samples were trawled at two stations close to Tongoa island: AT120 (latitude 15°41215′N, longitude 167°01701′E, between 431 and 445 m depth) and AT121 (latitude 15°39778′N, longitude 167°01639′E, between 275 and 290 m depth). Specimens from this cruise are named *Pectinodonta*-2006. A second set of specimens, used only for isotopic analyses, were collected with their host wood during the *BOA0* 2004 cruise aboard the *RV Alis*, in the Vanuatu archipelago. Samples were trawled between the Malekula and the Epi islands (station CP2304: latitude 16°37.4′N, longitude 167°59.8′E between 564 and 582 m depth). These specimens, labelled *Pectinodonta*-2004, have a morphology very similar to *Pectinodonta*-2006 specimens and were stored with the wood in ethanol 80%.

Upon recovery from trawl bags, *Pectinodonta* specimens were either fixed directly, or dissected in body parts: the gill, digestive mass (which comprises both the digestive tract and the digestive gland) and foot.

Samples for electron microscopy were fixed in a 2.5% glutaraldehyde–seawater solution and later postfixed in 1% osmium tetroxide. Samples for DNA extraction were either frozen or fixed in 95% ethanol. Samples for FISH were fixed in 4% formaldehyde in filtered seawater at 4 °C for 2 h, rinsed twice in filtered seawater and once in seawater/ethanol (1 : 1), and stored at 4 °C in ethanol 95%. Samples for enzymatic activity measurements were frozen in liquid nitrogen. Samples for isotopic analyses were either frozen in liquid nitrogen (*Pectinodonta*-2006) or stored in ethanol 80% (*Pectinodonta*-2004).

Light microscopy and transmission electron microscopy (TEM)

The gills, digestive tracts and digestive glands of two *Pectinodonta*-2006 specimens were prepared, using classical techniques, as described in Duperron *et al.* (2009).

Scanning electron microscopy (SEM)

The gills, digestive tracts, radulas and shells of two specimens, as well as wood pieces collected under (rasped) and away (nonrasped) from *Pectinodonta* specimens, were observed. Samples were prepared, using a classical protocol, as described in Duperron *et al.* (2009).

Amplification, cloning and sequencing of host and bacterial genes

DNA was extracted from the foot tissue of 11 *Pectinodonta*-2006 specimens using the QIAamp® DNA Micro Kit (Qiagen). The 5′-end of the host cytochrome *c* oxidase subunit I (COI) mtDNA-encoding gene was amplified using universal primers LCO 1490/HCO 2198 (Folmer *et al.*, 1994) and a standard PCR protocol described elsewhere (Samadi *et al.*, 2007). DNA was also extracted from dissected

gill tissue and digestive mass of two *Pectinodonta*-2006 specimens, and bacterial 16S rRNA-encoding genes were amplified during 35 cycles from each sample using universal primers 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GTTACCTTGTTACGACTT-3′) as described by Duperron *et al.* (2005). The PCR products obtained for host COI were sequenced directly. PCR products obtained for bacterial 16S rRNA genes were purified and cloned using the TOPO™ cloning kit (Invitrogen, Carlsbad, CA). Inserts from positive clones were sequenced after plasmid extraction. Sequencing was performed by Genoscreen (Lille, France).

Amplifications of fragments of genes linked to certain bacterial metabolisms were attempted, but failed to yield PCR products. The metabolisms tested were autotrophy (ribulose 1,5-bisphosphate carboxylase/oxygenase), sulfur metabolism (adenosine 5′-phosphosulfate reductase) and methane oxidation (particulate methane monooxygenase α subunit). Tissues of dual symbiotic *Bathymodiolus* mytilids, and of two thiotroph-associated *Myrtea* (Bivalvia: Lucinidae) and *Lamellibrachia* (Annelida: Siboglinidae), were used as controls.

Sequences were deposited at the EMBL database under accession numbers FN421451–FN421461 (host COI sequences from 11 specimens), FM994656–FM994674 (16S rRNA gene sequences from the gill) and FM994675–FM994682 (16S rRNA gene sequences from the digestive mass).

Gene sequence analysis

Bacterial 16S rRNA gene sequences were compared with the GenBank database. New sequences of this study, their best BLAST hits and a dataset containing representative sequences of all bacterial divisions recovered were aligned, representing a sum of 288 sequences. Alignments were performed using SINA webaligner (http://www.arb-silva.de/aligner/) and manually checked. Neighbor-joining methods were used to assess the preliminary phylogenetic relationships among these 288 bacterial 16S rRNA gene sequences. A final data set of 70 sequences was selected from these and analyzed using a Maximum Likelihood (ML) algorithm with a randomized input order of sequences using PHYLIP (Felsenstein, 2002). Bootstrap values were calculated based on 1000 ML replicates.

FISH

Four *Pectinodonta*-2006 specimens were used for FISH. Gill tissue and visceral mass were dissected and embedded separately in wax (90% poly-ethylene-glycol, 10% 1-hexadecanol). Sections, 10 μm thick, were cut using a microtome (JUNG, Heidelberg, Germany) and collected on Superfrost® Plus slides (Roth, Germany). After wax removal in ethanol and rehydration, each section was hybridized following the protocol described in Duperron *et al.* (2005), air dried and mounted using a SlowFade medium (Invitrogen).

FISH probes, summarized in Table 1, were labelled with Cy3, Cy5 or fluorescein isothiocyanate. Observations were performed on a fluorescence microscope (Olympus BX61, Tokyo, Japan) equipped with Optigrid™ (QiOptic, Rochester, NY). Image stacks were obtained by acquiring images on consecutive focal planes separated by 0.3 μm over the entire thickness of the sections. Stacks obtained for each fluorochrome were then merged to create an RGB image displaying the localization of each type of bacteria in the host tissue.

Cellulase activity assays

Two feet, two digestive masses and six gills (pooled in two samples) were homogenized separately on ice in 50 mM NaAc buffer (1 vol sample/1 vol buffer). Homogenates were centrifuged at 10 000 ***g*** (10 min) at 4 °C and the extracted proteins were quantified in the supernatant with the Bio-Rad protein assay using bovine serum albumin (Sigma) as the standard. Cellulase activity was measured using the microplate-based filter paper assay described by Xiao *et al.* (2004). Briefly, 20 μL aliquots of extracts were added to wells of a 96-well PCR plate containing 40 μL of 50 mM NaAc buffer, and a filter paper disk (Whatman No. 1 filters cut into 7-mm-diameter circles). After 60 min of incubation at 50 °C, 120 μL of dinitrosalicylic acid was added to each reaction and incubated at 95 °C for 5 min. Finally, a 36-μL aliquot of each sample was transferred to the wells of a flat-bottom plate containing 160 μL H_2O, and the absorbance was measured at 540 nm using a Microplate reader

Table 1. Oligonucleotide probes used in this study, position in the 16S rRNA gene of *Escherichia coli* and percentage of formamide used

Probe	Sequence (5′–3′)	Position	% Formamide	Target	Reference
EUB338	GCTGCCTCCCGTAGGAGT	338	20–40	Most eubacteria, positive control	Amann *et al.* (1990)
DEL495a	AGTTAGCCGGTGCTTSTT	495	30	Some *Delta Beta* and *Gammaproteobacteria*, some *Actinobacteria*	Loy *et al.* (2002)
GAM42	GCCTTCCCACATCGTTT	42	30	*Gammaproteobacteria*	Manz *et al.* (1992)
ARC94	TGCGCCACTTAGCTGACA	94		*Arcobacter*	Snaidr *et al.* (1997)
EPSY549	CAGTGATTCCGAGTAACG	549	20	*Epsilonproteobacteria*	Manz *et al.* (1992)
ALF968b	GGTAAGGTTCTKCGCGTT	968	20	Various bacteria (not highly specific)	Neef (1997)
CF319	TGGTCCGTGTCTCAGTAC	319	35	*Bacteroides*	Manz *et al.* (1996)

Target refers to the main groups targeted by probes as obtained from a 'probe match' search in ribosomal database project.

(Bio-Rad, France). Standard curves were obtained using glucose solutions (Sigma-Aldrich). A positive control for the reaction was also performed using Cellulase C-2605 from *Aspergillus* sp. (Sigma-Aldrich). Results are expressed as micromole glucose produced per minute per milligram protein.

Stable isotope analyses and sample preparation

Tissues from six *Pectinodonta*-2006 specimens were analyzed (six gills pooled in one sample due to the low amounts of dry material, foot tissue from six specimens, five digestive tracts and six digestive glands).

Pectinodonta-2004 specimens available in the lab were also investigated because the wood piece to which they were attached was also available (wood and animals were fixed and stored in ethanol 80%). Five whole specimens (without their shell), and dissected organs from five other specimens (five foot samples pooled in three samples, five digestive tracts pooled in two samples, five gills pooled in one sample, five digestive glands pooled in one sample) were analyzed. Three samples of the wood, identified to belong to the genus *Leucaena* sp. (Pailleret *et al.*, 2007), were also analyzed. All these samples were rinsed, dried at 60 °C for 30 h, ground to a powder and weighed (about 1 mg) before stable isotopes analyses.

The C/N atomic ratios of all samples were measured using a CHN elemental analyzer (EuroVector, Milan, Italy). The resultant gas was introduced into an isotope ratio mass spectrometer (IRMS) (GV IsoPrime, UK) to determine carbon and nitrogen isotopes. Stable isotopic data are expressed as the relative per mil (‰) differences between the samples and the conventional standard Pee Dee Belemnite for carbon and air N_2 for nitrogen.

Because ethanol fixation may induce a bias in the analyses of the isotopic ratio, in the form of increased $\delta^{13}C$ (Kaehler & Pakhomov, 2001), isotopic signatures of the foot of *Pectinodonta*-2004 and *Pectinodonta*-2006 were compared with test the effect of ethanol fixation using unbalanced one-way ANOVA (MINITAB 15).

Results and discussion

Characterization of *Pectinodonta* specimens

Among the plant remains collected from the Caribbean area (at depths below 1200 m), Wolff (1979) reported significant diversity and abundance of cocculinid gastropods, with at least seven species occurring in and on wood. All specimens recovered for the present study were assigned to the genus *Pectinodonta*, based on some group characteristics such as shell morphology (Fig. 1b) and the radula (Fig. 1e and f). Shells were between 0.9 and 1.8 cm in length. The radula, similar to a carpenter's saw, is characteristic of the genus *Pectinodonta*, as described by Marshall (1998). To test whether all the specimens represented a single species, mitochondrial COI mtDNA sequences were obtained from 11 *Pectinodonta*-2006 specimens from two trawls (AT120 and AT121) performed during the *SANTOBOA* cruise. The mean Kimura 2 Parameter (K2P) genetic distance measured among specimens, using the MEGA 4.0 software (Tamura *et al.*, 2007), was 0.1% (maximum value measured between two sequences: 0.3%). Such a low level of divergence is a strong indication that all specimens represented a single species, because COI displays a significant variation among species in Patellogastropoda (Nakano & Ozawa, 2007). Hence, the most similar COI sequences available in the GenBank database belong to *Pectinodonta rhyssa* and *Bathyacmaea nipponica*, and differ by mean K2P genetic distances of 19.0% and 18.4%, respectively. Given the fact that *Pectinodonta*-2006 specimens all come from the same location and similar depth, and that they are morphologically and genetically homogeneous, it is reasonable to assume that *Pectinodonta*-2006 specimens belong to a single species (Meier *et al.*, 2008). However, they cannot be assigned to any described species because sequences from very few species are available in databases.

Evidence for wood-feeding

Activities of marine wood-boring organisms (mainly bivalves of the *Teredinidae* and *Pholadidae* families, and crustaceans of the *Limnoridae* and *Sphaeromatidae* families) in shallow waters have been intensively studied because of their impact on human activities (wooden fishing boats, harbors, wharfs, piers, aquaculture facilities, etc.). Nevertheless, their deep-sea counterparts are much less known. A common question discussed about wood gribbles is whether animals bore into the wood for protection or to utilize the wood, or bacterial mats on its surface, as a food source. In the case of *Pectinodonta*, individuals do not bore inside the wood for protection as they are protected by their strong shell when firmly attached to the wood surface. Hence, they most probably ingest the wood for food, either to acquire nutrition from microorganisms on the wood, or from the wood itself. Wolff (1979) assumed that all cocculinids occurring on organic remains utilized them as food, grazing the microbial cover rather than the substrate. Considering the observations and analyses detailed below, we rather assume that the *Pectinodonta* specimens from this study are real wood-feeders.

Indeed, all our specimens were recovered from wood trunks, where they can be found at high densities (see Fig. 1a; from this wood piece, Pailleret *et al.* (2007) counted 248 individuals, corresponding to a density of 0.25 individuals cm^{-3}). The genus *Pectinodonta* seems to be present only on large trunks of hardwood, and was not seen on leaves, *Pandanus* stems or other soft wood (A. Warèn, pers. commun.; Marshall, 1985). They occur at the bottom of small

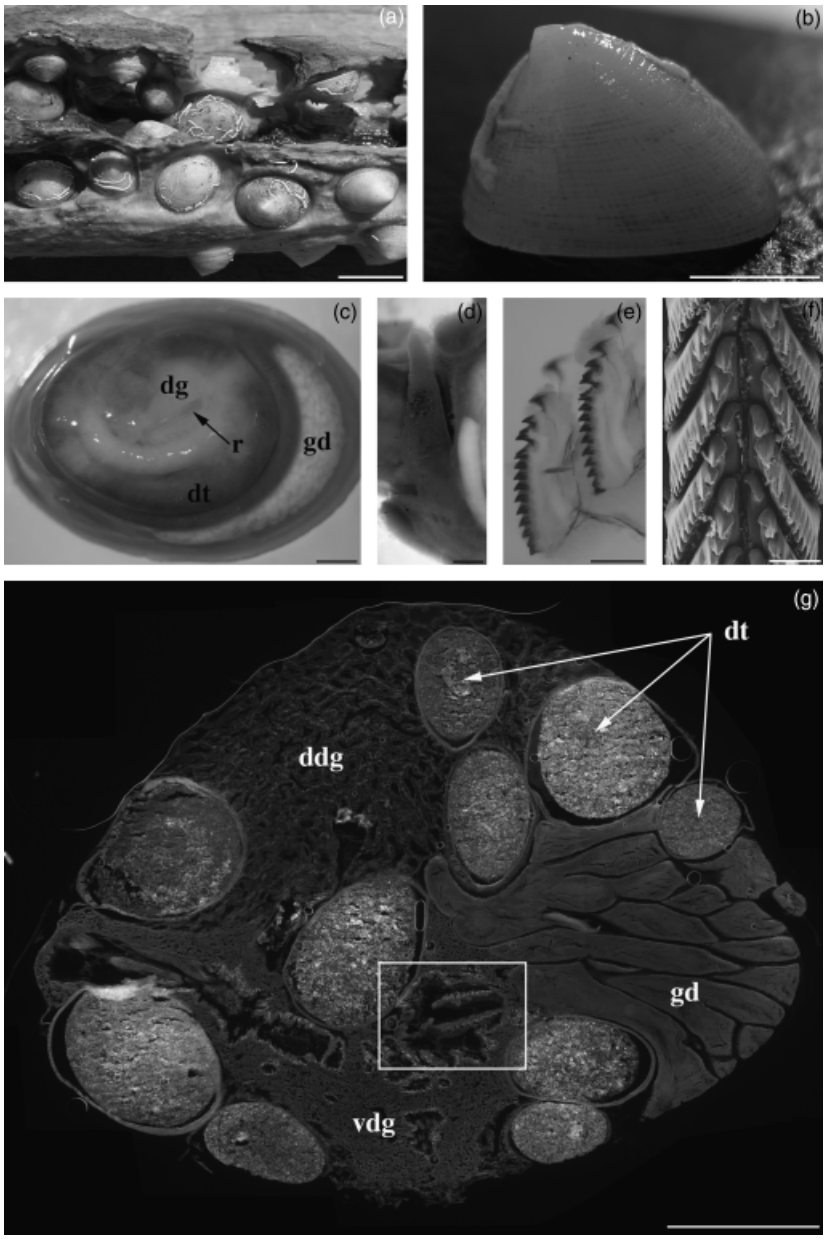

Fig. 1. *Pectinodonta* morphology and anatomy. (a) A densely colonized wood recovered during *BOA0*, showing *Pectinodonta* specimens located in the hollows. (b) *Pectinodonta* shell. (c) Dorsal view of a specimen without the shell, showing the digestive tract (dt), the radular ribbon (r), the dorsal part of the digestive gland (dg) and ventro-laterally the gonad (gd). (d) The gill extending anteriorly from the left to the right. (e–f), radula, photonic (e) and SEM (f) views. (g) Overview of a transversal section of a specimen (mosaic of 25 pictures of a specimen prepared for FISH), showing several sections of the digestive tract (dt), the dorsal (ddg) and ventral (vdg) digestive gland and the gonad (gd). White squares indicate the location of Fig. 6f and g. Scale bars: a = 1 cm, b = 0.5 cm, c = 1 mm, d = 0.5 mm, e, f = 50 μm, g = 1 mm.

depressions (see Fig. 1a) that they very likely dig beneath themselves, if we consider the shape concordance of the hollows and the shells. Microscopic observations indicate that the wood under the specimens is devoid of bacteria (data not shown). The occurrence of *Pectinodonta* in these depressions suggests that individuals always rasp the wood at the same place, which would preclude a regular recolonization by bacteria, making the bacterial biofilm grazing hypothesis (Wolff, 1979) unlikely.

Macroscopic, light and electron microscopic observations show that the gut is filled with tightly packed wood chips (Figs 1g and 2). Careful examination failed to reveal any conspicuous bacterial community associated with the wood fragments (Fig. 2c, d), free in the digestive tract or even attached to the epithelium. Very few prokaryotes were observed, sparsely distributed in the gut lumen (not shown).

Cellulase activity was assayed in the gill, foot and digestive mass of *Pectinodonta*. The activities, measured per milligram protein against filter paper in the different tissues, are summarized in Fig. 3. The highest activity was measured in the digestive mass. The value (48×10^{-3} μmol glucose min^{-1} mg^{-1} protein) is more than fourfold higher than that measured in the foot (10.7×10^{-3} μmol glucose min^{-1} mg^{-1} protein), and about sixfold higher than that in the gill (8.2×10^{-3} μmol

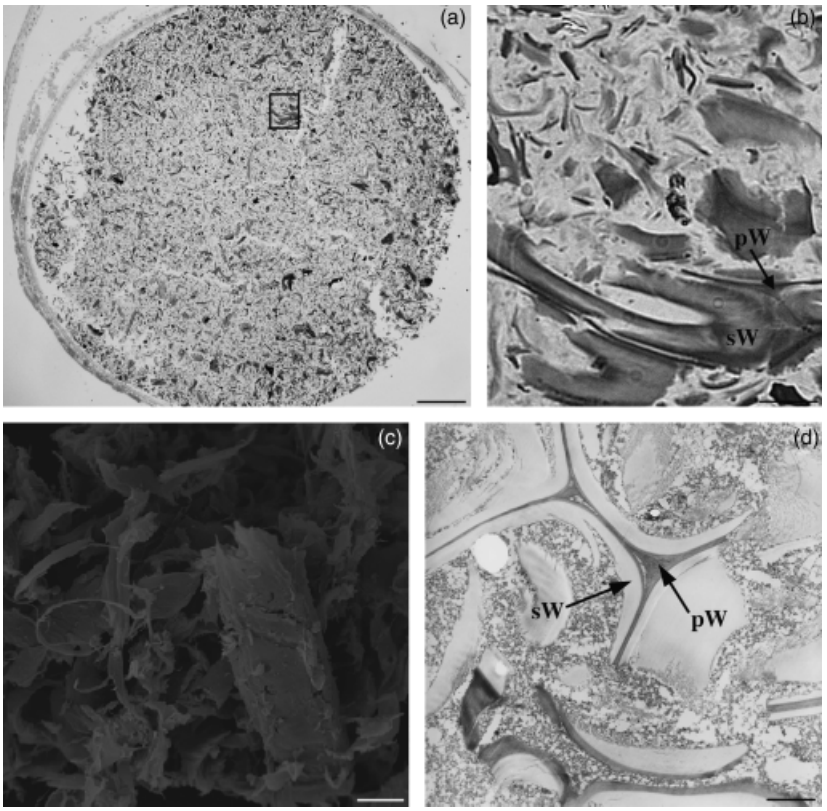

Fig. 2. *Pectinodonta* digestive tract. (a–b) Semi-thin section of the gut, full of wood fragments [square in (a) is enlarged in (b)]. (c–d) SEM and TEM views, respectively, of the gut content, full of wood fragments. Wood cell walls can be seen on the semi-thin and thin sections (sW, secondary wall; pW, primary wall). Note the absence of associated microorganisms. Scale bars: a = 100 μm, b = 10 μm, c = 5 μm, d = 2 μm.

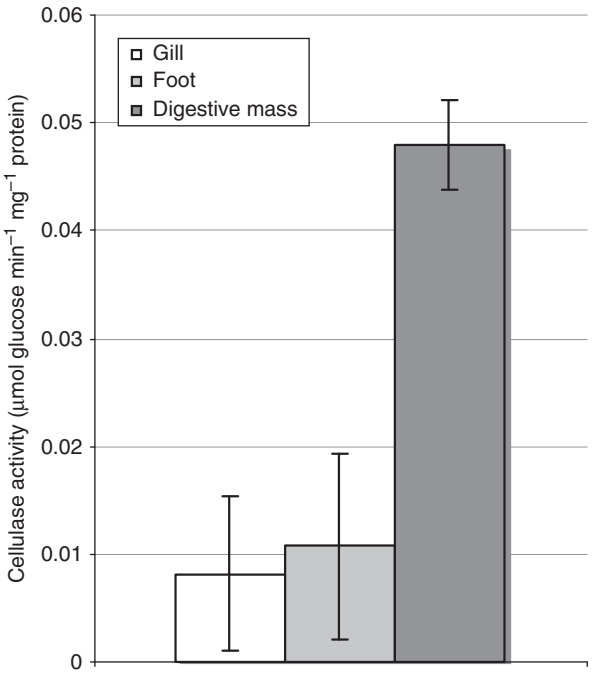

Fig. 3. Cellulase activity in *Pectinodonta* sp. tissues. Values are expressed as means ± SD.

glucose min^{-1} mg^{-1} protein). In comparison, the cellulase activity measured for the digestive mass is higher than that of a commercially available cellulase preparation from the cellulolytic fungi *Aspergillus* sp. (11.6×10^{-3} μmol glucose min^{-1} mg^{-1} protein). *Pectinodonta* are thus apparently able to degrade cellulose either with the help of symbiotic partners or using their own enzymes, as it occurs, respectively, in lower and higher wood-boring termites (Ohkuma, 2003).

The contribution of the wood to the carbon pool of *Pectinodonta* was supported by stable isotope analyses (Fig. 4). The $\delta^{13}C$ signature of *Pectinodonta*-2004 (-24.3 ± 0.3‰) was close to the $\delta^{13}C$ isotopic values of the wood (-27.0 ± 0.3‰) on which it was sampled (see Fig. 1a). These ^{13}C isotopic ratios are very similar to those published by Nishimoto *et al.* (2009) for the wood-boring bivalve *Xylophaga* sp. (-24.1 ± 0.4‰) and its host wood (-26.9 ± 1.1‰). Another potential carbon source in the ocean may be phytoplankton, but $\delta^{13}C$ isotopic ratios are less negative (-19‰ to -22‰, Fontugne & Duplessy, 1981; Rau *et al.*, 1982), suggesting that the wood is a better candidate as the main source of *Pectinodonta* diet. Among tissues of both *Pectinodonta*-2004 and *Pectinodonta*-2006, the most depleted values were consistently recorded for the digestive tract (-26.0 ± 0.1‰ and -25.4 ± 0.7‰, respectively) and the most enriched values were obtained for the

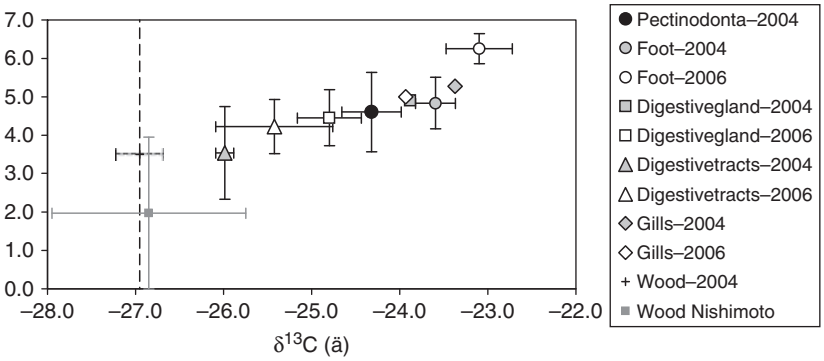

Fig. 4. Stable isotope signatures ($\delta^{15}N$ vs. $\delta^{13}C$) of isolated tissues (foot, digestive gland, digestive tract, gill), whole animal from *Pectinodonta*-2004 and its food source (wood). White symbols show the isolated tissues of *Pectinodonta*-2006 specimens, and the gray show those for *Pectinodonta*-2004 specimens. Black symbols are for the entire animal. As the nitrogen content of our wood sample was negligible, we only present the $\delta^{13}C$ value (-27‰) with its SD (± 0.3). For comparison, wood isotopic values from the Nishimoto *et al.*'s (2009) study are included in the figure.

gill (-23.4‰ and -24.0‰, respectively) and the foot (-23.6 ± 0.2‰ and -23.1 ± 0.4‰, respectively) (Fig. 4). Tissues such as muscles (foot) have a slow turnover, reflecting the food source integration of a long period of time, whereas tissues such as the digestive gland have a faster turnover, reflecting the diet of a shorter period of time (Lorrain *et al.*, 2002). This may explain the different ^{13}C isotopic ratios measured among the different organs for both *Pectinodonta* specimens (Fig. 4). No statistical difference was observed on the $\delta^{13}C$ isotopic signatures of the foot tissue between *Pectinodonta*-2006 (stored frozen) and *Pectinodonta*-2004 (stored in ethanol) specimens (ANOVA: $F_{(1,1)} = 4.33$; $P = 0.08$). The $\delta^{15}N$ signatures of the 2004 and 2006 specimens were between 3.5‰ and 6.3‰, the lowest value being measured from the digestive tract and the highest value being measured from the foot and the gill (Fig. 4). The nitrogen content within the wood was negligible (C/N ratio was very low; data not shown) and organisms that feed on wood in marine habitats, as seen in *Teredinidae*, obtain their nitrogen thanks to symbionts (Lechene *et al.*, 2007; Nishimoto *et al.*, 2009). Therefore, the missing data on the $\delta^{15}N$ signature of the wood in this study would not provide more insight into the fractionation of 3–4‰ usually observed between the primary producer and the primary consumer (De Niro & Epstein, 1978; Minagawa & Wada, 1984).

Considering only the *Pectinodonta*-2004 specimens, for which the $\delta^{13}C$ of the wood substrate was measured, a 3.5‰ shift was observed between the wood and the different tissues, well above the typical 1‰ difference documented for a single trophic level (De Niro & Epstein, 1978) (Fig. 4). This suggests that the wood is not directly, or not only, used by the limpet. Possible explanations are as follows: (1) an additional trophic intermediate may exist between the host and the wood that fractionates carbon and leads to a ^{13}C enrichment, as seen for some termites (Tayasu, 1998) and wood-borers (Nishimoto *et al.*, 2009); (2) the limpet also receives a second source of food in its diet besides the wood that is more enriched in ^{13}C (such as phytoplankton), yielding a $\delta^{13}C$ value that is a mean of $\delta^{13}C$ values from these two different food sources. In this second case, the animal could digest the wood using either its own cellulases or cellulases provided by symbiotic bacteria. Considering the absence of anything except for wood in the gut, and the occurrence of bacteria combined with the high cellulolytic activity measured in the digestive mass, the first hypothesis seems to be more likely.

Associated bacterial communities and their potential role

TEM and FISH (the FISH probes used are summarized in Table 1) revealed two distinct bacterial communities occurring in high densities in the ventral part of the digestive gland and on the gill surface (Figs 5–7).

A total of 40 and 38 partial 16S rRNA-encoding gene sequences (from the digestive mass and gill, respectively) were obtained from two specimens. Full sequences representative of the different phylotypes were obtained and used for phylogenetic reconstruction (Fig. 8). Three phylotypes were recovered from the digestive mass, affiliated with *Alphaproteobacteria* (phylotype B-DT-1, 36 clones), *Bacteroidetes* (phylotype A-DT-19, three clones) and *Gammaproteobacteria* (phylotype A-DT-24, one clone). The digestive gland is well developed and surrounds the intestine in the visceral mass (see Fig. 1g). The dorsal and ventral lobes displayed different structures. The dorsal part consisted of large flattened lobes (roughly 500×200 μm) (Fig. 5a and inset) surrounded by a thick epithelium constituted of secretory cells. Neither FISH nor TEM observations revealed the occurrence of bacteria in these structures. The ventral part of the gland consisted of smaller rounded lobes (25–50 μm diameter) (Fig. 5b). Strong, unambiguous, bacteria-specific FISH signals were seen associated with these sections using probe EUB338 (Fig. 5c; see Table 1 for probes' description). TEM observations showed that rounded lobes consisted of structures with a central lumen surrounded by abundant rod-shaped, gram-negative bacteria, roughly 0.5×2 μm in size, located extracellularly among folds of the gland wall (Fig. 5d, e). These bacteria only hybridized with probes EUB338 and ALF968b (Fig. 6a, c), indicating that they likely correspond to phylotype B-DT-1, the dominant

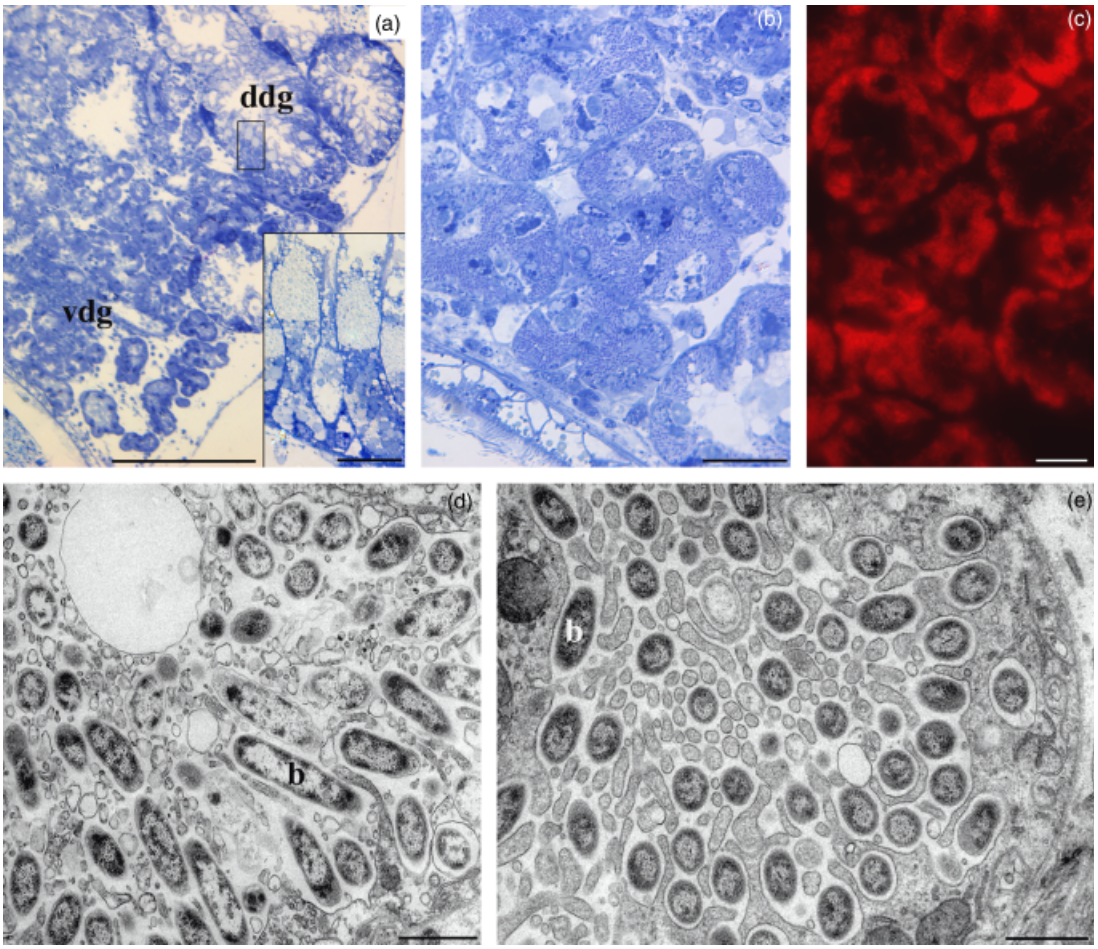

Fig. 5. *Pectinodonta* digestive gland. (a) Semi-thin section of the digestive gland, showing both large lobe sections of the dorsal digestive gland (ddg) and small lobe sections of the ventral digestive gland (vdg). Square in 'a' showing the secretory cells of the large lobes is enlarged in the inset. (b–c) Enlarged view of the ventral digestive lobe sections, (b, semi-thin section; c, FISH with EUB338). (d–e) TEM view of the ventral digestive gland, showing bacteria (b) in folds of the gland wall. Scale bars: a = 200 μm (inset: 20 μm), b = 20 μm, c = 10 μm, d and e = 1 μm.

phylotype recovered from the digestive mass. This alphaproteobacterial phylotype is moderately related (90% sequence identity) to several strains from seawater, a strain from the toxic dinoflagellate *Alexandrium tamarense*, several strains within genera *Ochrobactrum*, *Ahrensia* and *Labrenzia*, and to the heterotrophic denitrifying bacterium *Pseudovibrio denitrificans* (Shieh *et al.*, 2004).

The two other bacterial phylotypes, A-DT-19 and A-DT-24, cluster within the *Bacteroidetes* and *Gammaproteobacteria*, respectively. FISH using group-specific probes GAM42 and CF319 indicated that *Gammaproteobacteria* and *Bacteroidetes* were present within the gland, but only in restricted areas (Fig. 6b, e). They occurred in the ventral part of the gland, restricted to a thin layer delimiting large cavities (Fig. 6d, and see Fig. 1g for localization). Both types of bacteria were mixed, but no cohybridization was observed, indicating that the cooccurrence of the two bacterial types was real, and suggesting that they could function as a consortium. The closest relatives of A-DT-24 (95–96% sequence identity), *Psychromonas* sp., are obligate to facultative anaerobic psychrophilic heterotrophs, able to hydrolyze polymers such as starch (Auman *et al.*, 2006). Phylotype A-DT-19 is moderately related (92–93% sequence identity) to several *Bacteroidetes*, including uncultivated clones, as well as strains within the genera *Zobellia*, *Flexibacter*, *Arenibacter* and *Cellulophaga*, which are reported to be chemoorganotrophs and to be proficient in degrading various biopolymers such as cellulose, chitin and pectin (Kirchman, 2002). Such properties would make sense in bacteria associated with a digestive gland, and support the potential role of the bacterial community in wood digestion and measured cellulolytic activity. Because wood debris were not observed within the gland, the action of bacteria on wood is indirect, probably via extracellular enzymes transferred to the gut lumen through ducts. This has been suggested previously for *Teredinidae*, which harbor symbionts in their gill tissues. It is still unclear how the cellulolytic

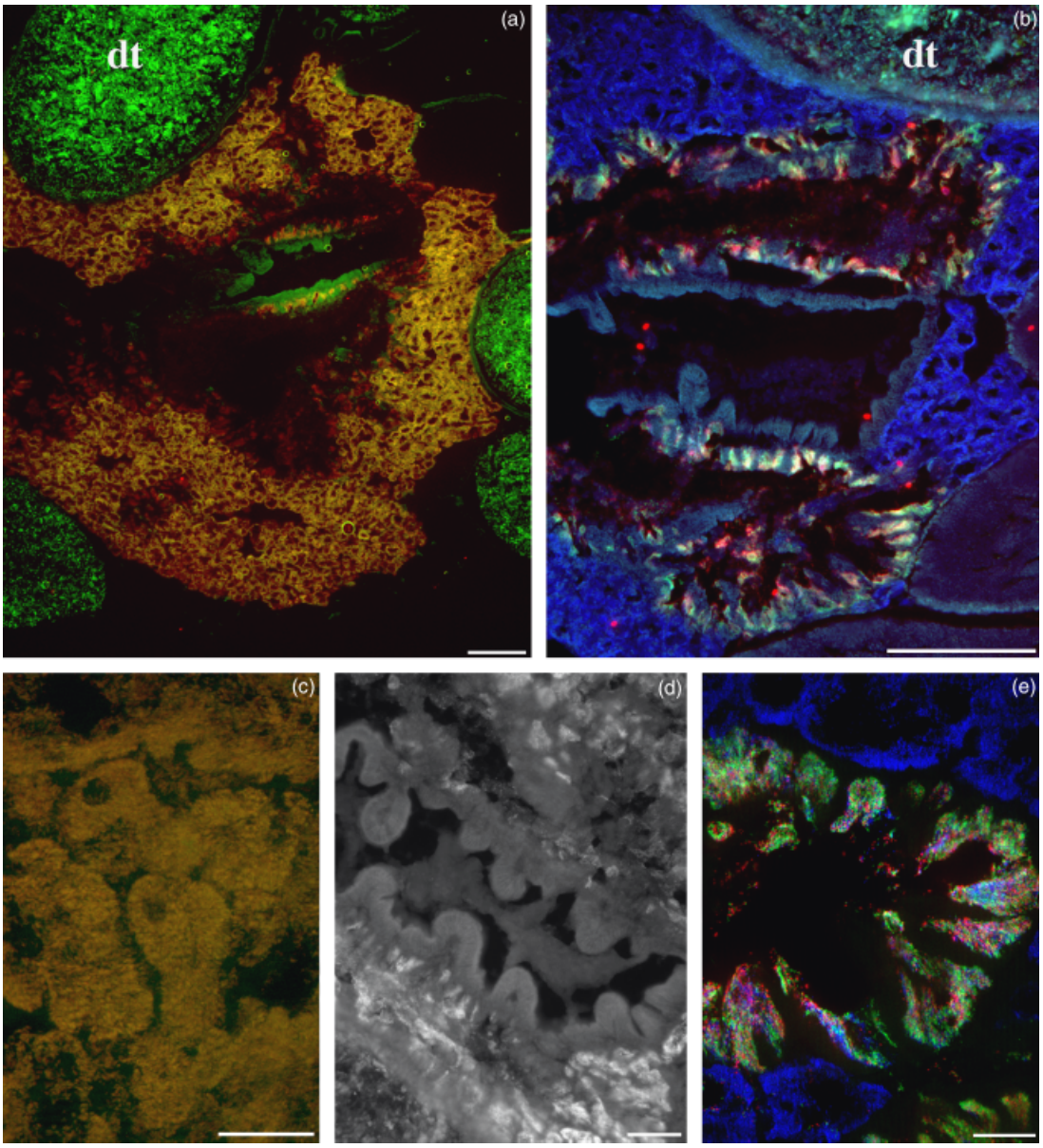

Fig. 6. *Pectinodonta* digestive gland: FISH observations of the ventral digestive gland (see localization on Fig. 1). (a) [enlarged in (c)], *Alphaproteobacteria* appear in yellow (composite view of a double hydridization: ALF968b signal in green, EUB338 signal in red). Wood in the digestive tract (dt) section appears green due to autofluorescence. (b) [enlarged in (e)], *Alphaproteobacteria* appear in blue (targeted only by the EUB338 probe), *Bacteroides* appear in cyan/green (CF319 probe signal in green) and *Gammaproteobacteria* appear in magenta/red (GAM42 probe signal in red). (d) View of the epithelium containing the *Bacteroides* and *Gammaproteobacteria* of a specimen prepared for FISH. Scale bars: a, b = 200 μm; c, d, e = 20 μm.

enzymes produced in the shipworm gills can be transported to the gut where the wood is degraded. Saraswathy (1971) suggested that a duct in the afferent branchial vein could connect the gills to the esophagus, but it has never been observed. The 'scaly-foot snail' (family *Peltospiridae*, gen. nov., sp. nov., Warén *et al.*, 2003) also houses a dense bacterial endosymbiotic population of thiotrophic *Gammaproteobacteria*, located in its esophageal gland. In this species, a hypertrophied circulatory system was highlighted between the gland and the foot, which seems to be the path for metabolic exchange between the bacteria and their host (Goffredi *et al.*, 2004). Apart from cellulose digestion, another possible role that deserves further attention is nitrogen fixation. Indeed, nitrogen-fixing bacteria are reported to be associated with many animals with a nitrogen-deficient diet, such as termites and *Teredinidae* bivalves (Waterbury *et al.*, 1983; Lechene *et al.*, 2007).

A second bacterial community, distinct from that of the digestive gland, was observed on the gill. Abundant

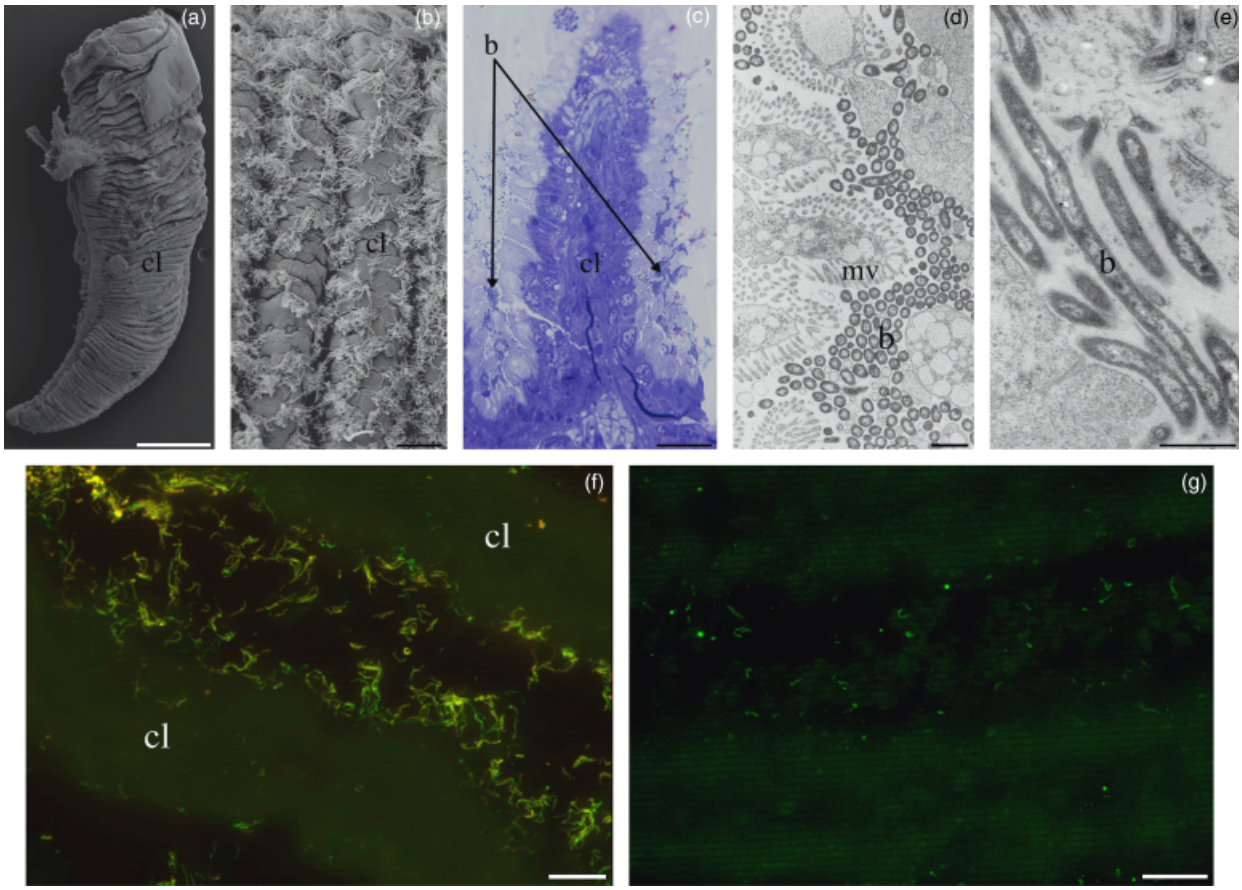

Fig. 7. *Pectinodonta* ctenidium. (a, b), SEM view of the whole gill (a) and a closer view on the lamella (b) showing the cilia. (c) Semithin sections of a ctenidial lamella, showing the bacteria (b) between the lamella. (d, e), TEM views of the gill, showing the bacteria (b) located close to microvilli (mv) of the lamella. (f, g) FISH observations of the gill showing numerous *Deltaproteobacteria* (f) [dual hybridization with probes EUB338 (green) and DEL495a (red), resulting in a yellow color], and only a few *Arcobacter* (g) (ARC94 probe signal in green). cl, ctenidial lamella. Scale bars: a = 500 μm; b, c = 20 μm; d, e = 1 μm; f, g = 10 μm.

filamentous bacteria were attached to the surface of host gill epithelial cells. The gill, a true ctenidium, located over the head and oriented from the left to the right (Figs 1d and 7a), is typical of the *Acmaeidae* family (Sasaki, 1998). The surface of the ctenidial lamellae is ciliated and bacterial filaments were located among the cilia, at the surface of animal cells (Fig. 7b–d). They seemed to be anchored on the cell surface (not shown). Each filament is a single, long and thin bacterium (0.3 μm in diameter and up to 5.5 μm in length, Fig. 7e) with a typical gram-negative membrane. All filaments hybridized with the Bacteria-specific probe EUB338.

Gill-associated phylotypes included *Epsilon-*, *Delta-* and *Gammaproteobacteria*, and *Bacteroidetes*. Based on FISH results (Fig. 7f and g), most of the gill bacteria hybridized with the *Deltaproteobacteria*-specific probe DEL495a. Two closely related deltaproteobacterial phylotypes were identified. B-G-64 and A-G-4 (0.2% divergence, 18 out of 36 clones) are not closely related to any known bacterium. Their closest relative, clone zEL76, a sequence obtained from a biofilm collected at a sulfidic cave, displayed only 87% sequence similarity. Because many *Deltaproteobacteria*, such as clone zEL76, live in sulfur-rich environments, it can be hypothesized that a metabolism involving sulfur compounds is likely. Many *Deltaproteobacteria* involved in sulfur metabolism are anaerobic and produce sulfide; hence, their occurrence in the gill appears to be a paradox. However, a different metabolism cannot be ruled out because of the low similarity to known bacteria. *Epsilonproteobacteria* were also present in the gill (phylotype B-G-52, 18 clones, Fig. 7g), related to a *Helicobacter* clone identified in the gorgonian *Muricea elongate* (91% sequence identity), whose role is unknown. Phylotypes B-G-4 and B-G-14, related to *Gammaproteobacteria* and *Bacteroidetes*, respectively, were detected in clone libraries (one clone each), but FISH did not confirm the occurrence of these groups (not shown).

Epi- or endosymbiotic associations were previously reported to be associated with the gills of several gastropod

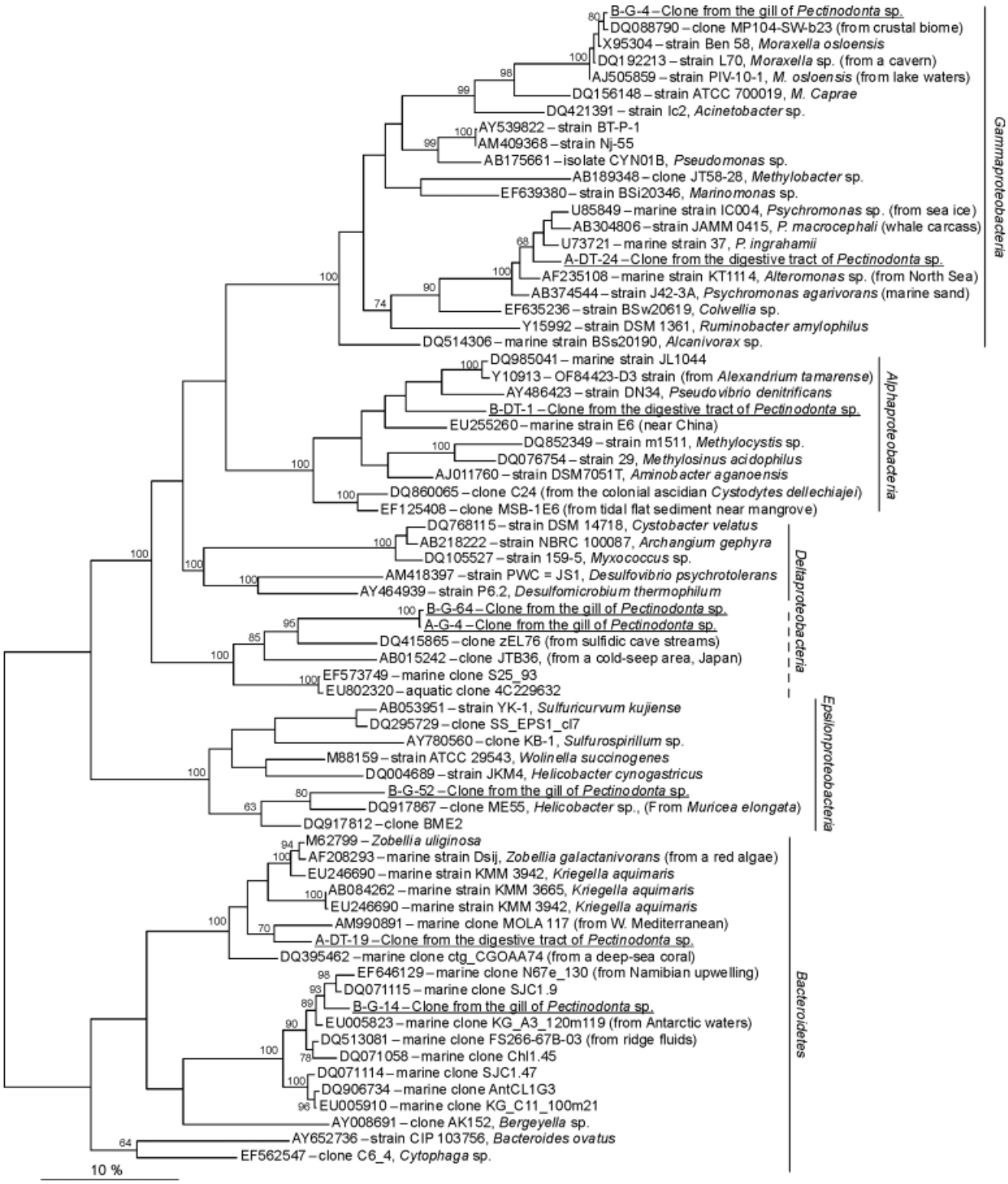

Fig. 8. Tree displaying the phylogenetic position of 16S rRNA-encoding gene sequences recovered from the gill and digestive tract of *Pectinodonta* (underlined). The tree was obtained using a ML algorithm. The value at the nodes represents percentage bootstrap support (1000 replicates, > 60% shown). *Bacteroidetes* are used as an outgroup. Scale bar corresponds to estimated 10% sequence divergence.

species, although not involving *Deltaproteobacteria* (*Lepetodrilus fucensis*, de Burgh & Singla, 1984; Bates, 2007a, b; several species of the genus *Alviniconcha*, Suzuki *et al.*, 2006; *Ifremeria nautilei*, Borowski *et al.*, 2002). These bacteria, with sulfur- or methane-linked metabolisms, are thought to contribute to host nutrition and also to protect the host

from the toxicity of sulfide. In the case of *Pectinodonta* however, the nutritional role of gill-associated bacteria is supported neither by stable isotope analyses nor by the low cellulolytic activity measured in that tissue.

Overall, bacteria identified in the digestive gland and on the gill are only distantly related to known bacteria. The closest documented relatives of *Pectinodonta* in terms of both the host phylogeny and the food source would be the shipworms, but limpet symbionts are not closely related to shipworm's symbiotic bacteria. Physiological assumptions based only on the phylogenetic position of *Pectinodonta*-associated sequence must be made with caution, and thus the exact role and contribution of each bacterial partner cannot be addressed as yet. In particular, the metazoan or the bacterial origin of cellulolytic activity measured in the digestive mass needs to be ascertained. The detailed study of sunken wood-associated organisms is an emerging field of deep-sea research and, except for shipworms, little information is available. *Pectinodonta*, with its bacterial populations associated with two organs, appears to be an interesting new model of interaction between metazoans and bacteria.

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