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Colwellia and sulfur-oxidizing bacteria: An unusual dual symbiosis in a *Terua* mussel (Mytilidae: Bathymodiolinae) from whale falls in the Antilles arc

Sébastien Duperron [a,b,*], Olivier Gros [c,d]

[a] Sorbonne Universités, UPMC Université Paris 06, UMR7208 Laboratoire biologie des organismes et écosystèmes aquatiques (UPMC CNRS MNHN IRD CAEN), 7 quai St Bernard, 75005 Paris, France
[b] Institut Universitaire de France, Paris, France
[c] Institut de Biologie Paris-Seine, UMR 7138 - Evolution Paris-Seine, Equipe Biologie de la Mangrove. Université des Antilles, UFR des Sciences Exactes et Naturelles, Département de Biologie, BP 592, 97159 Pointe-à-Pitre cedex, Guadeloupe, France
[d] C3MAG, UFR des Sciences Exactes et Naturelles, Université des Antilles, BP 592, 97159 Pointe-à-Pitre, Guadeloupe (French West Indies), France

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ABSTRACT

Seven individuals of a single morphotype of mussels (Bivalvia: Mytilidae) were found attached to a naturally sunken whale intervertebral disk collected in Guadeloupe (Caribbean) at 800 m depth. These specimens resemble small *Idas* mussels which are found worldwide at cold seeps and hydrothermal vents, and typically harbor ectosymbiotic bacteria on their gills upon which they depend for nutrition. Based on multi-locus gene sequencing, these specimens appear to belong to a new species closely related to two species now included within the genus *Terua*. Unexpectedly, its closest relatives are found in the Pacific, questioning how this species has reached the Antilles arc. Based on marker gene sequence analysis, electron and fluorescence microscopy, *Terua* n. sp. harbors two distinct and abundant extracellular bacterial symbionts located between microvilli at the apical surface of host gill epithelial cells. One is a sulfur-oxidizing bacterium similar to the symbionts previously identified in several deep-sea mussels, while the other is related to *Colwellia* species, a group of cold-adapted heterotrophic bacteria able to degrade organic compounds. This study provides the first evidence for the existence of a dual symbiosis in mussels from whale fall ecosystems in the Caribbean. The evolutionary history of *Terua* n. sp. and potential role of its *Colwellia* symbionts are discussed.

1. Introduction

Large mussels associated with deep-sea hydrothermal vents and cold seeps have been studied since their discovery in the late 1970s. Their abundance at many sites and their nutritional reliance on dense populations of gill-associated bacterial symbionts which use reduced sulfur or methane to produce their organic matter attracted interest from many researchers. They form the clade Bathymodiolinae, named after the genus *Bathymodiolus* in which most large mussels are included. Many papers have investigated how these symbiotic bivalves could thrive in *a priori* inhospitable habitats and how they had evolved (Dubilier et al., 2008; Duperron, 2010). Over the last decade smaller mussels, some described in the 19th century and overlooked since then, were shown to also belong to the Bathymodiolinae clade (Lorion et al., 2010, 2013; Thubaut et al., 2013). They live in reducing habitats including vents and seeps, but also on sunken carcasses of large animals and on wood falls, the decaying of which releases reduced compounds including sulfide and methane. The classification of small mussels is challenging because genus names do not overlap with gene phylogeny-based clades, but small species are of prime importance if we are to understand the evolution of deep-sea symbiont-bearing mussels. Indeed, large mussels cluster within a limited number of terminal clades, appearing as derived specialized forms, while the rest of the Bathymodiolinae phylogeny consists of various groups of small mussels. Biologically-speaking, apart from their small size, they share important features with large mussels, including the presence of bacterial symbionts in their gills (Deming et al., 1997; Gros and Gaill, 2007; Duperron et al., 2008b; Southward, 2008). However their symbioses seem to be more diverse and flexible. Large mussels usually have one to four types of bacteria located inside their gill epithelial cells. Small mussels on the other hand can display intra- or extracellular

* Corresponding author at: Sorbonne Universités, UPMC Université Paris 06, UMR7208 Laboratoire biologie des organismes et écosystèmes aquatiques (UPMC CNRS MNHN IRD CAEN), 7 quai St Bernard, 75005 Paris, France.
E-mail address: sebastien.duperron@upmc.fr (S. Duperron).

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bacteria, a greater diversity of symbionts with up to 6 distinct types co-existing in the gills of *Idas modiolaeformis*, not restricted to sulfur- and methane-oxidizers, and the composition of bacterial communities can vary between sites for a given species (Duperron et al., 2008a; Laming et al., 2015b). Unfortunately, because of the patchy and unpredictable occurrence of their habitats, small mussels are often collected by chance in very limited numbers. Nevertheless their study yields important information for our understanding of the diversity, biogeography and evolution of the fauna colonizing deep-sea reducing habitats.

Seven species of Bathymodiolinae mussels are currently reported from the Gulf of Mexico and Antilles arc, all from cold seep habitats. Four are large *Bathymodiolus* (*B. keckerae, B. boomerang, B. brooksi*, *B. childressi* and a yet unnamed species), and two are smaller species, namely *Tamu fisheri* usually collected from 540 to 700 m depth associated with vestimentiferan tubeworm bushes, and *Idas macdonaldi* from 650 m depth (Gustafson et al., 1998; Faure et al., 2015). Dell (1987) also mentions *Idas dalli* E.A. Smith 1885 sampled from the Culebra Island (Porto Rico, West Indies) during the Challenger expedition, but little data is available. Seven mussel individuals representing a single morphotype were recently found attached to an inter-vertebral disk of a naturally sunken whale collected near Guadeloupe (Caribbean) at 800 m depth. In this study we test whether these represent a new species, or one of the 7 previously documented from the region, using multiple marker gene sequencing and phylogeny. We also characterize associated bacterial symbionts using electron microscopy, marker gene sequencing and fluorescence *in situ* hybridization (FISH). Results are discussed with a special emphasis on mussel biogeography, and on the potential role of the identified symbionts. This study is the first investigation of bone-associated symbiotic mussels from the Antilles arc.

2. Materials and methods

2.1. Sample collection and preparation

One inter-vertebral disk from a whale carcass was collected using a beam-trawl 780–820 m depth during the Karubenthos2 cruise (June 2015, chief scientist: P. Bouchet) around Guadeloupe in the Caribbean [16°23′N, 60°46′W]. Mussel individuals up to 2 cm in length were found attached to the surface of a spinal disk obtained from a unique naturally submerged whale carcass. Mussel samples were processed onboard within 1 h after collection.

Six mussel individuals were stored in 100% ethanol for molecular investigation of bacterial diversity (see below) after severing adductor muscles. One individual was prepared for electron microscopy. The two adductor muscles were severed in order to open the bivalve, then the whole animal was pre-fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.2) adjusted to 900 mOsM with NaCl and $CaCl_2$ and stored at 4 °C. Two weeks later, after returning to the laboratory, the gills were dissected into three parts. Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) were performed to detect possible symbiosis. Samples for SEM observations were rinsed twice in the same buffer then dehydrated in graded concentrations of acetone, critical point dried in CO_2 and sputter-coated with gold before observation with a FEI Quanta 250 at 20 kV. For TEM observations, samples were rinsed in the same buffer, fixed for 45 min at room temperature in 1% osmium tetroxide in the same buffer with a final osmolarity adjusted to 1000 mOsm, then rinsed in distilled water and post-fixed with 2% aqueous uranyl acetate for one more hour at RT before embedding in epon-araldite resin and observation in a FEI Quanta 250 at 15 kV under the STEM mode.

2.2. Energy-dispersive X-ray spectroscopy (EDXs) analysis

In order to detect elemental compounds from the gill (such as sulfur), the gill fragment stored in the fixative solution was observed using an Environmental Scanning Electron Microscope (FEI Quanta 250) operating at 10 kV under an environmental pressure of 7 Torr at 8 °C. EDX spectra were obtained using a X-max 50 mm^2 Oxford SDD detector. Gills were rinsed briefly in distilled water (to remove salts) and observed without prior dehydration.

2.3. Gene sequence analysis

DNA was extracted from the gill tissue from two specimens according to the protocol previously described (Duperron et al., 2005). Fragments of host genes encoding H3 histone, 28S rRNA, mitochondrial cytochrome oxidase I (mtCOI) and mitochondrial 16S rRNA (mt16S), and fragments of bacterial genes encoding 16S rRNA and APS reductase were amplified by PCR. Products were cloned (products from 3 parallel PCR were pooled prior to cloning for bacterial 16S rRNA) using the PCR® 2.1-TOPO TA cloning kit (Invitrogen, CA), and inserts from selected clones were sequenced by GATC Biotech (Germany). PCR primers, cycling conditions and number of analyzed clones are summarized in Table 1. Sequences were deposited under GENBANK accession numbers KU747145-KU747153.

For each gene, representative sequences, their best hits according to BLAST (Altschul et al., 1990) and selected available sequences were aligned using ClustalX. Alignments were manually curated and phylogenetic reconstructions were performed using

Table 1
PCR primers, cycling conditions (temperature and number of cycles) and number of clones analyzed for each of the genes sequenced from *Terua* n. sp. Primers were obtained from: (Hassouna et al., 1984; Lane, 1991; Folmer et al., 1994; Colgan et al., 1998; Baco-Taylor, 2002; Meyer and Kuever, 2007; Ratnasingham and Hebert, 2007; Duperron et al., 2008a).

	Gene	T (°C) and cycles	Primer names	Primer sequences (5′3′)	Seq length (nt)	Reference	Clones analyzed
Host	COI mtDNA	50 °C (35)	H691	GTRTTAAARTGRCGATCAAAAAT	627	Duperron et al., 2008a	6
			LCO1490	GGTCAACAAATCATAAAGATATTGG		Folmer et al., 1994	
	28S rRNA	55 °C (35)	C1prime	ACCCGCTGAATTTAAGCAT	1128	Hassouna et al., 1984	9
			C4	TCGGAGGGAACCAGCTACTA		Hassouna et al., 1984	
	mt16S rRNA	55 °C (35)	16SA	GGARGTASGCCCTGCCCWATGC	466	Baco-Taylor, 2002	7
			LRJ	CTCCGGTTTGAACTCAGATCA		Ratnasingham and Hebert, 2007	
	H3	57 °C (35)	F1	ATGGCTCGTACCAAGCAGACVGC	351	Colgan et al.,1998	12
			R1	ATATCCTTRGGCATRATRGTGAC		Colgan et al., 1998	
Bacteria	16S rRNA	48 °C (31)	27F	AGAGTTTGATCCTGGCTCAG	~1500	Lane, 1991	271
			1492R	GGTTACCTTGTTACGACTT		Lane, 1991	
	APS	60 °C (30)	APS1-FW	TGGCAGATCATGATYMAYGG	400	Meyer and Kuever, 2007	21
			APS4-RV	GCGCCAACYGGRCCRTA		Meyer and Kuever, 2007	

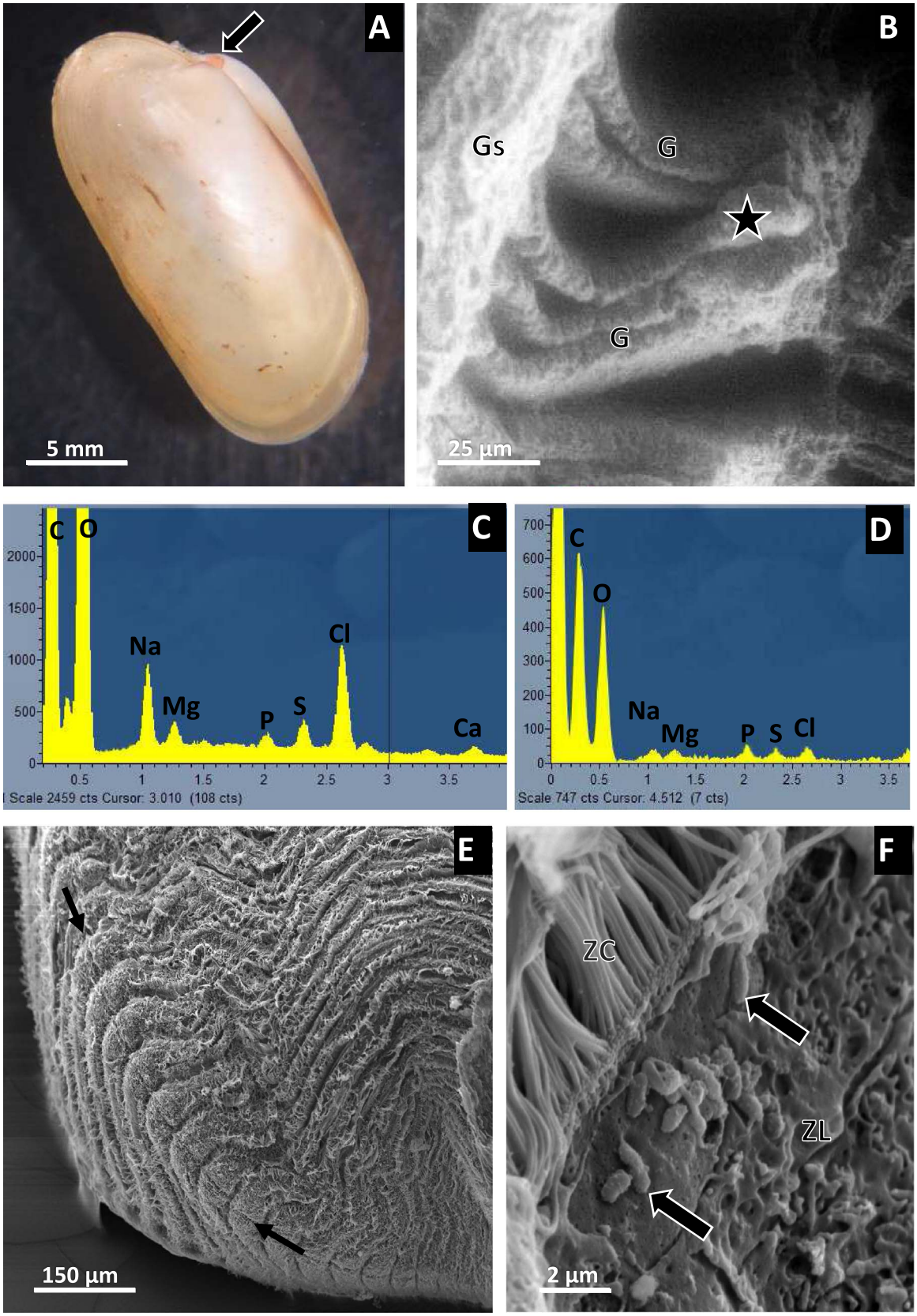

Fig. 1. Structural analysis of mussel specimens collected from sunken whale inter-vertebral disk. A: Light micrograph of a freshly collected mussel at a depth of 800 m. The shell appears smooth with a visible pink prodissoconch located at the umbo (arrow). **B:** ESEM image obtained from a piece of gill not dehydrated with solvents, showing seven gill filaments (G) attached to the gill axis (Gs). **C:** EDX spectrum obtained from one of these gill filaments showing a peak of elemental sulfur (11 times higher than in EDX spectra obtained from mantle or foot [D]) suggesting that gill filaments harbor sulfur-oxidizing bacteria (C: carbon, Ca: calcium, Cl: chloride, Na: sodium, Mg: magnesium, O: oxygen, P: phosphorous, S: elemental sulfur). E: SEM views show the faint marginal groove (arrows) along the ventral edge of the gill and the numerous cilia on the frontal surface (E-F). F: Just below the ciliated zone of each gill filament (ZC), some bacteria could be observed (arrows) attached to the apical pole of the cells forming the lateral zone (ZL) of each gill filament. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Mega v.6 using a Maximum Likelihood (ML) approach under a General Time Reversible (GTR) model. To account for different substitution rates among sites, gamma-distributed rates with 5 categories and invariants were included. For host genes, a multigene phylogeny was produced using concatenated sequences of mtCOI, mt16S and H3.

2.4. Fluorescence in situ hybridization (FISH)

Specimens were embedded in Steedman's wax or LR-White according to described protocols, and sectioned using a microtome (Thermo). FISH was performed using standard probes Eub-338 and Non-338 as positive and negative controls, respectively. Probe Bthio-193 displayed perfect match with the 16S rRNA-encoding sequence related to sulfur-oxidizing (SOX) bacteria and was used to test for its presence in the tissues (5′-CGAAGATCCTCCACTTTA-3′ (Duperron et al., 2007)). Probe Alt1413 (5′-TTTGCATCCCACTCCCAT-3′) showed no mismatch to the other, *Colwellia*-related abundant sequence in clone libraries and was used to test for the presence of this potential second bacterium (Eilers et al., 2000). It was designed to target the genus *Alteromonas* but also matches one third of available *Colwellia* sequences according to the ProbeMatch

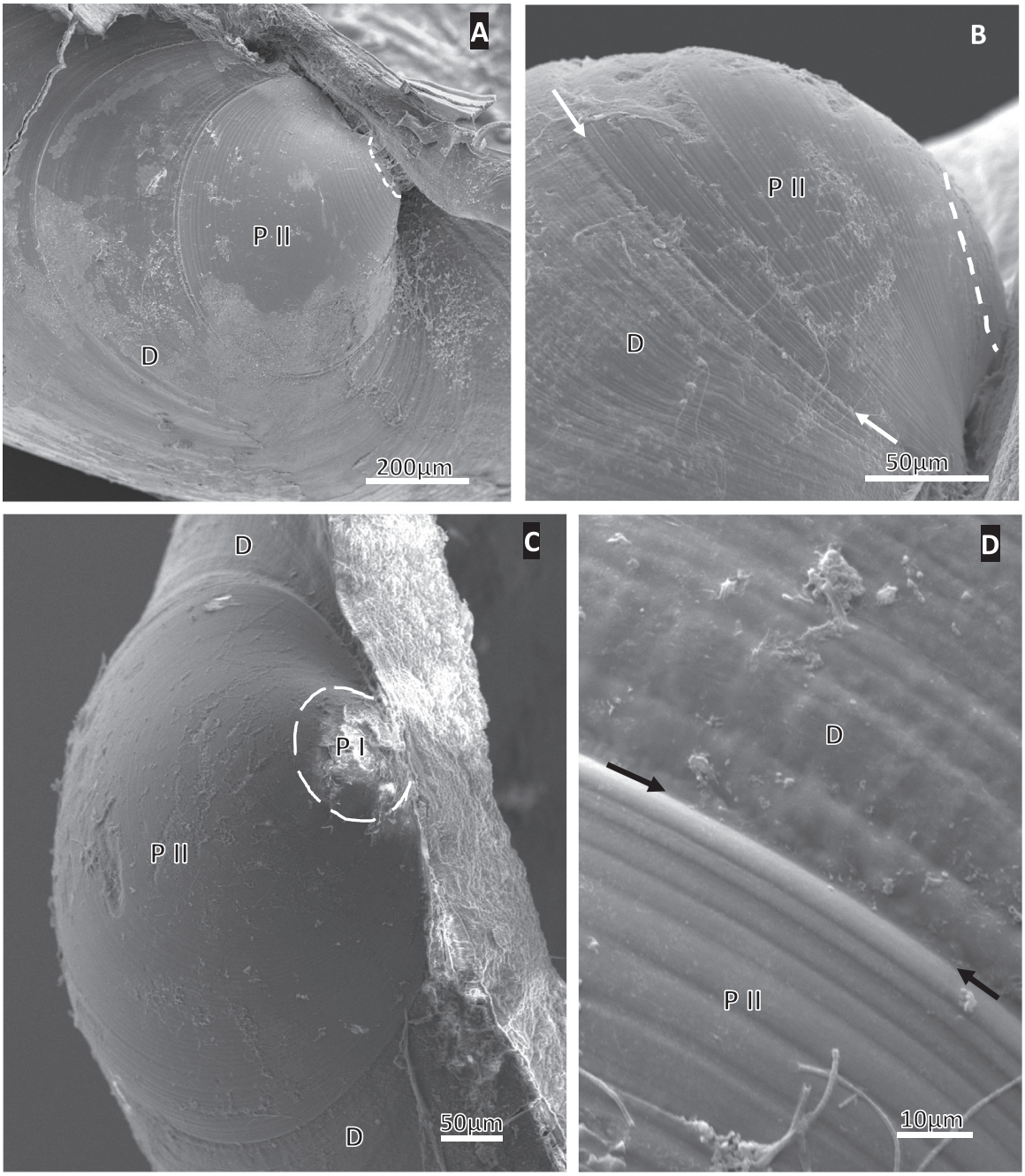

Fig. 2. SEM views of larval shell morphology. A: The larval shells (appearing in pink in Fig. 1A) correspond to a small prodissoconch I (PI delineated by a white dot line) and a larger prodissoconch II (P II) with a total mean length of 445 µm. In adult individuals, the umbo is curved and prevents the observation of the prodissoconch I. The separation between prodissoconch II and the adult shell (dissoconch, D) is obvious. **B:** In a lateral view, the numerous tight growth lines of the prodissoconch II clearly suggests a lengthy planktotrophic larval development. The metamorphic line separating prodissoconch and dissoconch (D) is indicated by straight arrows while the periphery of the prodissoconch I is delineated by a dot line. **C:** In this dorsal view, once the right valve is removed, the small prodissoconch I (dot line) appears smooth while the larval shell (P II) is characterized by numerous concentric growth lines. **D:** Higher magnification focusing on the metamorphic line (arrows) that delineates the dissoconch characterized by smoother growth lines compared to the prodissoconch II. Prodissoconch I (PI); prodissoconch II (PII); dissoconch (D).

function of Ribosomal Database Project (Cole et al., 2009).

Hybridizations were performed according to Duperron et al. (2005) using buffers containing 30% and 40% formamide, and probes were labelled with Cy-3 or Cy-5. Slides were counterstained with DAPI, and observed under an Olympus BX61 epifluorescence microscope (Olympus, Japan).

3. Results

3.1. Host features

The 7 small mytilids (shell length between 9 and 12 mm) displayed identical shell features, including a pink whole prodissoconch 445 ± 13 μm in length (Figs. 1A, 2A-D) and an adult dissoconch appearing smooth under a binocular microscope. According to SEM views, prodissoconch I was small (~92 ± 11 μm) and the prodissoconch II measured 445 ± 13 μm with several concentric growth lines (maximum length between two adjacent lines: ~9 μm). The visceral mass of two individuals (shell lengths of 10 and 12 mm) was filled with oocytes (not shown) indicating that they were adults, 6 fold above the size at maturity reported for the deep sea *Idas modiolaeformis* (Laming et al., 2014). Mean oocyte diameter was 63 ± 5.7 μm (not shown).

3.2. Molecular identification of mussels

The two specimens from which host genes were sequenced displayed identical sequences for all 4 genes based on 6–12 clones (Table 1). The sequence encoding 28S rRNA displayed only 1 out of 1002 base difference with sequences from *Adipicola pacifica* and ESU T, and above 99% identity with many sequences from various mussels. Due to this, 28S rRNA was not included in the concatenated analysis. Nucleotide sequences encoding mt16S, mtCOI and H3 all displayed ESU T as their best hit with 97%, 89% and 100% sequence similarity, respectively. *Adipicola pacifica* usually came second with 93%, 84% and 99%, respectively. Phylogenetic reconstructions obtained for each gene placed ESU T, collected from bones at depths between 800 and 1060 m near the Vanuatu (Lorion et al., 2013), as a sister group to the mussels from this study with high bootstrap values (above 95%, not shown). The tree based on concatenate gene sequences confirmed that our specimens were closely related to ESU sp. T, and that this group clustered with *Adipicola pacifica*, both nodes being supported by bootstrap values above 98% (Fig. 3).

3.3. Ultrastructural analysis of gill tissue

The overall structure of gill filaments in all specimens was comparable to that described previously in other mussel morphotypes (Gros and Gaill, 2007; Duperron et al., 2008b). According to SEM views, the creamy colored gills, which cover the visceral mass, are organized in consecutive gill filaments (Fig. 1B, D). The surface of the gills contains a dense covering of cilia that could carry food along a food groove (Fig. 1D-E) to the mouth region. The presence of such groove could suggest a mixotrophic feeding mode in this species. The ciliated zone is similar to that described in other mytilid species with typical frontal, latero-frontal, and lateral ciliated cells (Fig. 4A). In the ciliated zone, the core of each filament is occupied by a collagen axis which encloses few

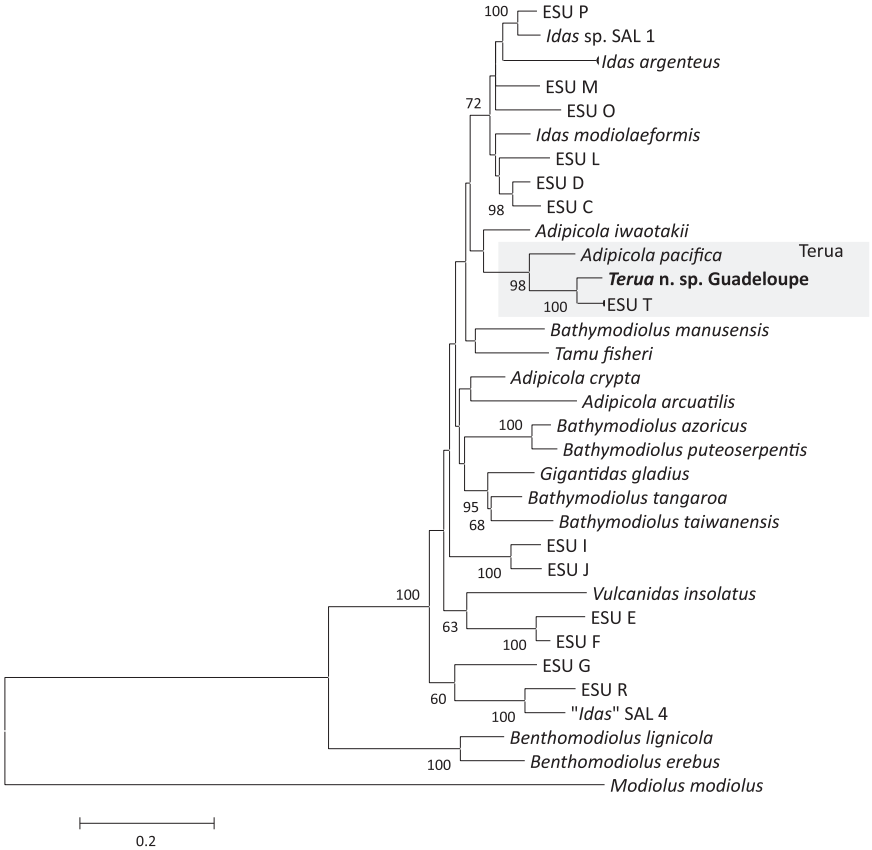

Fig. 3. Phylogeny of Bathymodiolinae based on concatenate sequences of fragments of genes encoding mtCOI, mt16S rRNA and histone H3. A total of 1099 nucleotide positions were analyzed, positions with gaps and missing data were removed. A ML approach was used (see Section 2 for detail). Scale bar represents 20% estimated sequence divergence. Only bootstrap values above 60 are displayed. The clade where sequences from this study cluster (in bold) and corresponding to the genus *Terua* sensu Thubaut 2013 is emphasized. Accession numbers of sequences used for this reconstruction are summarized in Supplementary material S1.

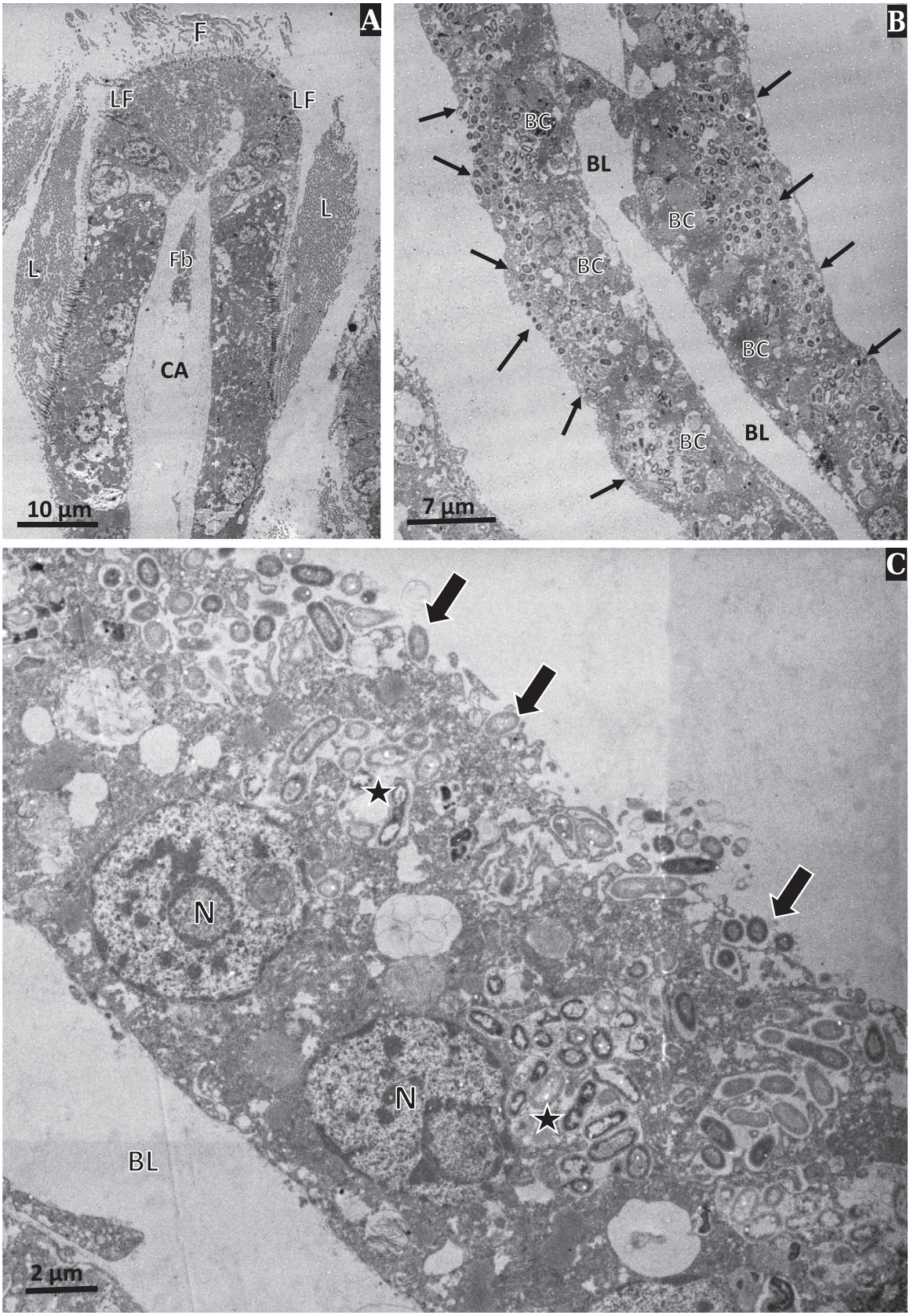

Fig. 4. Ultrastructural analysis (TEM) of the gill filaments. A: The ciliated zone of the gill filaments is free of bacteria and classically organized with several ciliated cells. This ciliated zone consists of a simple epithelium, which is in contact with a connective axis (CA), with cells harboring frontal cilia (F), latero-frontal cilia (LF), and lateral cilia (L). Fibroblasts (Fb). **B:** The lateral zone of each gill filament is organized as a pseudostratified epithelium organized around its blood lacuna (BL). Each epithelial cell (BC) harbors numerous bacteria (arrows) throughout the lateral zone of the gill filament. **C:** Higher magnification of the lateral zone focusing on two bacteriocytes. The various bacterial shapes observed (rod-shaped or ovoid-shaped figures) could be due to the section orientation. Note that two morphotypes are observable, one displaying higher density in the periphery of the cells (one almost black, the other one gray). Extracellular bacteria are located on the apical surface of the host cells in contact with microvilli (arrows). Bacteria are observed outside the bacteriocytes but also inside vacuoles (stars), each of which contains numerous bacteria with a shape similar to that of extra cellular ones. These bacteria probably became enclosed in the vacuoles by phagocytosis of extracellular bacteria. N: nucleus of the host cell.

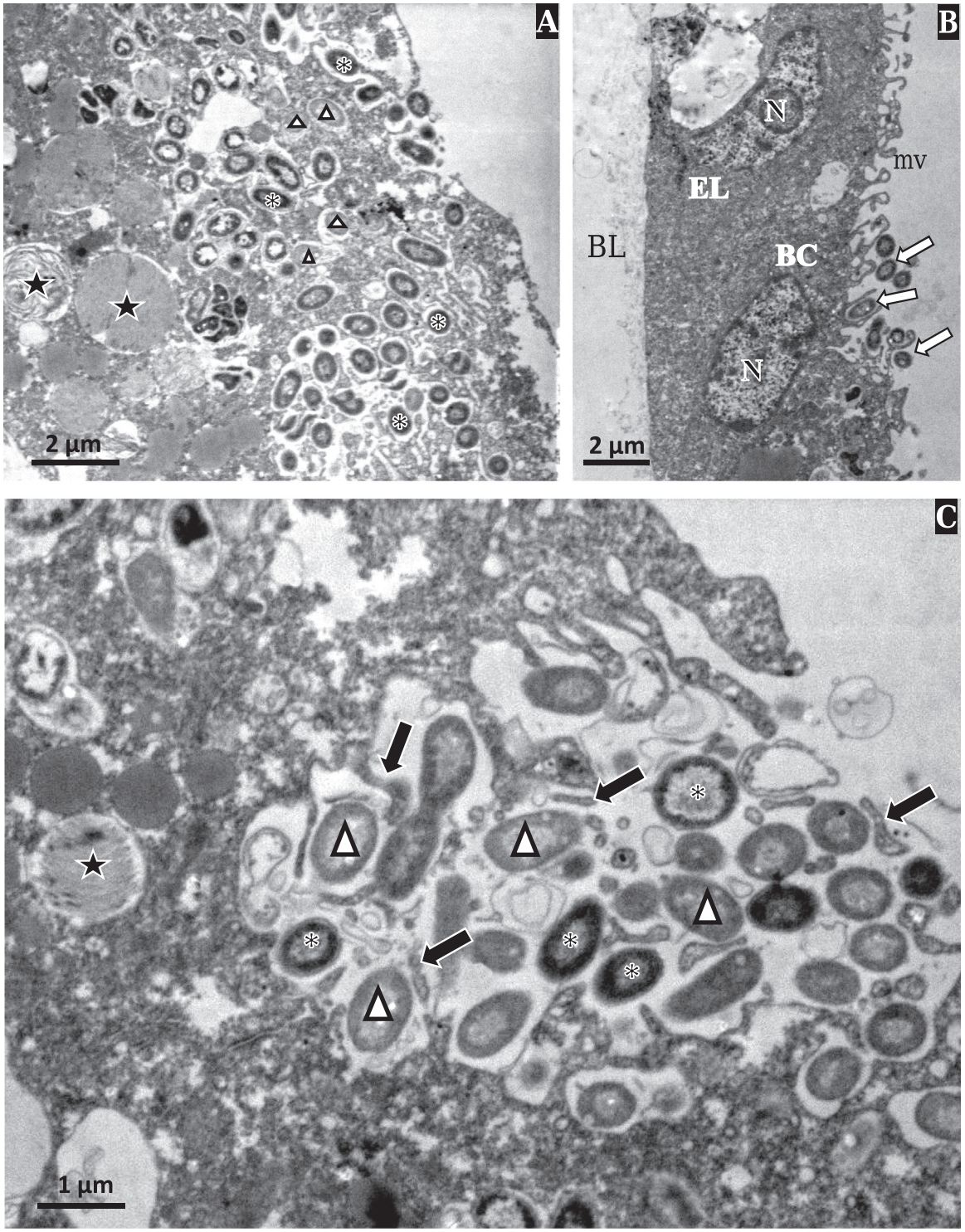

Fig. 5. Ultrastructural analysis of the bacteriocytes. A-C: The cytoplasm of the bacteriocytes contains residual bodies, characterized by whorls of membranes (stars), probably resulting from the intracellular digestion of extracellular bacteria as previously described in wood-mussels (Gros and Gaill, 2007). In these TEM views, most of the bacteria seem to be intracellular due to their location within large vacuoles resulting of phagocytosis. **B:** The first bacteriocyte (BC) of the lateral zone in contact with the last eulateral cell (EL) harbors only extracellular bacteria (arrows) located between the microvilli (mv). **C:** microvilli (arrows) are obvious inside the vacuole containing numerous bacteria, some of them dividing. Note the two bacterial morphotypes easily distinguishable (**A, C**); a dark one (asterisks) with a black periphery of the bacterial cell and a clearer one (white triangles). BL: blood lacuna. N; nucleus of host cells.

fibroblasts, whereas, in the lateral zone, it is occupied by connective tissue and a blood lacuna (Fig. 4B-C). The intermediary zone is short as the first cell in contact with the ciliated zone contained bacteria on its surface for all the samples studied (Figs. 1F, 4C). The ciliated zone always appeared devoid of bacteria.

A few gill filaments were analyzed in EDXs using an environmental SEM (Fig. 1B) searching for elemental compounds. The analysis showed that sulfur was one of the main elements detected from the gills where it was much more abundant than in mantle or foot (Fig. 1C and D), suggesting the putative presence of sulfur-oxidizing bacterial symbionts. Furthermore, ultrastructural analyses of thin sections of the specimens observed by TEM

confirmed that bacteria were associated with the gill tissue and were mostly extracellular (Figs. 4B-C, 5C). Moreover, bacteria with a similar morphotype compared to the extracellular ones could be present within vacuoles (Figs. 4C, 5A, C). Gill epithelial cells displayed a basal nucleus (Figs. 4C, 5B), few lysosome-like structures (Fig. 5A, C) and numerous bacteria inside vacuoles (Figs. 4B-C, 5A) or between the microvilli in their apical pole (Fig. 4C). The lateral zone of each filament consisted of a single cell type bearing the bacterial symbionts (Figs. 4 and 5). No intercalary cells and/or mucocytes could be detected in the samples analyzed.

Individually, the bacteria were small and rod shaped (1 μm length, 0.3 μm thick) with a cell wall typical of gram-negative bacteria (Figs. 4C, 5C). No stacked intracellular membranes typical of methanotrophic symbionts were seen. Differences in the shape of individual bacteria are due to section orientation. However, two morphotypes could be distinguished from the gill sections. The first one was characterized by a ribosome-rich cytoplasm appearing as a black area, localized at the periphery of the cell while the center of the cell containing the DNA was clearer (Figs. 4C, 5A-C). This morphology is unusual compared to previously described sulfur-oxidizing bacterial symbionts of bivalves. The second morphotype was characterized by a clearer cytoplasm (Figs. 4C, 5A-C). Both morphotypes seem to be equally (and randomly) distributed through the lateral zone of each gill filament. The first bacteriocytes contained extracellular, but no envacuolated bacteria, as shown by SEM (Fig. 1F) or TEM (Fig. 5B) views.

3.4. Molecular identification of associated bacteria

Two 16S rRNA-encoding gene sequences dominated clone libraries in both specimens investigated. The first phylotype displayed 10 out of 1501 base differences with *Colwellia* sp. ZS4-15 (FJ889666) cultured from Antarctic intertidal sediment (Yu et al., 2010), and above 99% sequence identity with various *Colwellia* including *C. psychroerythraea* 34H of which the full genome is available (CP000083 (Methe et al., 2005)) and *C. rossensis* from pack ice (AY167311 (Brinkmeyer et al., 2003)). The second phylotype displayed 99% identical positions with various sequences recovered from sulfur-oxidizing symbionts associated with deep-sea mussels, differing from clone M1.2 from *Idas modiolaeformis* by 11 out of 1492 aligned positions (Duperron et al., 2008a). The phylogenetic reconstruction confirmed these affiliations (Fig. 6). The *Colwellia*- and SOX-related phylotypes represented respectively 62.9% and 35.7% of clone sequences in specimen 1 (140 clones), and 41.2% and 58.0% in specimen 2 (131 clones). The three remaining clones (KU747151-3) corresponded to three distinct Epsilonproteobacteria related to various deep-sea bacteria. Several distinct sequences were obtained in clone libraries of the APS reductase-encoding gene. The most abundant, representing 13 of the 21 clones, was 93% and 98% (nucleotide and amino acids, respectively) similar to sequences previously obtained from mussel-associated SOX symbionts, suggesting that it could belong to the SOX-related bacterium identified herein.

3.5. FISH-based identification of potential symbionts

Hybridizations using the probe Eub-338 confirmed the presence of dense bacterial populations associated with the lateral zone of gill filaments in all 3 specimens investigated. Probe Bthio-193 targeting SOX symbionts labelled only a fraction of the bacteria labelled with the general probe Eub-338 (Fig. 7A-B). SOX signals could usually be seen spanning a number of neighbouring bacteriocytes, while large areas of the lateral zone were devoid of such signals. Probe Alt1413 targeting the *Colwellia*-related phylotype hybridized with those bacteria that were not labelled by Bthio-193 (Fig. 7C-D). Due to inappropriate fixation, FISH signals

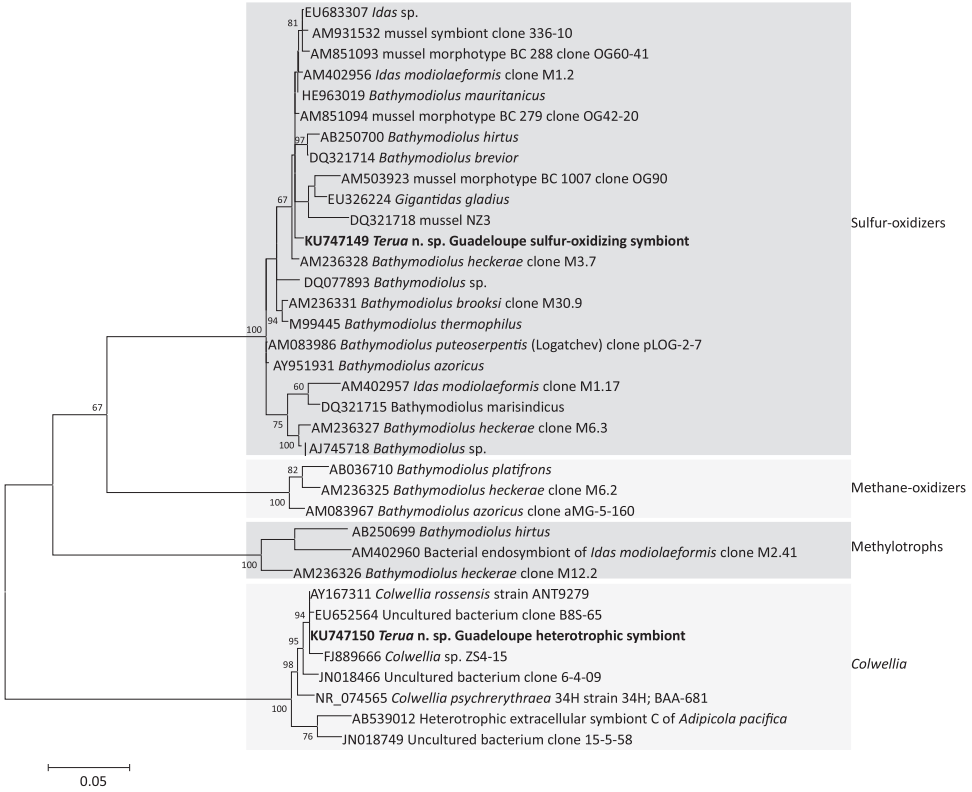

Fig. 6. Phylogeny based on 16S rRNA-encoding gene sequences from bacterial symbionts associated with Bathymodiolinae. Names refer to the host species, clades comprising sulfur-oxidizing, methane-oxidizing, methylotroph-related, and *Colwellia*-related bacteria are indicated. A total of 1197 nucleotide positions were analyzed. See Section 2 for detail. Scale bar represents 5% estimated sequence divergence. Bootstrap values above 60 are displayed. Sequences from this study in bold.

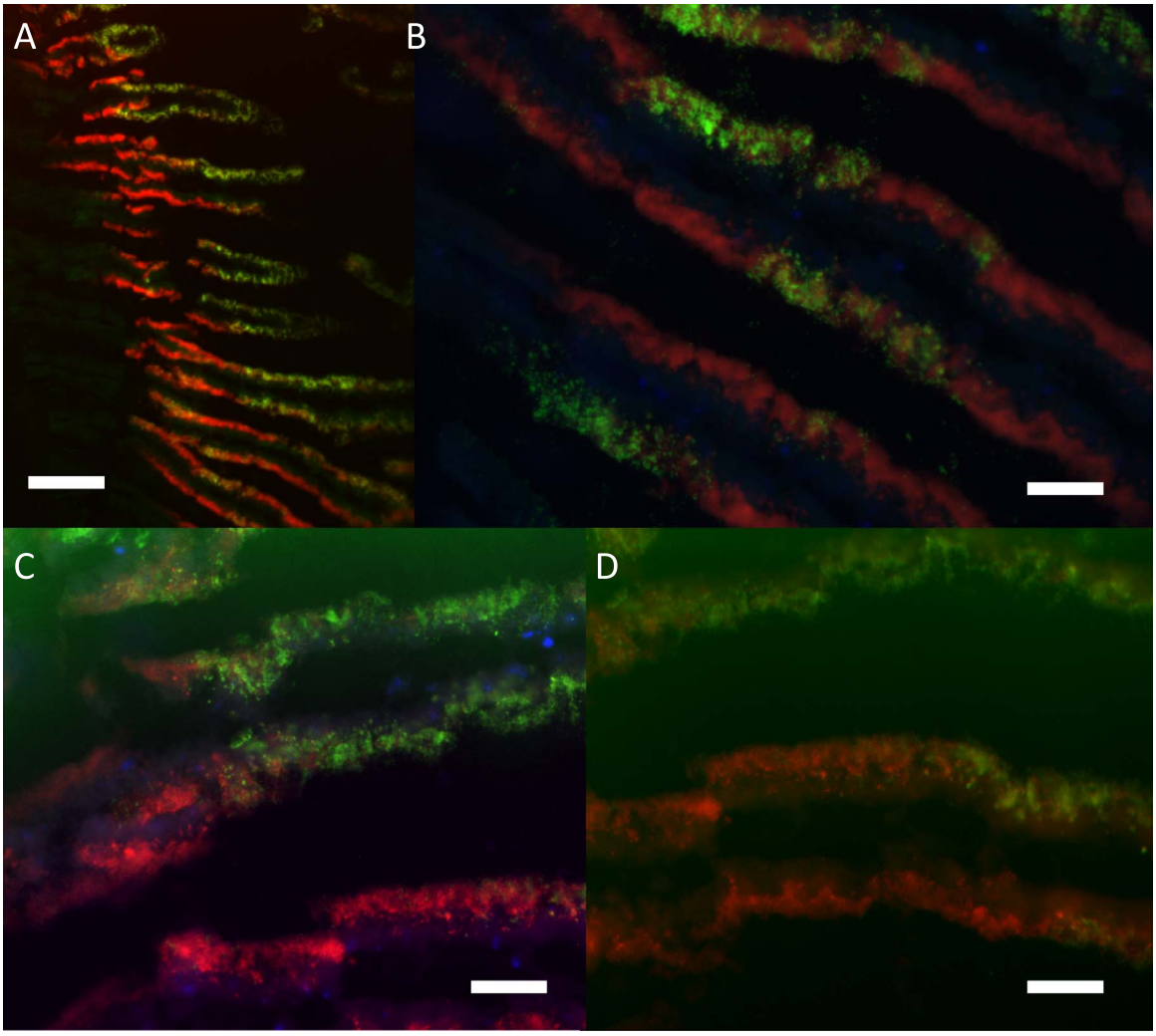

Fig. 7. Fluorescence *in situ* hybridization on sections of gill filaments of *Terua* n. sp. Guadeloupe. Signal from probe Bthio-193 is in green in pictures A to D (Cy-3 labelled in A-B, Cy-5labelled in C-D). Signal from probe Eub-338 is in red in A and B (Cy-5 labelled), signal from probe Alt1413 is in red in C and D (Cy-3 labelled). DAPI appear in blue in B and C. **A-B:** overview of several gill filaments, bacteria appear in red, sulfur-oxidizers in green or yellow. **C-D:** detail of gill filaments. Sulfur-oxidizing symbionts appear in green, *Colwellia*-related symbionts in red. Note the coexistence of the two symbionts in the gills. Scale bars represent 100 μm (**A**) and 25 μm (**B-D**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were not as sharp as could be, yet signals from probes Alt1413 and Bthio-193 did not seem to overlap, suggesting they succeeded in distinguishing the two main phylotypes identified in clone libraries.

4. Discussion

4.1. Unexpected occurrence of Terua n. sp. in the Antilles arc

According to various authors, the lack of specific shell features makes the latter poor predictors of species relatedness in small deep-sea mussels (Won et al., 2008; Lorion et al., 2010). Marker gene sequence analysis has thus proven to be the most useful tool to properly assign species. Sequence-based identification has revealed that most morphology-based genera within the Bathymodiolinae were not monophyletic. Genus names are thus of little help when describing mussel evolutionary relationships (Thubaut et al., 2013). Specimens from the present study clustered between *Adipicola pacifica* and ESU T. Their mtCOI nucleotide sequences differed by 16.4% and 11.4% from those of *A. pacifica* and ESU T, respectively, suggesting that they belong to a distinct, yet-to-be described species. Thubaut and co-workers recently suggested that *A. pacifica* and ESU T should be classified within the genus *Terua* initially described by Dall and his colleagues in 1938 (Thubaut et al., 2013). Following their recommendation we refer to our specimens as *Terua* n. sp. Guadeloupe in the following sections. Small Bathymodiolinae occur in the North West Atlantic and Gulf of Mexico, including *Idas macdonaldi* (sister species to *I. modiolaeformis* in Fig. 3) and *Tamu fisheri*, but they are not closely related to *Terua* n. sp. The closest relatives of *Terua* n. sp. are reported from the Pacific Ocean and are not related to any species recorded from the Antilles arc. Based on the 3 fossil-based calibrations used by Lorion et al. (2013) and using the RelTime method implemented in MEGA 6, *Terua* n. sp. Guadeloupe diverged from ESU T around 6.2 MYA. This is consistent with the closure of the Isthmus of Panama. A date around 8–12 MYA is often used as a time calibration in biogeography studies, however several studies question these dates and suggest final closure of the seaway later, possibly as late as 3 MYA (Bartoli et al., 2005). The divergence time estimated here suggests that colonization of the Gulf of Mexico by ancestors of *Terua* n. sp. could have happened before the seaway closed.

The shell provides another type of information. Here, the prodissoconch I (PI) measured 92 ± 11 μm, and the prodissoconch II (PII) measured $445\ \mu m \pm 13$ μm, within the range of values

published for several other small Bathymodiolinae including *Idas modiolaeformis, I. argenteus, I. iwaotakii* or *I. simpsoni* (74–96 μm for PI, ad 379–544 for PII, (Laming et al., 2015a)). Based on these values, it can be assumed that *Terua* n. sp. displays dispersal features similar to those reported in other clades within the Bathymodiolinae. The relatively small size of the oocytes (indeed with a low vitelline content) observed in 2 individuals, as well as the small PI size both confirm planktotrophic development in this new species of *Terua*. Similar oocyte diameter (41.5 ± 7.5 μm) was described in the small mytilid *Idas modioliformis* which displays a planktotrophic development (Gaudron et al., 2012). Based on much larger PII compared to PI sizes, these previous studies also inferred that larval life prior to settlement could potentially last for several months at low environmental temperatures.

4.2. An unusual type of dual symbiosis

According to clone libraries and *in situ* hybridizations on gill sections, two dominant bacteria occur in *Terua* n. sp. Guadeloupe. One is a sulfur-oxidizer closely related to various symbionts previously identified in mussels. Its capability to perform sulfur oxidation is further supported by identification of an APS reductase sequence displaying high similarity with those found in other SOX symbionts, and by the identification of abundant sulfur in the gills in EDXs analysis. Sulfur-oxidizing symbionts are reported in most Bathymodiolinae investigated to date in which they contribute to nutrition and, arguably, to sulfide detoxification (Distel et al., 1988; Dubilier et al., 2008). The second bacterium identified is a close relative of several *Colwellia* species. With a 16S rRNA sequence similarity above 99%, it can confidently be classified within this genus. Ultrastructural analysis indicates that bacteria are extracellular, a common feature of mussels associated with organic falls (Gros and Gaill, 2007; Duperron et al., 2008b). Clone libraries yielded similar amounts of the two 16S rRNA phylotypes, and FISH confirmed that both were abundant with possible dominance of the *Colwellia*-related bacterium in the investigated specimens. Both types of bacteria are thus likely to play a significant role in the association. In a study of mussels from whale falls near Japan, Fujiwara and colleagues also identified sulfur-oxidizing and *Colwellia*-related (symbiont C) bacteria as extracellular symbionts in the gills of *Adipicola pacifica* (Fujiwara et al., 2010). The 16S rRNA sequence from the latter symbiont (AB539012) is only 93.2% similar to the sequence reported herein and not its closest relative in the phylogenetic tree, and their FISH results indicated dominance of the sulfur-oxidizers. But overall it is reasonable to assume that the closely related *A. pacifica* and *Terua* n. sp. Guadeloupe which both live on whale carcasses share a similar type of dual symbiosis, different from the classical examples involving sulfur- and methane-oxidizers described in large vent and seep mussels (Fisher et al., 1993; Distel et al., 1995). Whether this unusual type of dual symbiosis is a peculiar feature of the *Terua* lineage or is strictly habitat-dependent needs to be further tested, notably on ESU T for which no data is currently available.

4.3. Potential significance of Colwellia-related bacterial symbionts in a dual symbiosis

The genus *Colwellia* currently comprises 13 described species, with many more sequences from environmental samples reported in databases. *Colwellia* are adapted to cold marine environments, and include no known pathogen. The two closest described relatives of the *Terua* n. sp. Guadeloupe symbiont are *C. rossensis* and *C. psychroerythraea*. The latter is considered an obligate psychrophile with optimal growth at 8 °C, congruent with recorded bottom water values (Methe et al., 2005). *Colwellia* are often reported attached to surfaces (Brinkmeyer et al., 2003) but the occurrence of a flagellum suggests that if a highly similar bacterium occurs as a symbiont, it probably also has a free-living stage during which the flagellum is used. The presence of genes encoding a flagellum is reported from various environmentally-transmitted symbionts including the sulfur-oxidizing bacterium associated with the vent annelid *Riftia pachyptila* (Robidart et al., 2008). Both *Colwellia* species related to the *Terua* symbiont able to degrade organic compounds (Methe et al., 2005). The 5.37 Mb genome from *C. psychroerythraea* strain 34H has been sequenced and possesses genes encoding various enzymes able to break down high molecular weight organic compounds. Many of these proteins seem to be extracellular. This capability could benefit the mussel host living on bone material, in particular inter-vertebrate disks, if we for example hypothesize a role in collagen degradation similar to what is reported for the *Osedax*-associated Oceanospirillales symbionts (Goffredi et al., 2014). It can be noted that two *Colwellia* species were cultured from animals, namely *C. asteriadis* from the skin of a starfish (Choi et al., 2010) and *C. meonggei* from a sea squirt (Kim et al., 2013). Potential interaction with their host was not investigated, but given that the 16S rRNA sequence identified here is above 99% identical to that of cultivated *Colwellia* species, it might be possible to attempt cultivation of the *Terua* n. sp. symbiont, should more become available in the future.

5. Conclusion

Terua n. sp. Guadeloupe is the first mussel species identified from whale fall ecosystems in the Antilles arc. Its absence from previous fauna samplings at various cold seeps in the Gulf of Mexico and western Atlantic suggests that it could be a true bone specialist, derived from ancestors found in the Pacific Ocean. Its dual symbiosis is unusual because most previously described bone-associated symbioses involved either only heterotrophic bacteria such as in the vestimentiferan *Osedax*, or sulfur-oxidizing autotrophs as in small mussels. Although the role of the *Colwellia*-related bacterium in the symbiosis is not ascertained, the fact that a similar dual symbiosis occurs in *Terua* n.sp. Guadeloupe's relative *Adipicola pacifica* suggests that this could be a particular adaptation to bone habitats.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr.2016.05.012.

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