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Microbial diversity associated with the hydrothermal shrimp *Rimicaris exoculata* gut and occurrence of a resident microbial community

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Abstract:

Rimicaris exoculata dominates the megafauna of several Mid-Atlantic Ridge hydrothermal sites. Its gut is full of sulphides and iron-oxide particles and harbours microbial communities. Although a trophic symbiosis has been suggested, their role remains unclear. *In vivo* starvation experiments in pressurized vessels were performed on shrimps from Rainbow and Trans-Atlantic Geotraverse sites in order to expel the transient gut contents. Microbial communities associated with the gut of starved and reference shrimps were compared using 16S rRNA gene libraries and microscopic observations (light, transmission and scanning electron microscopy and FISH analyses). We show that the gut microbiota of shrimps from both sites included mainly *Deferribacteres*, *Mollicutes*, *Epsilon-* and *Gammaproteobacteria*. For the first time, we have observed filamentous bacteria, inserted between microvilli of gut epithelial cells. They remained after starvation periods in empty guts, suggesting the occurrence of a resident microbial community. The bacterial community composition was the same regardless of the site, except for *Gammaproteobacteria* retrieved only in Rainbow specimens. We observed a shift in the composition of the microbiota of long-starved specimens, from the dominance of *Deferribacteres* to the dominance of *Gammaproteobacteria*. These results reinforce the hypothesis of a symbiotic relationship between *R. exoculata* and its gut epibionts.

Keywords: *Deferribacteres* • midgut epibiosis • *Mollicutes* • *Proteobacteria* • *Rimicaris exoculata* • starvation experiment

Introduction

Gut microbial communities are rather ubiquitous both in vertebrates and invertebrates. Symbioses between host and micro-organisms range from pathogenic to mutualistic, facultative to obligate relationships. Gut microbiotas have been characterized for insects such as termites (Breznak, 1982, Chaffron and Von Mering, 2007), isopods such as *Porcellio scaber* (Wang et al, 2004a, 2004b, 2007) and other invertebrates (Harris, 1993). These gut-associated microbial communities play a major role in the metabolism of the host, in

particular in the case of low level nutrient supply. Extreme environments such as deep-sea hydrothermal vents are oligotrophic and low oxygenated ecosystems enriched in numerous toxic compounds. Life under these conditions requires physiological adaptations of the fauna to low levels of nutrients and toxic environments. In such ecosystems, life is based on chemosynthetic primary production and microbial-invertebrates associations are widespread (Dubilier et al, 2008). Endosymbioses where bacteria are enclosed within bacteriocytes are well described in mussels such as *Bathymodiolus spp*. (Duperron et al, 2007) or vestimentiferan worms such as *Riftia pachyptila* (Dubilier et al, 1995), the latest even being deprived of an open digestive tract.

Rimicaris exoculata (Williams and Rona, 1986, Crustacea, Decapoda, Alvinocarididae) dominates the megafauna at several Mid-Atlantic Ridge (MAR) hydrothermal vent sites characterized by contrasted geochemical settings. End-member fluids from ultramafic-hosted sites, such as Rainbow, are usually enriched in methane (2.5 mM), hydrogen (16 mM) and iron (24.05 mM) but relatively depleted in sulphides (1.2 mM), in contrast to basalt-hosted sites like TAG (Trans-Atlantic Geotraverse, 0.124-0.147 mM CH_4, 0.15-0.37 mM H_2, 1.640 mM Fe, 6.7 mM H_2S; Charlou et al, 2002).

R. exoculata can form dense aggregates, of thousands individuals per square meter, living close to the active chimney walls, at temperatures between 10 to 20°C (Segonzac et al, 1993, Zbinden et al, 2004, Copley et al, 2007, Schmidt et al, 2008). They seem to “graze” the chimney walls, and are continuously in motion. *R. exoculata* has an enlarged gill chamber and hypertrophied mouthparts covered by thick microbial layers that could contribute to the shrimp nutrition and/or detoxification (Van Dover et al, 1988, Gebruk et al, 1993 and 1997, Segonzac et al, 1993, Polz and Cavanaugh 1995, Polz et al, 1998, Komaï and Segonzac, 2008, Zbinden et al, 2004 and 2008). Cephalothoracic epibionts of *R. exoculata* from Snake Pit first were affiliated to a single phylotype of *ε-proteobacteria* and assumed to be sulphide-oxidizers (Polz and Cavanaugh, 1995). In a recent study, shrimps from Rainbow displayed a

broader diversity of epibionts that may indicate an adaptation to the different geochemical conditions prevailing at this site (Zbinden et al, 2008).

Earlier observations have shown that *R. exoculata* is neither a predator, nor a scavenger. Although its gut seems to be functional (Casanova et al, 1993, Segonzac et al, 1993), the nutrition of this shrimp thus remains unclear. Three main nutrition strategies are currently suggested: (i) Micro-organisms from the chimney walls and the environment could constitute the main nutritional source (Van Dover et al, 1988), although $\delta^{13}C$ analyses revealed it was unlikely (Polz et al, 1998); (ii) The gill chamber epibionts could be the host main nutritional source, either by grazing on them (Gebruk et al, 1997, Casanova et al, 1993, Segonzac et al, 1993, Polz and Cavanaugh, 1995, Rieley et al, 1999, Gebruk et al, 2000), or by ingesting the bacteria and the exuviae after moult, or by trans-epidermal transfer of organic matter (Zbinden et al, 2004 and 2008, Corbari et al, 2008); (iii) Shrimps harbour a specific gut microbiota which could constitute an alternative nutritional source (Polz et al, 1998, Pond et al, 2000, Zbinden and Cambon-Bonavita, 2003). In a previous study of shrimps from Rainbow, no intact microbial cell was detected in the stomach, and the digestive microbial communities displayed a low diversity at the phylum level with mainly *ε-proteobacteria* (1/2), *Entomoplasmatales* (1/4) and *Deferribacterales* (1/4) (Zbinden and Cambon-Bonavita, 2003). However, as the gut was not empty, it was difficult to determine whether microbial communities were specific to the gut and/or ingested with minerals and surrounding seawater. Although the metabolisms and characteristics of these microbial communities are unknown, high carbon fixation rates have been measured in the gut (Polz et al, 1998), suggesting they could be involved in nutrition and/or detoxification processes.

The present study describes and characterizes the composition of *R. exoculata* gut-associated microbial communities, using 16S rRNA gene sequence analysis and microscopic observations. Shrimps from two MAR sites (TAG and Rainbow), representing contrasted geochemical settings and depths, were compared in order to test the influence of the hydrothermal habitat over the microbial gut community. To investigate the presence of a

108 resident microbial community, starvation experiments were performed, in order that shrimps completely expelled the bolus, by incubating live *R. exoculata* specimens during 8, 22 or 72
110 hours in sterile seawater within a pressurized incubator (IPOCAMP™, Shillito et al, 2001).

112 Materials and methods

Sample collection

114 Samples were obtained during the EXOMAR cruise in 2005 at the TAG (26°8'N-44°50'W, 3650 m depth) and Rainbow (36°14'N-33°54'W, 2320 m depth) hydrothermal vent sites on
116 the MAR. Shrimps were collected using the slurp-gun of the ROV *Victor 6000*, operated from the RV *L'Atalante*. Prior to each dive, the bowls of the slurp-gun used for collecting the
118 shrimps were aseptically washed with ethanol (96 %) before being filled with sterile seawater. Once on board, live shrimps were either immediately dissected under sterile
120 conditions and processed as reference samples, or placed into the pressurized incubator. The specimens were the same size and developmental stage (they were in the late
122 anecdysis stage). For DNA analyses, the stomach (foregut) was discarded to keep only the intestine-associated micro-organisms.

124 *In vivo experiments*

Live shrimps were incubated in a pressurized incubator (IPOCAMP™). The stainless steel
126 vessel (approximately 19 L) is a flow-through pressure system (Shillito et al, 2001). Pressure oscillations arising from pump strokes (100 rpm) were less than 1 bar at working pressure.
128 The temperature of the flowing filtered (0.4 µm) seawater was constantly measured and regulated, in the inlet and outlet lines (± 1°C).
130 Less than 2 hours after sampling, live and active animals were re-pressurized at *in situ* pressure: 230 bars at the Rainbow site and 300 bars at the TAG site (slightly less than the
132 TAG *in situ* pressure, i.e. 360 bars, due to instrumental limitations). The average incubation temperature was 15°C (Segonzac et al, 1993, Desbruyères et al 2001, Zbinden et al, 2004,

Schmidt et al, 2008). They were 20 shrimps per pressurized chamber. Previous *in vivo* experiments showed that the shrimps were under good physiological conditions when re-pressurized following this procedure (Ravaux et al, 2003, Zbinden et al, 2008). According to previous experiments, the specimens were starved in sterile seawater during 8, 22 or 72 hours, to eliminate the bolus and associated micro-organisms (Zbinden et al, 2008). The 8-hour starvation experiment was performed on Rainbow and TAG specimens whereas the 22- and 72-hour starvation experiments were conducted only for Rainbow shrimps. Live shrimps were dissected immediately after removal from the vessel and digestive tracts (Figure 1A) were stored for DNA analyses (frozen at -80 °C), fluorescent *in situ* hybridization analyses (FISH) and microscopic observations. No faeces could be collected in these experiments as they were completely dissolved in seawater.

16S rRNA gene sequence analysis

Phylogenetic analysis was done on six *R. exoculata* gut clone libraries (Rainbow site: 2 intestines from pooled reference samples, 1 from 8 hours-starved, 1 from 22 hours-starved, 1 from 72 hours-starved shrimps; TAG site: 1 from reference sample, 1 from 8 hours-starved shrimp) as follows: DNA was extracted on board on full length intestines with the FastDNA® SPIN kit for soil (QBIOgen, Santa Ana, CA, USA) following the manufacturer's instructions and kept at 4°C. Amplification of the bacterial 16S rRNA gene was performed with universal primers E8F/U1492R (respectively 5'-AGAGTTTGATCATGGCTCAG-3' and 5'-GTTACCTTGTTACGACTT-3', 1484 bp, annealing temperature 49°C) or E338F/U1407R (respectively 5'-ACTCCTACGGGAGGCAGC-3' and 5'-GACGGGCGGTGWGTRCAA-3', 1069 bp, annealing temperature 54°C) and for *Archaea* with A24F/A1492R (respectively 5'-CGGTTGATCCTGCCGGA-3' and 5'-GGCTACCTTGTTACGACTT-3', 1468 bp, annealing temperature 49°C). PCR were performed using a GeneAmp™ PCR System 9700 (Applied Biosystems, Forster City, CA, USA) under the following conditions: 3 min at 94°C, then 30 cycles including 1 min at 94°C, 1.5 min at the annealing temperature and 2 min at 72°C, and a final step of 6 min at 72°C. The PCR reaction mix (50 µL) was composed of 1 X Taq buffer,

0.8 µM dNTP mix (QBIOgen), 10 pmol of each primer (Eurogentec, Liège, Belgium), 2.5 U
162 Taq polymerase (QBIOgen) and approximately 100 ng DNA template. PCR products were cloned using the TOPO® TA Cloning kit (Invitrogen, Carlsbad, CA, USA) following the
164 manufacturer's instructions. Positive clones were sequenced at the "Plateforme Biogenouest" (Roscoff, France, http://www.sb-roscoff.fr/SG/) on an Abi prism™ 3100 GA using the Big-Dye
166 Terminator V3.1 chemistry (Applied Biosystems).

Sequences were analyzed using the NCBI BLAST search program within the GenBank
168 database (Altschul et al, 1990). They were aligned using CLUSTALW (Thompson et al, 1994) and edited using SEAVIEW (Galtier et al, 1996). Phylogenetic trees were constructed
170 using PHYLO-WIN (Galtier et al, 1996). The robustness of inferred topologies was tested using 500 bootstraps re-sampling of the trees (Felsentein, 1985) calculated by the neighbor-
172 joining algorithm (Saitou and Nei, 1987) with Kimura two parameters correction matrix. Sequences displaying over 98 % similarity were considered to belong to a single phylotype
174 (OTU) and were clustered together. Only homologous positions were included in the final alignment.

176 Sequences were named as: R or T for Rainbow or TAG specimens respectively, the number in the clone library, and R for reference shrimp, SW, S and LS for an 8, 22 and 72-hour
178 seawater starvation experiments respectively. They are available from the EMBL nucleotide sequence database under accession numbers FM863726 to FM863780 and FM865857 to
180 FM865858.

For each library (one reference sample from TAG, two references pooled from Rainbow, one
182 8 hours-starved shrimp from each TAG and Rainbow, one 22 hours and one 72 hours-starved specimens from Rainbow only), rarefaction curves were drawn using the RarFac
184 program available online at http://www.icbm.de/pmbio/downlist.htm. The library clone coverage was estimated using the formula [1-(n1/N)] (Good, 1953) were n1 is the number of
186 OTUs represented by only one clone and N is the total number of clones. The Shannon index was calculated using the formula $H' = -\sum_{i=1}^{S} pi \ln pi - [(S-1)/2N]$ were S is the

number of OTUs, N is the total number of clones and p_i is the relative abundance of each OTU (calculated as the proportion of clones of a given OTU to the total number of clones in the community) (Shannon, 1948, Krebs, 1989).

Fluorescence in situ hybridization

In situ hybridization analyses were performed in order to study the distribution of phylotypes identified in the clone libraries. Samples (n = 8: one reference and one 8 hours-starved shrimps from TAG; two references, one 8 hours-starved, one 22 hours-starved specimen and two 72 hours-starved shrimps from Rainbow) were fixed for 2 hours in formaldehyde 3 %-sterile seawater solution and rinsed with PBS 2X-sterile seawater buffer (1:1). Samples were stored in absolute ethanol-PBS 2X solution (1:1) at -20 °C until use. Samples were embedded in polyethylene glycol distearate-1-hexadecanol (9:1) blocks (Sigma, St. Louis, MO, USA) after being dehydrated and soaked (water-ethanol and ethanol-resin series at 37°C) (Duperron et al, 2007). Blocks were cut in 6 to 10 µm sections using a RM 2165 microtome (Reichert-Jung, Germany). Resin was eliminated in ethanol and re-hydrated sections were hybridized in a reaction mix containing 0.5 µM of each probe in a 10%, 20%, 30% or 40 % formamide hybridization buffer [0.9 M NaCl, 0.02 M Tris-HCl, 0.01 % sodium dodecyl sulphate (SDS), 10, 20, 30 or 40 % deionized formamide; see Table 1] and hybridized for 3 h at 46°C. Sections were washed at 48°C for 15 min in a washing buffer (respectively 0.450, 0.215, 0.102 or 0.046 M NaCl, 0.02 M Tris-HCl, 0.005 M EDTA, 0.01 % SDS) and rinsed briefly. Sections were covered with Slow Fade® Gold antifade reagent containing DAPI (Invitrogen), and a cover slip. The universal probes (Eurogentec) were Eub338 (targeting most of *Eubacteria,* Amann et al, 1990), Arch915 (targeting *Archaea*, Stahl and Amann, 1991), GAM42a (targeting *γ-proteobacteria*, Manz et al, 1992) and EPSY549 (targeting *ε-proteobacteria*, Lin et al, 2006). New probes were also designed according to our gut clone sequences: Molli352 (targeting gut-associated *Mollicutes* of *R. exoculata*, modified from Wang et al, 2004b), Def576 (targeting gut-associated *Deferribacteres* of *R. exoculata*, modified from Kumaraswamy et al, 2005), clo4/Epsi653 and clo15/Epsi653 (targeting gut-

associated *ε-proteobacteria* of *R. exoculata*, modified from Polz and Cavanaugh 1995), and
216 LBI32/130 (targeting *R. exoculata* cephalothorax methanotrophic *γ-proteobacteria*, modified from Duperron et al, 2008) (Table 1). Each probe was used on every sample. Observations
218 were performed on an Olympus BX61 microscope (Olympus Optical Co., Tokyo, Japan) equipped with a U-RFL-T UV light (Olympus Optical Co.) and using a Retiga 2000R camera
220 (Qimaging, Surrey, BC, Canada). Micrographs were analyzed using the Qcapture Pro program (Qimaging).

222 *Light and electron microscopic observations*

Samples for LM (Light Microscopy, n = 3 digestive tracts: Rainbow reference, 22 hours-
224 starved and 72 hours-starved shrimps), SEM (Scanning Electron Microscopy, n = 5: Rainbow reference, 8 hours-starved and 72 hours-starved shrimps and two TAG reference shrimps)
226 and TEM (Transmission Electron Microscopy, n = 4: Rainbow reference, 8 hours-starved and 72 hours-starved shrimps and TAG reference shrimp) microscopic observations were fixed
228 for 16 h in 2.5 % glutaraldehyde-sterile seawater solution and stored at 4°C in a NaN_3-sterile seawater buffer (Sigma, final concentration 6.7 mM). Samples for SEM were then
230 dehydrated by ethanol series and desiccated with HMDS (Hexamethyldisilazane, Sigma) during 45 min and 5 h in a critical-point dryer CPD 020 (Balzers union, Balzers,
232 Liechtenstein). The digestive tracts cut longitudinally were displayed on a specimen stub, before desiccation and gold-coated with a SCD 040 (Balzers union). Observations were
234 performed with a Quanta 200 MK microscope (FEI, Hillsboro, OR, USA) and the Scandium acquisition program (Soft Imaging System, Munster, Germany). For light and TEM
236 observations, samples were post-fixed in 1 % osmium tetroxide, dehydrated in ethanol and polypropylene oxide series before they were embedded in an epoxy resin (Serlabo, Paris,
238 France). Semi-thin (800 nm) and ultra-thin (50 to 75 nm) sections were cut with an ultramicrotome Ultracut E (Reichert-Jung) using a diamond knife. Semi-thin sections were
240 stained with toluidine blue for observations by light microscopy (Nikon Optiphot-pol microscope and Zeiss Opton photomicroscope). Ultra-thin sections were displayed on copper

grids and contrasted with uranium acetate and lead citrate. Observations were carried out on a Philips 201 electron microscope, operating at 80 kV.

Results and discussion

In vivo experiments and sample considerations

The shrimps analyzed were actively swimming before, during and after the incubation experiments. 16S rRNA genes were successfully amplified and cloned from the total DNA extracted from midgut and hindgut (Figure 1A). Semi-thin sections and macroscopic observations of the digestive tracts indicated that the 8-hour or 22-hour starvation incubations were not long enough to completely expel the bolus, as all guts observed were still full of minerals (Figure S2A). However, after the 72-hour starvation experiment, guts were empty. In order to identify a possible resident microbial community, the gut microbial composition of reference shrimps (TAG and Rainbow) and animals incubated for 8 and 22 hours was therefore compared with 72 hours-starved shrimps.

Microscopic observations of the R. exoculata gut anatomy

The anatomy of the *R. exoculata* digestive tract showed a shorter foregut (mouth, stomach and pyloric chamber) and hindgut (Figure 1A) compared to other crustaceans (Komaï and Segonzac, 2008). The gastric mill usually has a crushing function. The microscopic observations showed that spinules and setae on the stomach wall of *R. exoculata* were less abundant than for scavenger hydrothermal shrimps such as *Chorocaris chacei* and *Mirocaris fortunata* (data not shown). *R. exoculata* had more developed setae at the beginning of the pyloric chamber than *C. chacei* and *M. fortunata*. It suggests that *R. exoculata* may have a low mechanical digestive activity.

The midgut of *R. exoculata*, the central digestive absorption zone deprived of cuticle, represented two thirds of its total gut length while it represents only between one sixth and one third of the total gut length in most crustaceans (Milne-Edwards, 1840). Cross-sections

of *R. exoculata* midgut also revealed large exchange surfaces for all specimens (reference and starved animals), as the epithelium displayed numerous invaginations and cells with dense microvilli. These cells were typical active digestive cells, with large nuclei (Figure 1D) and many mitochondria (MET observations, Figure S2C). The microscopic observations of the bolus revealed it was mainly composed of organic matter (few cuticle fragments and probably degraded micro-organisms) and minerals (Figure S2A, no total organic matter analysis of the bolus was performed), which may also suggest a seawater intake activity.

Microscopic observations of the microbial communities

In LM, SEM and TEM observations, the numerous mineral particles representing most of the bolus made the micro-organisms detection difficult. All our observations of the bolus have shown rare disc-shaped bacteria, which are reported on the vent chimney walls (Van Dover et al, 1988) and in the gill chamber (Polz and Cavanaugh, 1995, Zbinden et al, 2004). These rare disc-shaped cells were intact, apparently not submitted to rapid digestion. In a previous work it was shown that the cephalothoracic filamentous mat was not scraped (Zbinden et al, 2004, Corbari et al, 2008a). Moreover, the seawater flow in the gill chamber enters the chamber towards the gills and exits bathing the filamentous epibiotic bacteria (Casanova et al, 1993, Zbinden et al, 2004). So the seawater flow is at the opposite of the mouth which probably does not allow epibiont ingestion. The observation of microbial cells associated with the bolus was easier using fluorescence microscopy. A positive signal using the Eub338 probe (*Eubacteria*-specific) revealed the bacterial cells within the bolus (single rods or rods in small aggregates, cocci and rare disc-shaped cells). Part of the rods, and a few cocci were detected using the *γ-proteobacteria*-specific probe Gam42a. A few cocci hybridized using the LBI32/130 probe (data not shown). This probe was designed to target methanotrophic *γ-proteobacteria* associated with *R. exoculata* gill chamber epibionts (Zbinden et al, 2008). Few rods and all disc-shaped bacteria successfully hybridized using the *ε-proteobacteria*-specific probe EPSY549. These rods were also positively labelled using the two *R. exoculata* gut clones-specific probes clo4/Epsi653 and clo15/Epsi653. The rod-shaped bacteria in small

aggregates successfully hybridized using the *Deferribacteres*-specific probe Def576 (Figure S2D and E). Few *Deferribacteres* were also retrieved associated with the bolus all along the gut, regardless of the site. No signal was ever obtained using the Molli352 probe on the gut content.

In the midgut, the microscopic observations showed numerous long (up to 15 µm) and thin (0.2-0.3 µm) filaments, corresponding to individual cells without any visible septum, inserted between the microvilli of the epithelial gut cells (Figure 1 D to F). These filamentous bacteria were observed in dense populations within the gut of all specimens (Figure 1 B to E), with no visible difference in their distribution and abundance, regardless of the collection site and starvation treatment. *In situ* hybridizations using the *Eubacteria*-specific probe Eub338 indicated that all these filaments were active bacteria (Figure 1 B and C). No *Deferribacteres* were observed associated with the gut epithelium. No clear *in situ* hybridization signals were obtained from the filaments using *γ-proteobacteria*-specific probes (listed in Table 1) or using the Molli352 probe. A weak positive signal was observed from these epithelium-associated communities using the *ε-proteobacteria*-specific probe EPSY549, even after the 72-hour starvation experiment. These bacterial communities were clearly separated from the bolus by the peritrophic membrane (Figure S2B), a natural barrier that preserves the gut epithelium of arthropods from mechanical abrasion and microbial infections (Mercer and Day, 1952, Brandt et al, 1978). The filamentous morphology of these bacteria associated with the large surfaces they occupy thanks to the numerous invaginations of the gut epithelium, yield a large exchange surface between microbial communities and their environment. As the midgut is not subject to exuviation, this probably favours long-term microbial colonization and interactions of a resident microbial community with its host.

This community may play a significant role in the detoxification of compounds present in the bolus, such as minerals or heavy metals. Detoxification has been described for *Limnoria tripunctata*, a wood-boring marine isopod (Zachary and Colwell, 1979; Zachary et al, 1983). The resident gut microbiota of *L. tripunctata* was only observed in specimens inhabiting

creosote-treated wood, but it was absent if reared on non-treated wood. Therefore it suggested that these gut microbial communities could contribute to the creosote resistance of the isopod. Analogously, as the *L. tripunctata* bacterial community was also in close association with the intestinal epithelium within the peritrophic space, the *R. exoculata* gut microbial communities might participate in mineral/metal detoxification.

Archaeal communities of the gut

The composition of archaeal communities from the gut of reference and starved *R. exoculata* at the TAG and Rainbow sites was investigated using 16S rRNA gene clone libraries (detailed in Tables 2, 3 and S1). The archaeal sequences from the gut of TAG shrimps (34) were affiliated to the *Euryarchaeota* lineages DHVE 2 (Deep-sea Hydrothermal Vent *Euryarchaeota*, 23), *Thermococcales* (6) and to *Crenarchaeota* Marine Group I (5). In contrast, all archaeal-related clone sequences from the gut of Rainbow shrimps (39) were affiliated to *Methanococcales*. Although these archaeal lineages are usually found at hydrothermal vents (e.g. Takai and Horikoshi, 1999, Reysenbach et al, 2000, Nercessian et al, 2003 and 2005, Schrenk et al, 2003, Roussel and Cambon-Bonavita unpublished data), the TAG and the Rainbow gut-associated archaeal populations were clearly different (Roussel, unpublished data)., probably due to the contrasted geochemical conditions characterizing both sites. Interestingly, no archaeal cells were ever detected using *in situ* hybridization with the general *Archaea*-specific probe Arch915 (Stahl and Amann, 1991) whatever the condition used (Table 1), and no archaeal sequences were retrieved after long starvation incubations. These results suggest that these archaeal communities were probably rare and/or not active. Therefore they probably do not belong to the gut microbiota but more likely have been ingested with chimney particles and fluids.

The gut bacterial distribution and composition

Six bacterial 16S rRNA gene clone libraries were constructed (Table 2), representing a total of 376 clone sequences from reference shrimps (154) and from starved shrimps (222).

348 Although the analysis of clone libraries is only partially quantitative, as the sampling methods and molecular techniques introduce biases (e.g. Bent and Forney, 2008, Quince et al,
350 2008), the phylogenetic diversity of the microbial communities associated with *R. exoculata* midgut for each starvation incubation time can be compared, though caution is needed. As
352 the number of clones per sample was low, rarefaction analyses were conducted for each library. Results indicated that clone libraries adequately represented the composition of the
354 communities in the gut contents (see Figure S1 for rarefaction curves), because curves reached a plateau for the number of clones investigated. The clone coverage was satisfying
356 for the six clone libraries: 92 % for Rainbow and TAG reference shrimps, 96 % for the Rainbow 8 hours-starved specimen, 97 % for the TAG 8 hours-starved specimen, 95 % for
358 the 22 hours-starved shrimp and 94 % for the 72 hours-starved shrimp. On average, the six 16S rRNA bacterial clone libraries were mostly dominated by 4 phyla affiliated to
360 *Deferribacteres* (63 %), *Mollicutes* (12 %), *γ-proteobacteria* (10 %) and *ε-proteobacteria* (9 %). Although sequences affiliated with *Firmicutes*, *Cytophaga-Flavobacter-Bacteroides*
362 (*CFB*), *Verrucomicrobiae*, *δ-proteobacteria* and *β-proteobacteria* were also detected, they represented a small fraction of the clones (6 %). The overall bacterial communities
364 composition, detailed in Tables 2 and S1, was consistent with a previous molecular analysis of the *R. exoculata* gut (Zbinden and Cambon-Bonavita 2003). Clone libraries from the
366 Rainbow and TAG reference shrimps showed a similar bacterial community composition. In both, the clones were mainly related to *Deferribacteres* (75 %), *Mollicutes* (10 %), *ε-*
368 *proteobacteria* (6 %), *γ-proteobacteria* (3 %) and other groups (*CFB* and *Firmicutes*, 6 %). Composition of gut bacterial clone libraries after the 8-hours starvation experiment (on both
370 sites) and the 22-hours starvation incubation (on Rainbow site) were also similar and still dominated by *Deferribacteres* (81 % and 96% respectively). In contrast, the 72-hours
372 starvation clone library was dominated by *γ-proteobacteria* (40 %), *Mollicutes* (23 %), *ε-proteobacteria* (23 %), and other minor lineages (11 %) whereas the *Deferribacteres*
374 represented less than 3 % of the sequences (Table 2 and S1).

All sequences affiliated to the *Deferribacteres* phylum (238) represented a single phylotype previously detected from *R. exoculata* gut (Table S1 and Figure 2A; *R. exoculata* gut clone 62 and 91, Zbinden and Cambon-Bonavita, 2003). To date, the *R. exoculata* gut clone-related genera, i.e. *Mucispirillum* and *Geovibrio*, are only detected from gut microbiota, sediments and oil reservoirs and were never reported from hydrothermal vents, suggesting that this phylotype could be specific to *R. exoculata*. Moreover, the high similarity level between all *Deferribacteres*-related sequences (> 99%) associated with shrimps regardless of the site, suggests a long-term, specific association between these bacteria and their host. Hence, the *Deferribacteres* from this cluster probably are part of *R. exoculata* specific gut microbial community. The difference in the proportion of *Deferribacteres*-related sequences between the 72 hour-starved specimen clone library (< 3 %) and all the other clone libraries (at least 70 %) may be explained by several hypotheses: (i) *Deferribacteres* could have been free-living in the gut (not attached to the gut wall) and eliminated with the bolus after the 72-hour starvation experiment (ii) Some *Deferribacteres* use iron and sulphides as energy sources (Miroshnichenko et al, 2003), and therefore would not survive if deprived of these compounds during long starvation periods. (iii) Some *Deferribacteres* are heterotrophic bacteria, therefore using organic matter of the bolus, such as ingested microorganisms or cuticle fragments. (iv) All *Deferribacteres* known so far are strict anaerobes. During the starvation experiment, the reduced minerals are evacuated with the bolus. The gut is then probably under aerobic conditions which could impair the *Deferribacteres* growth and maintenance.

Sequences related to *Mollicutes* also represented a significant proportion of the clone libraries (13 % from reference shrimps) and this even after a 72-hour starvation experiment (23 %, Figure 2B and Table 2). Three clusters were identified and affiliated to *Entomoplasmatales* and *Mycoplasmatales*. Clusters B and C were detected in specimens from both TAG and Rainbow sites, and after all starvation experiments (Table S1), suggesting they were not a site-specific community and so reinforcing the hypothesis of a

402 specific symbiotic relationship. *Entomoplasmatales* and *Mycoplasmatales* bacteria are usually pathogens, commensals or symbiotic bacteria associated with vertebrates
404 (mammalians or fishes), insects, crustaceans or plants (Razin, 1978 and 1998, Clark, 1984). The cluster A (24 sequences, Figure 2B) was closely related to a sequence detected from a
406 starved Mediterranean shrimp (98 % similarity), *Pestarella tyrrhena*, suggesting that *Mollicutes* are ubiquitous of crustaceans gut and so could also be part of the resident
408 digestive tract microbiota. The cluster A-affiliated sequences were retrieved only in Rainbow specimens (Table S1). This microbial population could be site-dependant or this could be
410 due to the number of sequences analyzed. The cluster B (16 sequences, Figure 2B) was closely related to *R. exoculata* gut clones within the *Mycoplasmatales* order (99 % similarity;
412 Zbinden and Cambon-Bonavita, 2003). Cluster C (4 sequences, Figure 2B) was affiliated to epibiotic phylotypes from crustaceans (isopods) and fish guts, suggesting they could also
414 represent resident microbial phylotypes. Interestingly, all the *R. exoculata* gut sequences related to *Mollicutes* were affiliated to epibionts from other crustaceans such as *Porcellio*
416 *scaber*, which harbours long or spherical stalked microorganisms inserted between the microvilli of midgut glands (Wang et al, 2004b and 2007). Although microscopic observations
418 showed that the morphology and size of *R. exoculata* gut epibionts (long thin filamentous single cells) were similar to some *Spiroplasmas* (long thin bacteria, 0.1-0.35 µm diameter, up
420 to 5 µm length, Garnier et al, 1981), suggesting they could be *Mollicutes* (Figure 1 B to F), no clear *in situ* hybridization signal was ever detected using the *Mollicutes*-specific probe for
422 each stringency condition and sample tested (Table 1). However, Wang and collaborators reported a very low hybridization signal on the *Mollicutes* symbionts of the midgut glands of
424 *P. scaber* (Wang et al, 2004b). Moreover, as even the DAPI staining of the filaments was difficult to observe, the very low *Mollicutes* hybridization signals could also be due to the size
426 of these very thin bacteria (~0.3 µm diameter). *Mollicutes* are polymorphic micro-organisms (spheroid, filamentous, ramified, helicoïdal) characterized by an absence of cell wall, and
428 therefore poorly resistant to extreme environments. Usually, *Mollicutes* have a reduced

genome and depend on host nutrients (Maniloff and Morowitz, 1972, Razin, 1978 and 1998, Clark, 1984, Regassa and Gasparich, 2006). The midgut of *R. exoculata* does not moult and is relatively independent of environmental conditions, suggesting it could be a sufficiently stable habitat to harbour *Mollicutes*. Therefore *Mollicutes* could be a part of the resident microbial community associated with the gut wall, able to survive to a long-term starvation.

Sequences related to *ε-* and *γ-proteobacteria* from the gut of the reference specimens were dominant in the clone library of the 72-hour starvation experiment (63 %) (Tables 2 and S1, Figures 2C and 2D). The values of the Shannon index are low after 8- and 22-hours starvation experiment (1.01 for Rainbow and 0.65 for TAG 8 hour-starved shrimps, 0.27 for Rainbow 22 hour-starved shrimp). This may be explained by the low number of clones treated per sample. But we also suggest that microbial density is low and so, molecular approaches favour only the dominant population. After the 72-hours starvation experiment, the Shannon index value is high (4.97) as *γ-proteobacteria*-related sequences were highly diverse. Their dominance could be a possible consequence of the lesser quantity of detectable 16S rRNA genes related to *Deferribacteres*. We also suggest the *γ-proteobacteria* were in a latent state in reference shrimps and between 8 and 22 hours starvation, and were actively dividing at 72 hours starvation. This could be explained by the switch of the physiological conditions in the empty digestive tract. The elimination of the bolus led to the evacuation of reduced minerals and intake of oxygen. This could have been favourable to the *Proteobacteria* and deleterious to the *Deferribacteres* which are strict anaerobes micro-organisms. Regarding the *γ-proteobacteria*-related sequences retrieved in the Rainbow reference shrimp clone library, they were all affiliated to the gill chamber clone LBI32 (AM412518, Zbinden et al, 2008). This sequence clusters in the methanotrophic *γ-proteobacteria* symbionts group. Positive *in situ* hybridization signals were observed on methanotrophic-like bacteria. So, these *γ-proteobacteria*-related sequences are probably site-dependant which could be due to the geochemical conditions prevailing on this ultramafic site.

One third of the *ε*-proteobacterial sequences from starved shrimps were affiliated to *R. exoculata* gut sequences (99 % similarity, Zbinden and Cambon-Bonavita, 2003) and to the *R. exoculata* ectosymbiont thought to be a sulphide-oxidizing chemoautotroph (Polz and Cavanaugh, 1995). Other *ε-proteobacteria* and part of the *γ-proteobacteria* sequences from starved shrimps were affiliated to autotrophic epibiont-like microorganisms mainly associated with a hydrothermal gastropod (Goffredi et al, 2004). Other *γ-proteobacteria* were affiliated to heterotrophic micro-organisms (e.g. *P. entomophila* CT573326 and *Alteromonas sp.* AB078014). Moreover, the sequences closely clustering within the *ε-proteobacteria* cluster A (99% similarity), were detected in the reference shrimps and also in the 22 and 72 hours-starved shrimps (Figure 2C). Hence, as previously suggested, specific *Proteobacteria* phylotypes could also be part of the local midgut resident microbiota (Polz et al, 1998, Zbinden et Cambon-Bonavita 2003). From all our libraries no sequence was affiliated to the gill chamber clones except one (clone R32R) related to clone LBI32 (96% of similarity). This indicates that in our experiments, on both sites, the gill chamber epibionts were little ingested.

New insights in the shrimp nutrition and role of the microbial communities.

Observations of the shrimps behaviour indicated they were healthy before and after treatments. Microscopic observations of brush cells showed they were intact in all specimens suggesting that the long filaments inserted between microvilli are probably not pathogenic.

Part of the community associated with the bolus was probably ingested non-specifically with minerals from chimney walls and surrounding seawater (*Archaea* and some *Proteobacteria* identified within the bolus) and expelled in the faeces. The intake of environmental seawater and chimney particles could play a role in the diet of the host.

The gill epibiont communities are thought to represent the second major nutritive contribution (Gebruk et al, 1993 and 1997, Segonzac et al, 1993, Polz and Cavanaugh 1995). In our analyses, cephalothoracic epibionts do not seem to be that much ingested. So, trans-

epidermal exchanges could be the main nutritional source from the gill epibionts as proposed before (Zbinden et al, 2004 and 2008).

Microscopic observations, 16S rRNA gene clone libraries analyses and *in situ* hybridizations indicated that gut-associated bacterial communities were rather similar in all specimens from TAG and Rainbow, except for *γ-proteobacteria*, and presented a limited diversity of phylotypes. Most of the bacterial sequences were related to eukaryote-associated bacteria usually considered as symbiotic, rather than to free-living vent environmental bacteria. Taken all together, these data clearly reinforce the hypothesis of a symbiotic relationship between *R. exoculata* and at least certain members of gut-associated bacterial communities.

A previous study had measured autotrophic carbon intake in the gut (Polz et al, 1998). Some of the sequences retrieved in this study were affiliated to the *Proteobacteria* lineage and clustered with sequences from autotrophic bacteria. *Mollicutes* are usually heterotrophic bacteria and may be involved in organic matter degradation. The *Deferribacteres* species are usually heterotrophic micro-organisms involved in sulphur compounds and iron cycles. So they may be implicated in the nutrition of the shrimp and in detoxification processes. These results suggest that several metabolic pathways likely co-occur within the epibiotic community.

Culturing attempts are in progress in the laboratory. But as the epibionts are usually refractory to cultures metagenomic approaches will be conducted in order to better understand the epibionts roles.

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Tables and figures legends

TABLE 1. Fluorescent probes used in this study.

TABLE 2. Distribution of the bacterial 16S rRNA gene clones from Rainbow and TAG reference and starved shrimps

TABLE 3. Closest match of representative 16S rRNA gene clone sequences

TABLE S1. Distribution of the bacterial 16S rRNA gene clones per OTU

FIG. 1. Representation of *Rimicaris exoculata* digestive tract (A). Bacterial gut epibionts inserted between microvilli of the gut epithelium of a Rainbow long-starved shrimp observed in fluorescence *in situ* hybridization (B, C), semi-thin section (D), scanning electron microscopy (E) and transmission electron microscopy (F). Hybridizations have been done using the Eub338 Cy3-labelled probe (in red, Amann et al, 1990) and DNA was stained with DAPI (in blue). m = mouth; pc = pyloric chamber; s = stomach. In circles: b = bacteria; mv = microvilli; n = nucleus; bl = basal lamina

FIG. 2. Phylogenetic trees based on 16S rRNA gene sequences from the digestive tract of reference and starved *R. exoculata* from TAG and Rainbow hydrothermal sites. They represent the main bacterial phylogenetic groups of micro-organisms associated with the shrimp gut: *Deferribacteres* (2A, calculated on 882 bp), *Mollicutes* (2B, calculated on 796 bp), *ε-proteobacteria* (2C, calculated on 841 bp) and *γ-proteobacteria* (2D, calculated on 842 bp). The robustness was tested using 500 bootstraps re-sampling of the trees calculated by the neighbor-joining algorithm with Kimura two parameters correction matrix. Sequences were named as: R or T for Rainbow or TAG specimens respectively, the number in the clone

library, and R for reference shrimp, SW, S and LS for an 8, 22 and 72-hour seawater
784 starvation experiments respectively.

786 FIG. S1. Rarefaction curves of the bacterial 16S rRNA genes of epibionts associated with the *Rimicaris exoculata* gut.

788

FIG. S2. Digestive tract of *Rimicaris exoculata*

790 (A, B) Semi-thin sections showing bacterial gut epibionts localized between the gut epithelium and the peritrophic membrane and clearly separated from the bolus (Rainbow
792 reference shrimp).

(C) Transmission electron microscopy micrograph of numerous apical mitochondria in gut
794 epithelial cells and epibionts inserted between microvilli (Rainbow long-starved shrimp).

(D, E) Fluorescence *in situ* hybridization micrographs of microbial aggregates in the gut
796 content of a Rainbow reference shrimp. *Deferribacteres* epibionts hybridized with the Def576 Cy3-labelled probe (white, this study).

798 In circles: b = bacteria; bl = basal lamina; cf = cuticle fragments; mch = mitochondria; mp = mineral particles; mv = microvilli; n = nucleus; pm = peritrophic membrane.

TABLE 1. Fluorescent probes used in this study

Phylotype	Probe	Probe sequence (5' to 3')	Fluorescent dye	Position (rRNA genes)	% formamide	References
Archaea	Arch915	GTGCTCCCCCGCCAATTCCT	Cy3	915 (16S)	10 – 20 – 30 %	Stahl and Amann, 1991
Eubacteria	Eub338	GCTGCCTCCCGTAGGAGT	Cy3 or Cy5 or ATTO488	338 (16S)	10 – 20 – 30 – 40 %	Amann et al, 1990
Mollicutes *R. exoculata* gut clones	Mollic352	GTGAAAAATTCCTTACTGCTG	Cy3 or ATTO488	352 (16S)	10 – 20 – 30 – 40 %	This study
Deferribacteres *R. exoculata* gut clones	Def576	CACTGACTTGACAAACCT	Cy3	576 (16S)	10 – 20 – 30 – 40 %	This study
ε-*proteobacteria*	EPSY549	CAGTGATTCCGAGTAACG	Cy3	549 (16S)	20 – 30 %	Lin et al, 2006
ε-*proteobacteria* *R. exoculata* gut clones	clo4/Epsi653	ATCTTCCCCTCCCAGACTCT	Cy3	653 (16S)	10 – 20 – 30 – 40 %	This study
ε-*proteobacteria* *R. exoculata* gut clones	clo15/Epsi653	ATCTTCCTCTCCCTCACTCT	Cy5 or ATTO488	653 (16S)	10 – 20 – 30 – 40 %	This study
γ-*proteobacteria*	GAM42a	GCCTTCCCACATCGTTT	Cy3	1027 (23S)	20 – 30 %	Manz et al, 1992
Methanotrophic γ-*proteobacteria* *R. exoculata* clones	LBI32/130	TCCTGGCTATCCCCCACTAC	ATTO488	130 (16S)	10 – 20 – 30 %	This study

TABLE 2. Distribution of the bacterial 16S rRNA gene clones from Rainbow and TAG reference and starved shrimps. In bold, the main phylogenetic group per sample

Phylogenetic groups	Number of clones						
	Reference samples		Starvation 8 h		Starvation 22 h	Starvation 72 h	Total
	Rainbow (2 guts)	TAG	Rainbow	TAG	Rainbow	Rainbow	
Deferribacteres	**51**	**65**	**43**	**32**	**45**	2	238
Mollicutes	10	5	1	9	0	19	44
γ-proteobacteria	4	0	0	0	0	**33**	37
ε-proteobacteria	7	2	3	0	2	19	33
β-proteobacteria	0	0	0	0	0	1	1
δ-proteobacteria	0	0	1	0	0	0	1
CFB	2	1	2	1	0	0	6
Firmicutes	1	1	0	1	0	8	11
Verrucomicrobiae	0	5	0	0	0	0	5
Total	75	79	50	43	47	82	376

TABLE 3. Closest match of representative 16S rRNA gene clone sequences

Phylogenetic group	Representative clone sequences	Hit of BLAST (accession no.)	Similarity	No. of clones
Bacteria				
Deferribacteres	R36S, R28SW, R82R	*R. exoculata* gut clone 62 (AJ515723)	99 %	238
γ-proteobacteria	R67LS	Endosymbiont of *Alviniconcha sp.* type 1 clone SyA1-P1 (AB235235)	95 %	4
	R57LS	*Pseudomonas entomophila* strain L48 (CT573326)	98 %	1
	R32R	*R. exoculata* gill chamber clone LBI32 (AM412518)	96 %	4
	R48LS	γ-proteobacterium clone Belgica2005/10-ZG-8 (DQ351804)	93 %	2
	R53LS	*Alteromonas sp.* strain SHY1-1 (AB078014)	99 %	3
	R16LS	Zebrafish gut clone aab28h07 (DQ819366)	99 %	11
	R68LS	Iron-reducing enrichment clone Cl-A2 (DQ676994)	99 %	1
	R56LS	*Photobacterium phosphoreum* strain RHE-01 (AY435156)	98 %	9
	R19LS	Endosymbiont of *Acanthamoeba sp.* Ac309 (AY549549)	98 %	1
ε-*proteobacteria*	R3R, R30LS	Hydrothermal vent gastropod clone SF_C23-F4 (AY531582)	98-97 %	6
	R62LS	Hydrothermal vent gastropod clone SF_C23-C8_shell (AY531600)	96 %	2
	R69LS	*R. exoculata* gut clone 4 (AJ515714)	99 %	12
	R28R, R23LS	*R. exoculata* gut clone 11 (AJ515717)	99 %	13
Mollicutes	R67SW, R2R	*R. exoculata* gut clone 42 (AJ515720)	99 %	24
	T8SW	*R. exoculata* gut clone 69 (AJ515722)	99 %	16
	T28SW	*Gillichthys mirabilis* gut clone C13 (DQ340200)	86 %	4
Firmicutes	T17R	Mammals gut clone Saki_aaj62c07 (EU461853)	87 %	1
	R8R, R2LS, T17SW, R26LS	*Gillichthys mirabilis* gut clone C13 (DQ340200)	81-91 %	8
	R5LS	*'Candidatus Bacilloplasma'* isopod gut clone P10 (DQ485976)	80 %	2
CFB	R21R, R21SW, R39SW	Arctic sediment clone SS1_B_01_16 (EU050905)	94-97 %	4
	T70R	Hydrothermal sediments clone p816_b_3.23 (AB305587)	92 %	1
	R14R	*Bacteroidetes* clone C319a-R8C-D4 (AY678514)	94 %	1
Verrucomicrobiae	T72R	Hydrothermal *Verrucomicrobia* clone pItb-vmat-33 (AB294943)	87 %	1
	T20R	*Paralvinella palmiformis* clone P. palm C 136 (AJ441224)	92-96 %	4
β-proteobacteria	R64LS	*Ralstonia sp.* clone EMP_AD31 (EU794311)	99 %	1
δ-proteobacteria	R65SW	*Geothermobacter sp*. Fe30-MC-S (AB268315)	93 %	1
Archaea				
Methanococcales	R5R	*Methanococcus aeolicus* strain Nankai-3 (DQ195164)	97 %	39
DHVE 2	T48R, T4R	Hydrothermal clone met43 (DQ082955)	93-98 %	23
Thermococcales	T38	*Thermococcus siculi* strain DSM 12349 (AY099185)	99 %	6
Marine Group 1	T24R	Hydrothermal clone pEPR624 (AF526982)	99 %	5

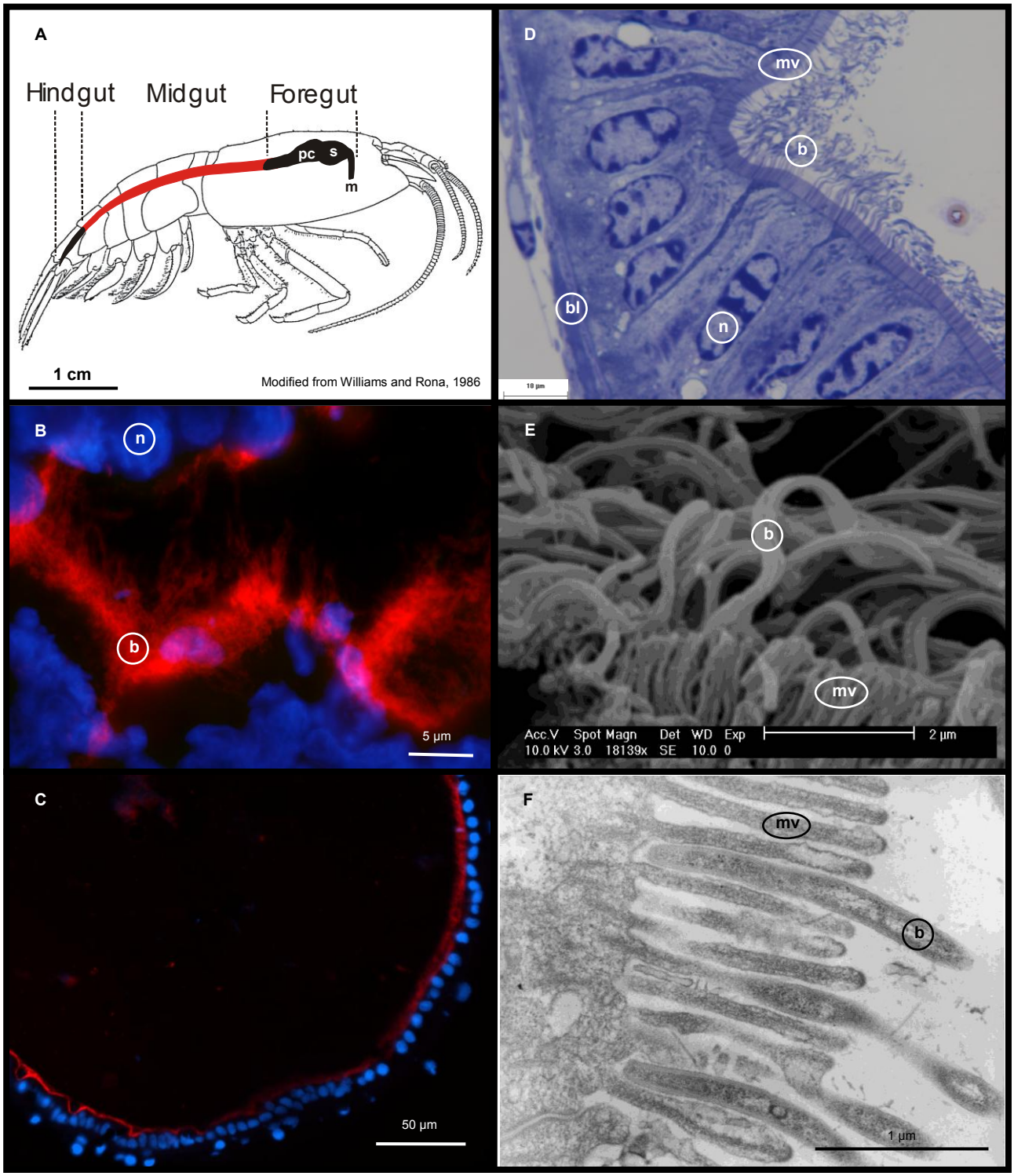

FIG. 1. Representation of *Rimicaris exoculata* digestive tract (A). Bacterial gut epibionts inserted between microvilli of the gut epithelium of a Rainbow long-starved shrimp observed in fluorescence *in situ* hybridization (B, C), semi-thin section (D), scanning electron microscopy (E) and transmission electron microscopy (F). Hybridizations have been done using the Eub338 Cy3-labelled probe (in red, Amann et al, 1990) and DNA was stained with DAPI (in blue). m = mouth; pc = pyloric chamber; s = stomach. In circles: b = bacteria; mv = microvilli; n = nucleus; bl = basal lamina

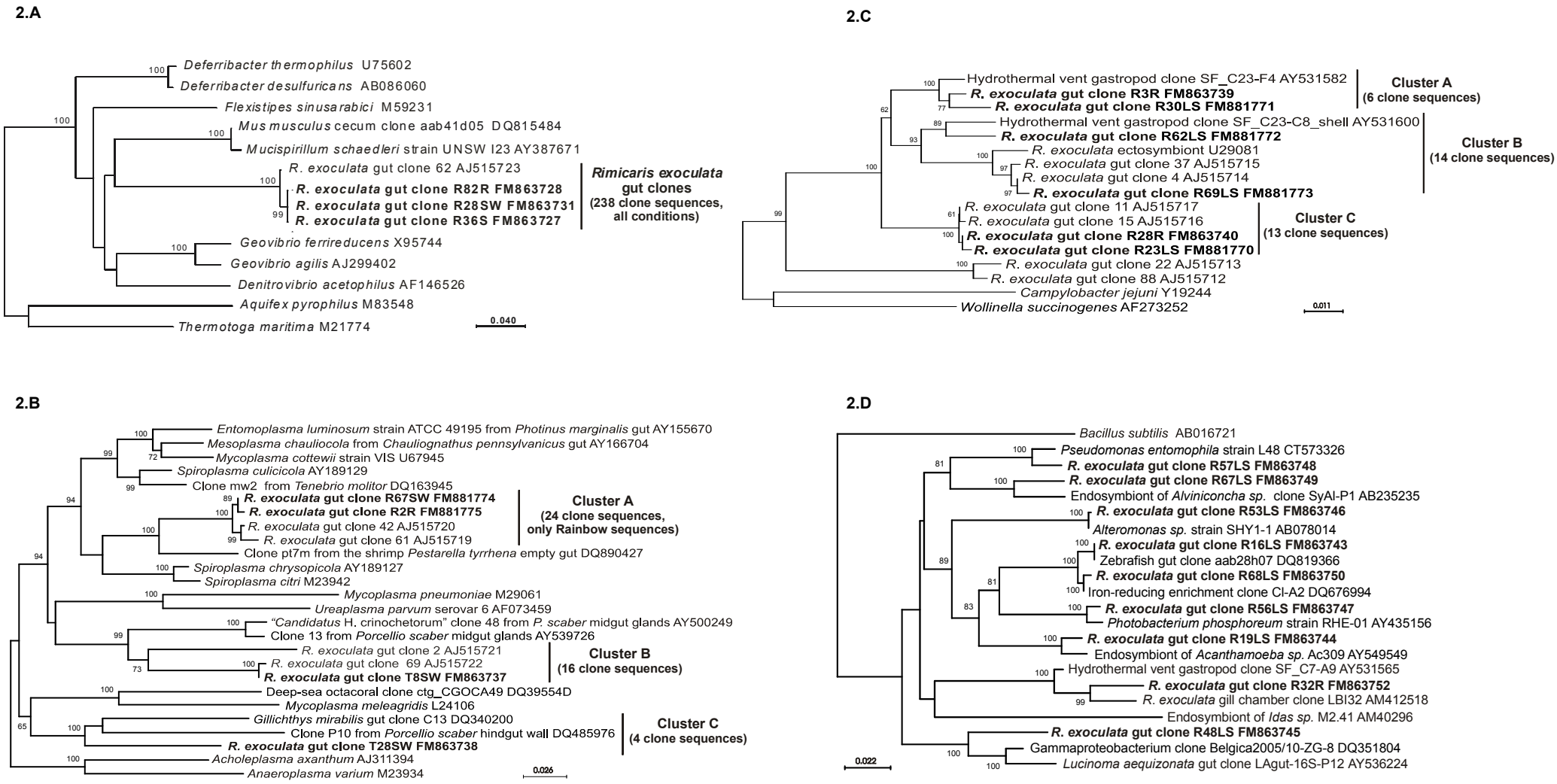

FIG. 2. Phylogenetic trees based on 16S rRNA gene sequences from the digestive tract of reference and starved *R. exoculata* from TAG and Rainbow hydrothermal *Mollicutes* (2B, calculated on 796 bp), *ε-proteobacteria* (2C, calculated on 841 bp) and *γ-proteobacteria* (2D, calculated on 842 bp). The robustness was tested using 500 bootstraps re-sampling of the trees calculated by the neighbor-joining algorithm with Kimura two parameters correction matrix. Sequences were named as: R or T for Rainbow or TAG specimens respectively, the number in the clone library, and R for reference shrimp, SW, S and LS for an 8, 22 and 72-hour seawater starvation experiments respectively. In bold, our clones.

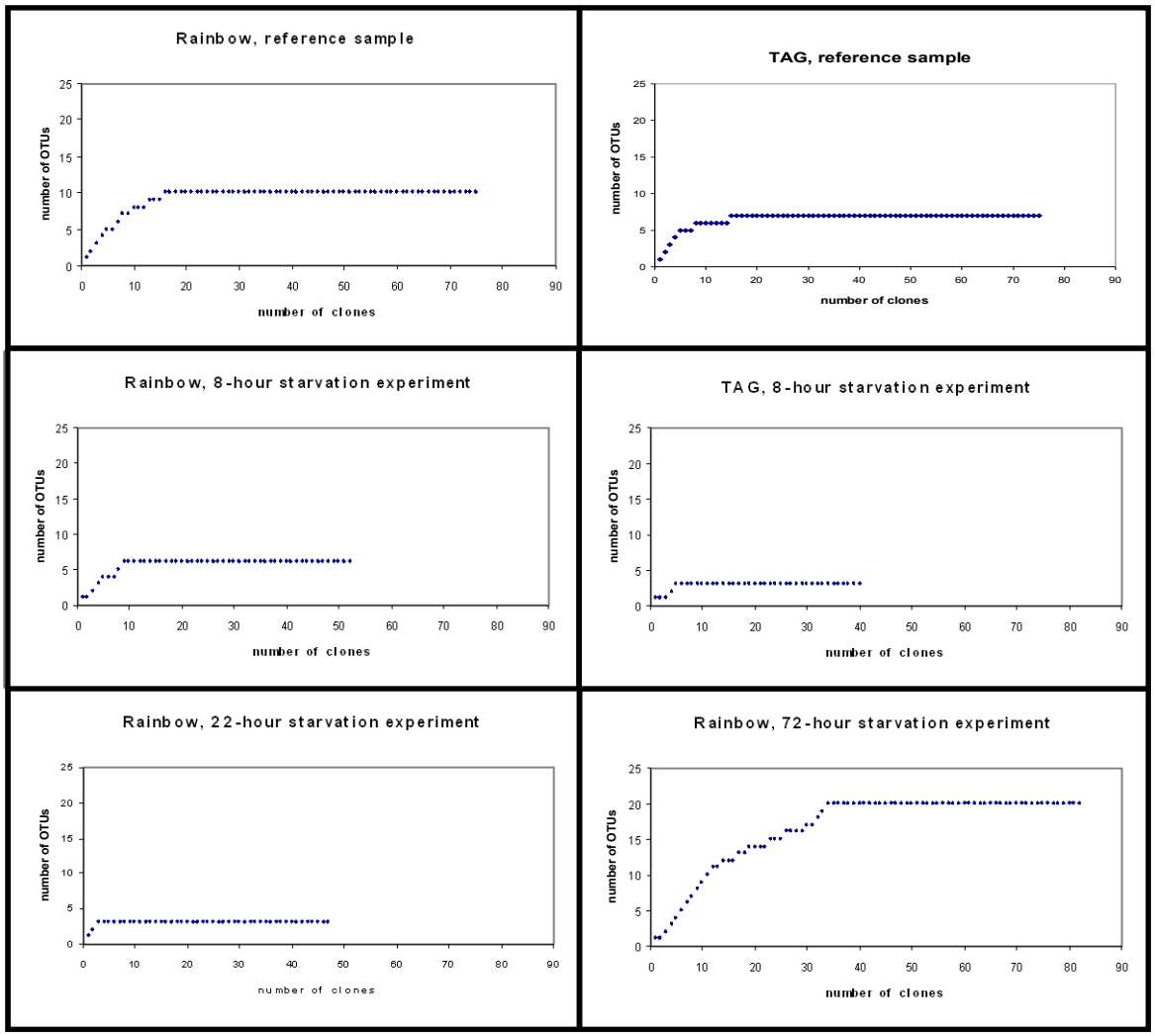

FIG. S1. Rarefaction curves of the bacterial 16S rRNA genes of epibionts associated with the *Rimicaris exoculata* gut.

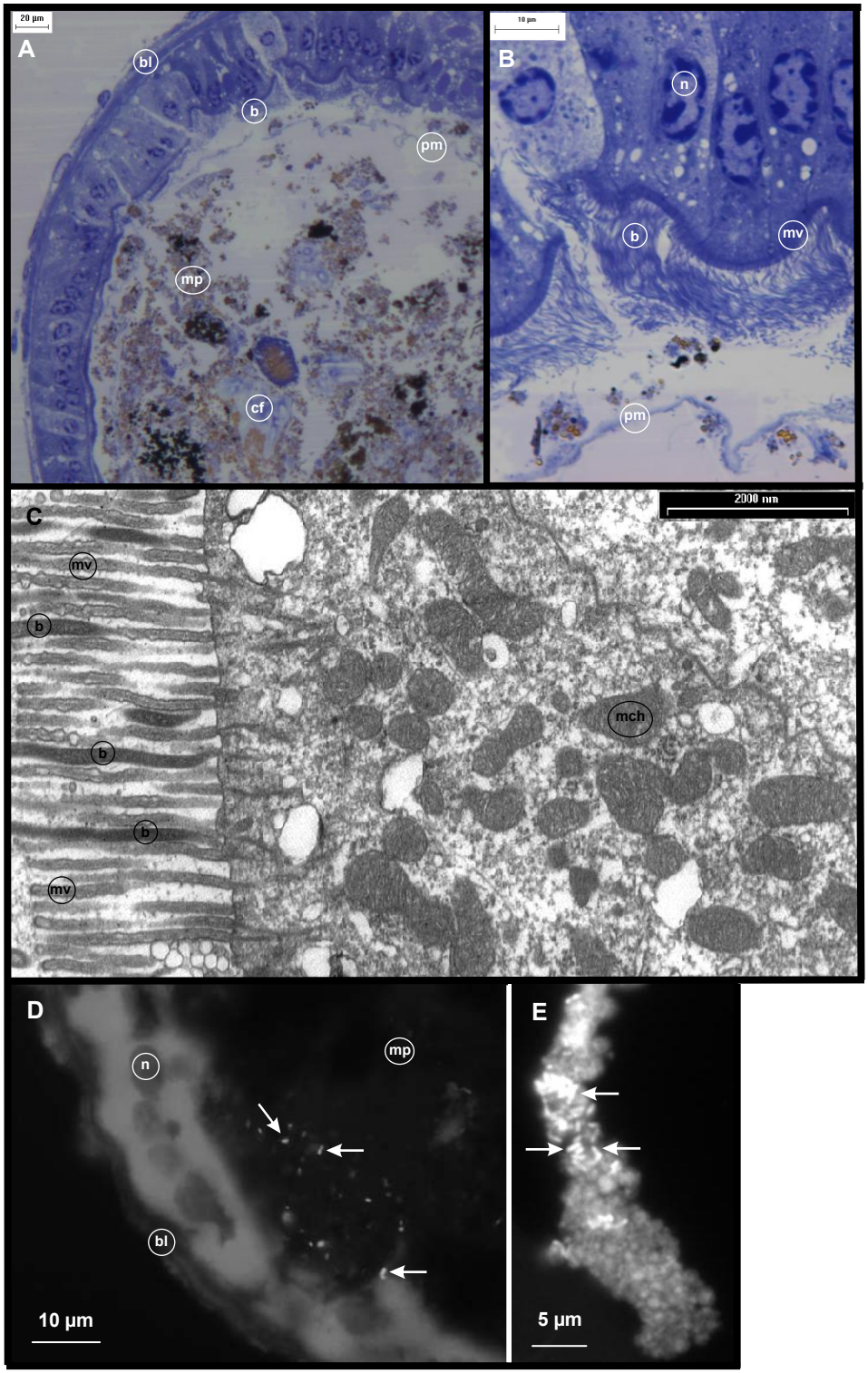

FIG. S2. Digestive tract of *Rimicaris exoculata*

(A, B) Semi-thin section showing bacterial gut epibionts localized between the gut epithelium and the peritrophic membrane and clearly separated from the bolus (Rainbow reference shrimp).

(C) Transmission electron microscopy micrograph of numerous apical mitochondria in gut epithelial cells and epibionts inserted between the microvilli (Rainbow long-starved shrimp).

(D, E) Fluorescence *in situ* hybridization micrographs of the digestive tract of a Rainbow reference shrimp. *Deferribacteres* epibionts hybridized with the Def576 Cy3-labelled probe (white, this study).
In circles: b = bacteria; bl = basal lamina; cf = cuticle fragments; mch = mitochondria; mp = mineral particles; mv = microvilli; n = nucleus; pm = peritrophic membrane.