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Occurrence of Deferribacterales and Entomoplasmatales in the deep-sea Alvinocarid shrimp Rimicaris exoculata gut

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Abstract: The phylogenetic diversity of the gut microbial population of the hydrothermal shrimp Rimicaris exoculata was determined. The presence of micro-organisms in the hindgut of the shrimp was determined, and their morphology illustrated for the first time by transmission electron microscopy. DNA was extracted from the fore-, mid- and hindgut of shrimps collected at the Rainbow site, at the Mid-Atlantic Ridge, and the sequences of the small-subunit rRNA (16S rDNA) were analyzed. Seven different bacterial phylotypes were identified from the 90 clones screened. The sequences were affiliated to three major groups: small epsilon, Greek-Proteobacteria (48,8%), Entomoplasmatales (23.3%) and representatives of the new phylum Deferribacteres (26.6%). These results show that the diversity in the shrimp gut is very low compared to that of the surrounding medium. Furthermore, the presence of groups that are not found in the external medium (Spiroplasma sp. and Geovibrio sp.) suggests the existence of a local microflora. The potential roles of these bacteria are discussed, involving the shrimp diet and metal bioremediation.

Keywords: Hydrothermal vent; Shrimp; Genetic diversity; Indigenous gut microflora; Metal bioremediation

1. Introduction

Caridean shrimps belonging to the family Alvinocarididae are the dominant megafauna at the Mid-Atlantic Ridge (MAR) vent sites [1]. *Rimicaris exoculata* [2] is particularly abundant, forming dense, motile swarms around the chimney walls [3,4]. A striking characteristic of these shrimps is that they have an unusually enlarged gill chamber housing epibiotic bacteria, which are most abundant on mouthparts, bacteriophore structures and on the inner surface of the gill chamber [5]. Although three different morphotypes were observed for these bacteria (two filamentous and one rod-shaped [4]), they consist entirely of one single bacterial phylotype, forming a distinct branch within the *ε-Proteobacteria* [6].

As for the East Pacific Rise polychaete *Alvinella pompejana* Desbruyères & Laubier [7], which exhibits similar (i.e. filamentous *ε-Proteobacteria*) bacterial epibiosis [8], a trophic role has largely been discussed for the shrimp epibiotic bacteria, since bacteria have also been found in the gut [5,9]. The observation of large quantities of sulphide particles in the stomach and gut contents of *Rimicaris exoculata* first led Van Dover and collaborators [9] to propose that the primary food source of the shrimp was bacteria living on the surface of the chimney, with a secondary contribution of the epibiotic bacteria to the diet. An alternative hypothesis proposed that the diet of *Rimicaris exoculata* may rely totally on its epibiotic bacteria [4,10,11,12]. A third hypothesis has emerged from different studies [13,14]: a local, highly active autotrophic bacterial population in the shrimp's gut may serve as a source of nutrition.

However, knowledge of the bacterial microflora associated with the gut is still poor. Polz et al. [14] have reported that about 20% the bacteria of the gut (cardiac stomach and combined mid- and hindgut) belong to the phylotype found in the gill chamber epibionts. The aim of this study was to determine the shrimp gut bacterial diversity, based on the 16S rRNA distribution, in order to assess the 80% of unknown microflora.

2. Materials and methods

2.1. Collections

Specimens of *Rimicaris exoculata* were obtained during the French ATOS cruise (June 2001) on the Rainbow vent site (36°14.0’ N, MAR, 2300 meters depth). Animals were collected with the slurp-gun of the ROV «Victor», operated from the RV «Atalante». The bowls of the suction sampler were aseptically washed with ethanol 95 and then filled with distilled water before dives. Immediately after recovery, live specimens were dissected under sterile conditions to sample stomachs and digestive tracts. These tissue samples were either fixed in 3% glutaraldehyde - sodium cacodylate buffer solution, and later post-fixed in osmium tetroxide for microscopy, or frozen for DNA extraction.

2.2. Light and Transmission Electron Microscopy (TEM)

For TEM observations, fixed samples were dehydrated in ethanol and propylene oxide series, and further embedded in epoxy resin (Serlabo). Semi-thin and thin sections were obtained from a Reichert-Jung Ultramicrotome (Ultracut E). Semi-thin sections were stained with Toluidine blue for observations by light microscopy (using a Nikon Optiphot-pol microscope). Thin sections were laid on 200 mesh copper grids and stained with uranyl acetate and lead citrate. Observations were carried out on a Philips 201 TEM, operating at 80 kV.

2.3. DNA extraction

Samples of the digestive tract (fore-, mid- and hindgut see Fig. 1) of 2 specimens were centrifuged (4000 *g*, 40 min, 4°C) and resuspended in TE-Na-1X lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA, pH 8). 500 µl of 10% lauryl sarkosyl and 500 µl of 10% sodium dodecyl sulphate (SDS) were added after homogenization. The samples were treated

with proteinase K (20 mg/ml, Eurobio, France) at 40°C for 3 h. Digested samples were centrifuged for 5 min (6000 *g*) and supernatants were extracted three times with cold phenol-chloroform-isoamyl alcohol (24:24:1 vol/vol/vol) and once with chloroform (mixed in a 1:1 ratio with lysates). Nucleic acids corresponding to the microbial community were obtained by overnight precipitation in –20°C absolute ethanol (2:1 ratio with lysates) and centrifugation for 1.5 h (12000 *g*). They were dried, resuspended in 500 µl TE-1X (10 mM Tris/HCl, 2 mM EDTA, pH 7.5) and absorbance was determined at 230, 260, 280 and 320 nm, on a GenQuantII spectrophotometer (Pharmacia Biotech Inc., Uppsala, Sweden) to check the purity of the nucleic acids and to determine their concentration.

2.4. PCR and cloning

Clone libraries were produced from community 16S rDNA by amplifying approximately 100 ng of bulk DNA in 50 µl reaction mixtures containing (final concentrations) 1 X PCR buffer (QBIOgene, France), 200 µM of each deoxyribonucleoside triphosphate, 100 µM of each *Bacteria* specific forward and reverse primer, and 0.5 µl of *Taq* polymerase (QBIOgene). Reaction mixtures were incubated in a Robocycler Gradient 96 (Stratagene, La Jolla, CA.). For *Bacteria*, the cycling program was as follows: 94°C for 3 min, 30 cycles of 94°C for 1 min, 48°C for 1.5 min, 72°C for 2 min and a final extension period of 6 min at 72°C. The forward primer specific for the bacterial 16S rDNA was 27F (5'-AGAGTTTGATCATGGCTCAG-3') and the reverse primer was the universal 1492R (5'-GTTACCTTGTTACGACTT-3'). The PCR products were cloned directly to separate them from each other, with the TA cloning method, using the pCR2.1 vector according to the manufacturer's instructions (Invitrogen Corp., San Diego, CA). Clone libraries were constructed by transforming *E. coli* TOP10F'.

2.5. Screening of rDNA clones by ARDRA (Amplified Ribosomal DNA Restriction Analysis)

Each recombinant colony was suspended in 100 µl of sterile deionised water. Ribosomal DNA inserts from recombinant clones were amplified by PCR from 5 µl of colony suspension, using the reaction mixtures described above. The PCR program was the same as that for the initial amplification of 16S rDNA (see above), except that the initial step lasted 10 minutes (for cell lysis). Three to 5 µl of the PCR products was digested with 3 U of the 4-base-specific restriction endonuclease *Hha*I (New England Biolabs) in 1X NEB buffer 4 supplemented with 100 µg ml^{-1} of bovine serum albumin (BSA), in a final volume of 20 µl for 3 h at 37°C. The digested fragments were separated by electrophoresis in a 3% agarose (Sigma) gel stained with ethidium bromide and the bands were visualized by UV transillumination (Fluor-S-multi-imager, Biorad, Hercules, CA.). ARDRA patterns for each library were normalized and grouped with «Molecular Analyst» software (Biorad). One gene representative of each pattern was selected for sequencing. Where patterns indicated dominance (more than 12 clones per pattern), 3 clones were partially sequenced to check for the homogeneity of the group. ARDRA screening was stopped when no new pattern was observed after 30 successive clones.

2.6. Sequencing of rDNA clones

Representative rDNA inserts were sequenced by Genome Express (Grenoble, France) with automatic sequencers (Perkin Elmer, Sanger method [15]). Two sequence reactions per insert with specific primers were required to determine the bacterial 16S rDNA sequence (about 1300 bp). The primers used for sequencing included the universal primer 27F (see above), and an internal bacterial primer EUBINT (5'-GCGCCAGCAGCCGCGGTAA-3') (position 520-539 on *E. coli* 16S rDNA sequence). Sequences were then assembled using DNASTAR sofware (Madison, Wis., USA).

2.7. Phylogenetic analysis and chimera detection

Sequences were compared with those available in databases using the BLAST (Basic Local Alignment Search Tool) network service [16], in order to determine approximate phylogenetic affiliations. Chimeric sequences were detected by using the BLAST program (with the 5' or 3' partial sequences) and confirmed by phylogenetic reconstruction on partial sequences (unstable sequences were removed from the study). Sequences were compiled in MEGALIGN (DNASTAR) and aligned with the rDNA sequences from the Ribosomal Database Project, using the CLUSTALW program [17]. Alignments were refined manually, using the SEAVIEW program [18]. All the trees presented were constructed by using the PHYLO-WIN program [18], on the basis of evolutionary distance neighbor-joining [19] and maximum likelihood methods [20]. The robustness of inferred topologies was tested by the bootstrap resampling [21] of trees calculated on the basis of evolutionary distance (neighbor-joining algorithm with Kimura two-parameters correction [22]) and maximum likelihood. If ARDRA patterns were not identical, while related sequences displayed over 98% sequence similarity, then only one of the sequences was retained for phylogenetic analysis.

2.8. Nucleotide sequence accession numbers

The EMBL accession numbers of the 13 sequences used in this study are AJ515712 to AJ515724

3. Results

3.1. Light and Transmission Electron Microscopy

Semi-thin sections of the stomach and the gut were observed. Both of these parts of the digestive tract were full of black and brown (respectively iron sulphides and iron oxides, Zbinden, unpubl. data) mineral particles (Fig. 2). TEM observations (Fig. 2) revealed the presence of bacteria in the gut, whereas none were observed in the stomach. Morphotypes observed in the hindgut indicate that bacteria are either cocci or rods, of 0.35 µm in diameter, and at least 1.5 µm in length. They seem to be undamaged (i.e. not degraded by a digestion process), since the cell wall, composed of two membranes (as in gram-negative bacteria), is readily visible and intact (see Fig. 2c).

3.2. ARDRA

When one ARDRA pattern contained more than 12 clones, 3 of them were partially sequenced. Most of the clones from the same ARDRA pattern displayed more than 99% sequence similarity, showing that ARDRA screening with *Hha1* was efficient. One exception was *Entomoplasmatales*, for which clone 2 and clone 69 displayed the same ARDRA pattern but had different sequences.

3.3. Phylogenetic analysis

Among the 100 recombinant colonies, 92 contained an insert and 8 were false positive clones (no insert). The ARDRA screening for these 92 clones evidenced 16 different patterns, with 7 patterns containing only one clone. Among these 7 patterns, 2 were removed from the study as they were probably chimeric. The 14 "positive" patterns represented 90 clones. Sequences of these clones were associated with 3 phylogenetic groups: ε-*Proteobacteria*, *Spiroplasma* and *Geovibrio*, from a new phylum described in 1997 [23].

Forty four clones (47.8% of the study) were related to ε-*Proteobacteria* (see Fig. 3a) and could be divided in two subgroups [24]. Twenty four clones (representing 4 ARDRA patterns) were related to the epibionts of *R. exoculata* group (called Hydrothermal vent ε–groupII) while 20 clones were related to cold seep environmental clones (Cold seep ε–groupII), 11 (2

patterns) being related to BD21 [25] and 9 (2 patterns) related to NKB11 [26]. Twenty one clones (3 patterns) were related to the *Entomoplasmatales* and can be divided in two lineages, both related to *Spiroplasma* spp. and *Mycoplasma* spp. clusters (see Fig. 3b). Twenty four other clones (representing 2 ARDRA patterns) showed 97% sequence similarity and are related to the genus *Geovibrio* of the new phylum Deferribacteres (Fig. 3c). One clone was related to γ-*Proteobacteria*, closely related to a Red sea clone [27] (Fig. 3a).

4. Discussion

The presence of bacteria in the digestive tracts (particularly the hindgut) of Crustacea is widespread, occurring across the taxa, feeding types and habitats [28,29]. These associations range from pathogenic to mutualistic, and from facultative to obligate [30], but in general the roles that these bacteria play in the nutrition of their hosts are unknown.

Our TEM observations showed for the first time the presence of micro-organisms in the hindgut of the shrimp, and illustrate their morphology and integrity. As we were unable to observe bacteria in the foregut on our thin sections, they are probably much less numerous, if present, in the stomach than in the mid- and hind-gut. The presence of large amounts of bacterial cell-material in the cardiac stomach of *Rimicaris exoculata* has been evidenced by lipopolysaccharide assays [9]. However, their presence could not be confirmed by scanning electron microscopic observations, suggesting that only bacterial cell-wall remains were present in this part of the gut. The bacteria observed in the hindgut had intact membranes, which suggested that they were resident and not submitted to a digestion process. This hypothesis is reinforced by the "high molecular weight" of the extracted DNA, which was not lysed, indicating bacterial activity in the gut, or at least suggesting that the DNA (and thus the bacteria) was not degraded. The physiological state of the bacteria should nevertheless be tested on fresh samples to confirm this. These assumptions are consistent with the hypothesis of the occurrence of a local microflora [13,14].

The phylogenetic diversity of microbial communities of the MAR was reported for the Snake Pit and "Les Ruches" sites [31,32]. Bacterial diversity revealed the presence of numerous phylotypes (27) related to the ε and β subclasses of the *Proteobacteria*, the *Aquificales*, the genus *Desulfurobacterium* and the *Cytophagales*. Although these analyses were not performed on samples from the same origin site as our samples, comparison of the results from both studies indicates that the microbial populations present in the hydrothermal fluid and in the shrimp gut are different. Two main differences are to be noticed: (1) the hydrothermal fluid displayed an important phylogenetic diversity which did not exist in the *R. exoculata* gut, where only 3 major groups were present; (2) occurrence of *Deferribacterales* and *Entomoplasmatales* was observed in the gut, which was not found in the outer medium.

Deferribacterales: Species from the order *Deferribacterales* was first described from submarine petroleum reservoirs [23] and more recently from hydrothermal vents (from MAR: R. Miroshnichenko, pers. comm. and from a seamount in the Japanese sea [33]). To our knowledge, no *Geovibrio* species has yet been described from the Atlantic hydrothermal sites. If they are present, they are probably very rare. As they represent 25% of our clones, they are unlikely to come from the outer medium, and most probably represent a part of the local microflora of the gut. Most of these bacteria are iron-reducing. Furthermore, although a new species of the Deferribacteres phylum (under description, R. Miroshnichenko, pers. comm.) is reported to be lithoautotrophic, the rest of the group is strictly heterotroph. Physiological assumptions based only on the phylogenetic position of a sequence must be made with caution. However, it is reasonable to think that the Deferribacteres occurring in the *R. exoculata* gut are heterotrophs and iron-reducing bacteria, the presence of iron oxides in the

shrimp gut [10, Zbinden, unpubl. data] suggesting the existence of metal-reducing micro-organisms. They would most probably be involved in detoxification processes or bioremediation of the mineral compounds found in the environment, rather than in primary production used as food source by the host.

Entomoplasmatales: Almost all members of the genus *Spiroplasma* that have been described are associated with arthropods, but are mainly found in terrestrial insects [34,35]. Most *Spiroplasma* spp. are known or suspected to be parasitic, although the degrees of pathogenicity may be extremely diverse between different species, ranging from evidently detrimental to sometimes slightly beneficial [36,37]. From available data, it is difficult to speculate on the roles of *Spiroplasma* species in the gut of *R. exoculata*. Nevertheless, as they were recovered from apparently healthy animals, they are probably non toxic for the shrimp.

ε-Proteobacteria: In all deep-sea vent microbial communities studied, *ε-Proteobacteria* have been found to be dominant, accounting for 40% to 98% of the bacterial clones libraries [38]. Among these bacteria, several are capable of autotrophic growth [14,38,39].

Genetic analysis of the bacterial populations associated with *R. exoculata* [6,14] showed that the population of bacterial epibionts consisted entirely of a single phylotype, which was also found to contribute to 60% of the detectable 16S rRNA amplified from the sulphide chimney samples, and to more than 20% of the 16S rRNA amplified from the shrimp gut (which is consistent with our results). Furthermore, very high carbon dioxide fixation rates were reported [14] in homogenates of total shrimp guts. From these results, Polz and collaborators suggested that there should be additional, highly active bacterial populations in the shrimp gut that could serve as a previously unsuspected source of nutrition. Considering that most *Deferribacterales* and *Entomoplasmatales* are heterotrophs, it is reasonable to think that the high CO_2 fixation rates measured in the shrimp [14] are due to the *ε-Proteobacteria* activity. This suggests that the putative nutritional symbiosis with a specific gut microflora, if existing, occurs through bacteria from this group.

In conclusion, it appears from our results that most of the bacteria from the surrounding medium are not found in the gut. *Geovibrio* sp. and *Entomoplasmatales* sp., which are found in the gut, do not seem to be present in the surrounding medium [31,32, Cambon-Bonavita, unpubl. data]. These results lead us to propose the following hypotheses: the foregut of *R. exoculata* is highly active and the ingested bacteria are rapidly degraded. Extracted DNA from the mid- and hind-gut belongs to a local microflora. Some of these bacteria (the *ε-Proteobacteria*) could provide the shrimp with their metabolism by-products as a source of nutrition. A trans-epidermal transfer of dissolved organic matter from the bacteria has already been suggested for the filamentous bacteria present on the mouthparts [5]. Gebruk and collaborators [12] rejected this hypothesis on the basis of studies on other Crustacea, which show the active transport of dissolved organic matter to be unlikely. But recently, absorption of hydrolysates resulting from gut bacterial activity has been suggested to occur through hindgut cuticle of a deposit-feeding shrimp [30]. Another explanation for the low bacterial diversity found in the gut is that the physico-chemical conditions existing in the gut are very specific and not suitable for numerous micro-organisms. This could explain why most of the bacteria found in the external medium are not able to develop in the gut environment.

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Table 1 : Clone library description.

Clone number (Fig. 3a, 3b, 3c)	Closest relative	Number of ARDRA patterns	Number of clones represented	% similarity between clones	% similarity with database sequences
Clone11, 15	Clone BD2-1 *ε-Proteobacteria*	2	11	98 %	96%
Clone 37, 4	*Rimicaris* exosymbiont *ε-Proteobacteria*	4	24	98 %	96%
Clone 22, 88	Clone NKB11 *ε–Proteobacteria*	2	9	98%	94%
Clone 26	γ *-Proteobacteria*	1	1	-	90%
Clone 42, 61	New lineage *Entomoplasmatales*	2	2	93%	85%
Clone 69, 2	New lineage *Entomoplasmatales*	1	19	92 %	75%
Clone 62, 91	New lineage *Deferribacterales*	2	24	99%	81 %

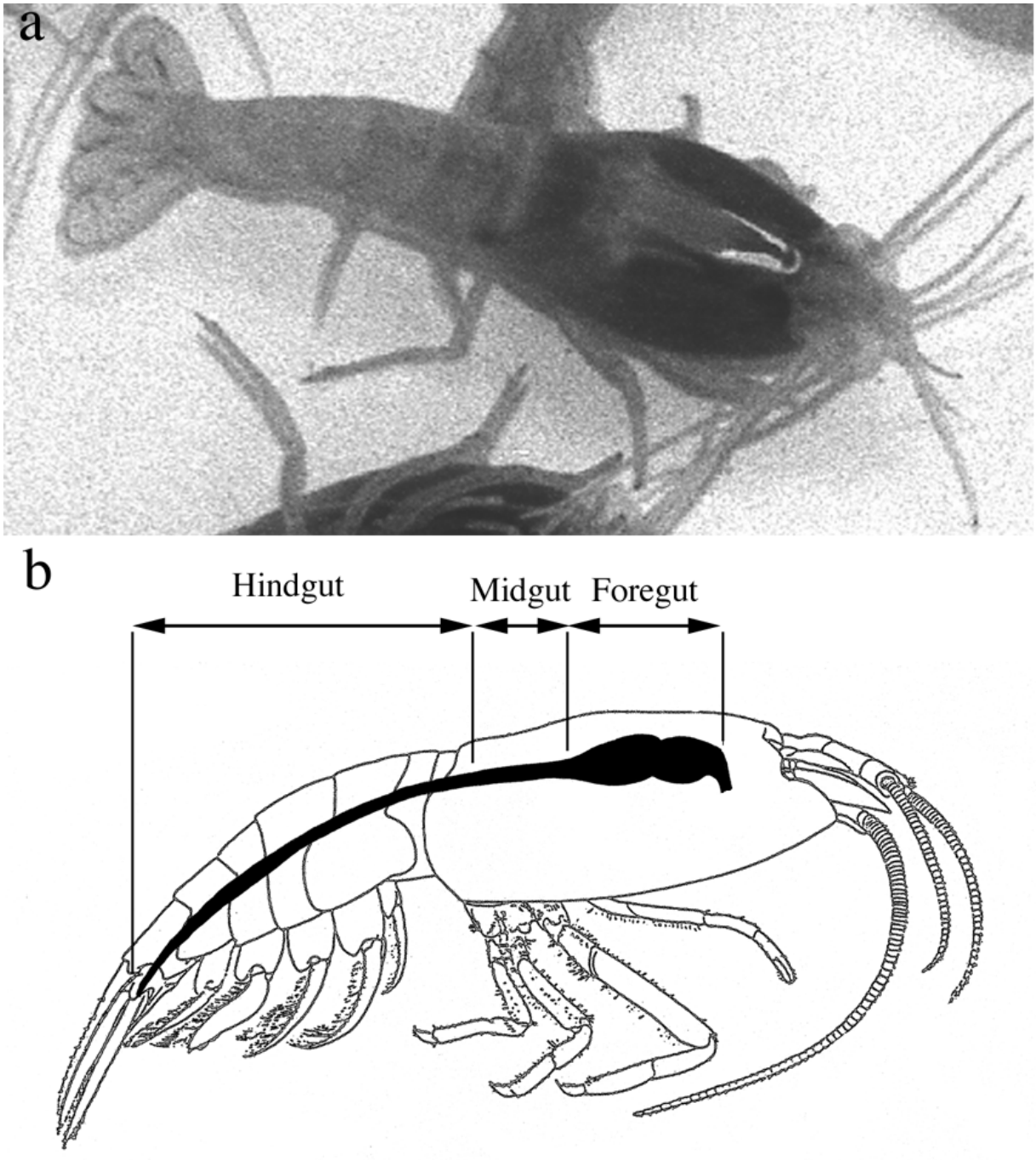

Figure 1: Morphology of *Rimicaris exoculata* and anatomy of its digestive tract. (a) On board just after recovery. (b) Schematic drawing of the shrimp (adapted from Williams and Rona, 1986) on which the digestive tract is represented. All the tract was sampled for the analyses, i.e. the foregut, midgut and hindgut.

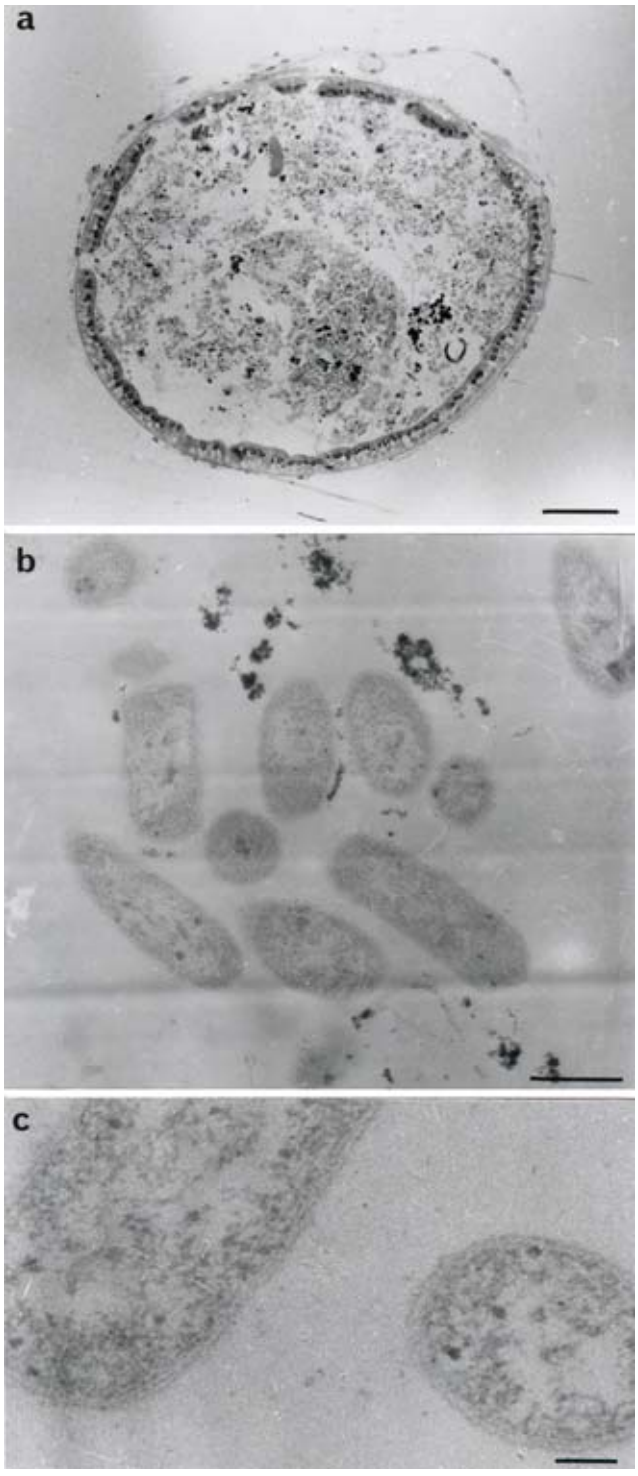

Figure 2: *Rimicaris exoculata* hindgut content (a) Photonic observation of a semi-thin section of the digestive tract (hindgut). The gut is full of mineral particles, generating stria on the sections. Scale bar = 100 µm. (b) Bacteria observed in the hindgut in TEM. Scale bar = 0,5 µm. (c) Higher magnification of the membrane of the bacteria. Scale bar = 10 nm.

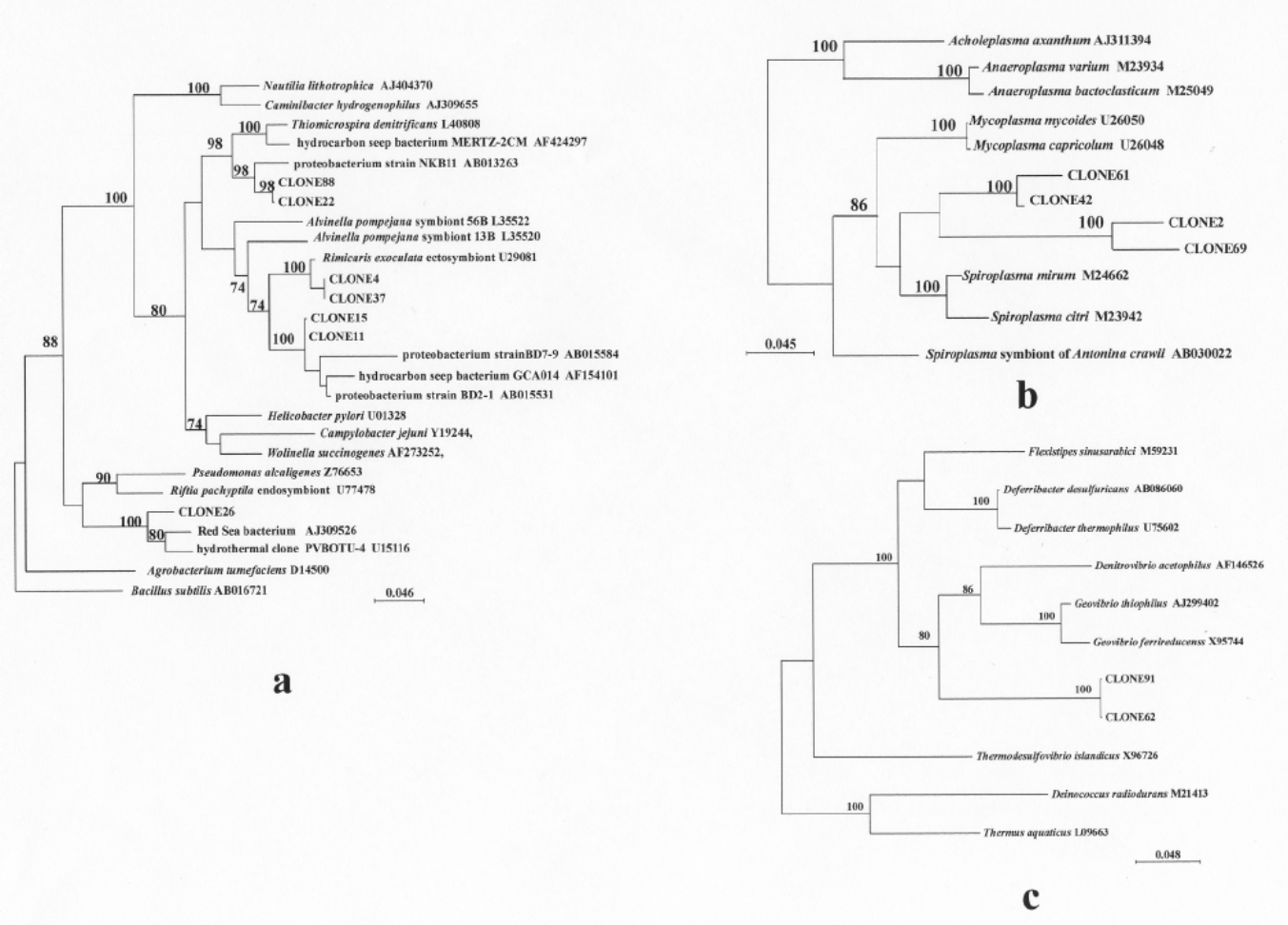

Figure 3: Phylogenetic trees obtained using Maximum Likelihood analysis with bootstrap resampling. Topologies were confirmed with Maximum Parsimony and Neighbor Joining methods. Bootstraps values are indicated on nodes above 70%. Accession numbers of the sequences used are indicated on trees. a) Tree *a* was based on 27 species and 673 sites were analysed. *Bacillus subtilis* was chosen as outgroup. b) Tree *b* was based on 13 species and 1060 sites were analysed. *Achoplasma* spp. were chosen as outgroup. c) Tree *c* was achieved with 11 species and 1121 sites were analysed. *Deinococcus radiodurans* and *Thermus aquaticus* were chosen as outgroups.