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1 **New insights on the metabolic diversity among the epibiotic microbial community of the**  
2 **hydrothermal shrimp *Rimicaris exoculata*.**

3

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6

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14

15 *Key words:* high pressure experiments, hydrothermal vent shrimp, intracellular granules,  
16 iron, methane, sulfur

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17

**18 Abstract.**

19 The shrimp *Rimicaris exoculata* (Williams and Rona, 1986) dominates the megafauna of  
20 some of the Mid Atlantic ridge hydrothermal vent sites. This species harbors a rich  
21 community of bacterial epibionts inside its gill chamber. Literature data indicate that a single  
22 16S rRNA phylotype dominates this epibiotic community, and is assumed to be a sulfide-  
23 oxidizing bacteria. However attempts of cultivation were not successful and did not allow to  
24 confirm it. The aim of our study was to test the hypothesis of sulfide oxidation in the gill  
25 chamber, by a multidisciplinary approach, using *in vivo* experiments at *in situ* pressure in the  
26 presence of sulfide, microscopic observations and a molecular survey. Morphology of  
27 microorganisms, before and after treatment, was analyzed to test the effect of sulfide  
28 depletion and re-exposure. Our observations, as well as molecular data indicate a wider  
29 diversity than previously described for this shrimp's epibiotic community. We observed  
30 occurrence of bacterial intracellular sulfur- and iron-enriched granules and some  
31 methanotrophic-like bacteria cells for the first time. Genes that are characteristic of methane-  
32 oxidizing (*pmoA*) and sulfide-oxidizing (APS) bacteria were identified. These results suggest  
33 that three metabolic types (iron, sulfide and methane oxidation) may co-occur within the  
34 epibiont community associated with *Rimicaris exoculata*. As this shrimp colonizes chemically  
35 contrasted environments, the relative abundance of each metabolic type could vary according  
36 to the local availability of reduced compounds.

37

**38 1. Introduction**

39 Hydrothermal vent communities along the Mid-Atlantic Ridge (MAR) are dominated by large  
40 populations of caridean shrimps. Found in dense clusters of 40 000 individuals per m<sup>3</sup>  
41 (Segonzac et al., 1993), *Rimicaris exoculata* is the most abundant species on some of these  
42 sites. This shrimp has been found to host a dense bacterial epibiosis on the internal walls  
43 (branchiostegites) of its branchial chamber and on its mouthparts (scaphognathites and  
44 exopodites of the first maxillipeds) (Van Dover et al., 1988; Casanova et al., 1993; Segonzac  
45 et al. 1993; Zbinden et al., 2004). This indicates an intimate association between these  
46 organisms. The main source of dietary carbon could originate: 1) from bacteria ingested with  
47 the sulfide scraped from the chimney (Van Dover et al., 1988), 2) from their epibiotic bacteria  
48 (Segonzac et al., 1993; Gebruk et al., 2000) or 3) from an autotrophic bacterial population  
49 living in the shrimp's gut (Pond et al., 1997; Polz et al., 1998; Zbinden and Cambon-  
50 Bonavita, 2003). Fatty acid abundances and carbon isotopic composition recently provided  
51 strong evidence that mature *R. exoculata* gain most of their carbon from the epibiotic bacteria  
52 within their carapace rather than from bacteria grazed on the chimney walls (Rieley et al.,  
53 1999). For shrimps sampled from the Snake Pit site, three bacterial morphotypes were  
54 described (Segonzac et al., 1993) which all belonged to the same phylotype of  
55 *Epsilonproteobacteria* (Polz and Cavanaugh, 1995). Although attempts to cultivate these  
56 microorganisms failed until now, they were hypothesized to acquire their metabolic energy  
57 from sulfide oxidation (Gebruk et al. 1993; Wirsen et al., 1993). Chemosynthetic activity of  
58 the filamentous bacteria from the inner cephalothorax surface has been shown (Wirsen et al.,  
59 1993), but no significant increase of CO<sub>2</sub> incorporation was observed in the presence of  
60 reduced sulfur compounds (Polz et al., 1998).

61 More recently, Zbinden et al. (2004) suggested that another metabolic pathway, iron  
62 oxidation, could be involved at the iron-rich Rainbow ultramafic site. Unlike most active  
63 hydrothermal sites known to date, the hydrothermal circulation at Rainbow is hosted on  
64 mantle rocks. As a result, its fluid composition departs from the common range of  
65 hydrothermal end-members, and is relatively depleted in H<sub>2</sub>S and enriched in H<sub>2</sub>, FeII and  
66 CH<sub>4</sub>, as a result of the serpentinization processes (Charlou et al., 2002; Douville et al., 2002).  
67 During the ATOS cruise shrimps were all collected from the Rainbow site. The main  
68 objective of our work was to test the hypothesis that all the shrimp epibionts were sulfide-  
69 oxidizers. To overcome the inability to cultivate the epibionts, we performed *in vivo*  
70 experiments. For the first time, pressurized aquaria were used to gain information on the  
71 bacterial epibionts' metabolism. The aspect and ultrastructure of the bacteria were checked  
72 after incubations at 230 bars (*in situ* pressure), at 15°C (*in situ* temperature) with or without  
73 sulfide-enriched seawater (thereafter called sulfide pulses), and compared to *in situ* reference  
74 shrimps. A molecular survey was undertaken to get new insights on possible metabolic type  
75 of the epibiotic microbial communities of *Rimicaris exoculata*, particularly thiotrophy using  
76 the 5'-adenylylsulfate (APS) reductase gene.

77

## 78 **2. Materials and methods**

### 79 **Animal collection and selection**

80 Specimens of *Rimicaris exoculata* were collected during the French ATOS cruise (June  
81 2001), on the Rainbow vent site (36°14.0' N, Mid-Atlantic Ridge, 2320 meter depth).  
82 Shrimps were collected with the suction sampler of the ROV "*Victor 6000*", operated from

83 the R/V “*L’Atalante*”. Once on board, some live specimens were either immediately dissected  
84 into body components. These samples are referred to as “reference shrimps” further in the  
85 text. Alternatively, some shrimps were placed in pressure vessels (IPOCAMP™) for *in vivo*  
86 experiments (see below) and in this case dissected immediately after removal from the vessel.  
87 Scaphognathite samples were fixed in a 2.5% glutaraldehyde - sodium cacodylate buffered  
88 solution and later post-fixed in osmium tetroxide for morphological observations. Samples for  
89 X-ray analyses were not postfixed. For each treatment, shrimps in anecdysis were selected for  
90 observation according to the moult-staging method of Drach and Tchernigovtzeff (1967), by  
91 examination of bristle-bearing appendages (uropods) under a lightmicroscope. The moulting  
92 stage was later confirmed by examination of the branchiostegite integument by light  
93 microscopy and Transmission Electron Microscopy (TEM). For molecular studies, shrimps  
94 were frozen immediately after recovery under sterile conditions. Once in the lab, the  
95 scaphognathites and branchiostegites were dissected and DNA extraction was performed.

96

#### 97 **Pressurized incubator IPOCAMP™**

98 The stainless steel pressure vessel has an internal volume of approximately 19 liters (see  
99 Shillito et al., 2001 for detailed description and diagrams). This is a flow-through pressure  
100 system, with flow rates that can reach 20 l.h<sup>-1</sup>. Pressure oscillations arising from pump strokes  
101 (100 rpm) are less than 1 bar at working pressure. The temperature of the flowing seawater  
102 (filtered at 1 µm mesh) is constantly measured, at pressure, in the inlet and outlet lines  
103 (±1°C). Temperature regulation is powered by a regulation unit (Huber CC 240) that

104 circulates ethylene-glycol through steel jackets surrounding the pressure vessel and around  
105 the seawater inlet line.

106

107 ***In vivo* experiments.** Re-pressurization at 230 bars was achieved in about 2 min after closure  
108 of the vessel. As the shrimps were sampled at the end of the dive, less than 2 h passed  
109 between the time the samples began decompression (submersible ascent) and the moment  
110 they were re-pressurized. At atmospheric pressure, just after the submersible recovery, the  
111 shrimps (except for some individuals, which may have been damaged by the suction sampler)  
112 were alive and active. Pressure vessel experiments were carried out at *in situ* pressure (230  
113 bars) and at 15°C, according to the literature data: 10-15°C (Segonzac et al., 1993) ; 3.8-  
114 14.7°C (Zbinden et al., 2004) ;  $13.2 \pm 5.5^\circ\text{C}$  (Desbruyères et al., 2001). Previous *in vivo*  
115 experiments showed a good physiological state of the shrimps when re-pressurized at these  
116 temperature and pressure conditions (Ravaux et al., 2003). Only alive and active shrimps after  
117 treatment were used for the present study.

118 Two experiments at 230 bars were performed:

119 (1) Sample incubation at 15°C in surface seawater, to investigate the effect of depletion of  
120 electron donors on the shrimps and their epibionts. Twelve shrimps were placed in the  
121 pressure vessel, for 30 h. The seawater was regularly (5 times) renewed, by replacing a  
122 quarter of the total volume. Surface seawater oxygen level (253  $\mu\text{M}$ ) lies slightly above the  
123 concentration measured in the environment of the shrimps (Schmidt et al., in press). These  
124 samples are referred to as "non-sulfide shrimps" further in the text.

125 (2) Incubation at 15°C, with exposure to sulfide pulses. Nine shrimps were placed in the  
126 pressure vessel for 32 h. During the 32 h of the experiment, we first maintained the shrimps in  
127 normal sea-water for 8 hours. Then, 4 pulses were performed as follows : i) the inlet of the  
128 flow-through pressure system was fed with a reservoir containing 20 l of a solution of 25 µM  
129 sulfide in natural surface seawater. This concentration roughly corresponds to the maximum  
130 of estimated from the shrimps environment at Rainbow (Schmidt et al., in press). This  
131 moderate concentration also ensured that the oxygen is not fully depleted from the medium.  
132 When the reservoir was almost empty, the outlet line was connected to the inlet line, in order  
133 to recirculate the sulfide-enriched seawater; ii) After an exposure of one hour, seawater was  
134 then pumped into the vessel for 2h ; iii) finally the vessel was closed for 3h before the next  
135 pulse started with a freshly prepared 25 µM sulfide solution. These samples are referred to as  
136 "sulfide shrimps" further in the text. The term "re-pressurised shrimps" englobes both "non  
137 sulfide" and "sulfide" shrimps.  
138 Survival of the re-pressurized shrimps was determined at the end of the pressure experiments,  
139 by identifying each individual and witnessing its movements.

140

#### 141 **Light microscopy and transmission electron microscopy (TEM)**

142 Samples were dehydrated in ethanol and propylene oxide series and then embedded in an  
143 epoxy resin (Serlabo). Semi-thin and ultra-thin sections were made on a Reichert-Jung  
144 Ultramicrotome (Ultracut E) using a diamond knife. Semi-thin sections were stained with  
145 toluidine blue for observations by light microscopy (using a Nikon Optiphot-pol microscope  
146 and a Zeiss Opton photomicroscope). For ultrastructural observations, thin sections were laid



147 on copper grids and stained with uranyl acetate and lead citrate. Observations were carried out  
148 on a Philips 201 TEM, operating at 80 kV.

149

#### 150 **Energy dispersive X-ray microanalyses (EDX)**

151 Microanalysis was carried out using a JEOL JEM 2100F transmission electron microscope,  
152 operating at 200 kV, and acquired with an energy dispersive X-ray detection system (Tracor  
153 5400 FX), equipped with a Si(Li) diode, using a 2.4 nm probe.

154

#### 155 **Ultrastructural analyses and enumeration of bacteria**

156 Exhaustive analysis and enumeration of bacteria and their intracellular granules were  
157 undertaken on one individual for each treatment. For each shrimp, bacteria associated to 5  
158 setae of the scaphognathite were analyzed. For each seta, an overall picture was taken and  
159 picture of all the bacteria were then captured at a magnification of 20000. Bacteria cells were  
160 then counted and described. The occurrence of intracellular granules was noted for each cell.  
161 Granules were defined as electron-dense spots larger than 1.5  $\mu\text{m}$  on the pictures (i.e. 75 nm),  
162 as numerous dark spots of various sizes occur in the cells. Due to their small size, spots  
163 smaller than 75 nm cannot be analyzed by EDX and were not taken into account in this study  
164 because of the uncertainty on their nature.

165

#### 166 **Statistical analyses**

167 A one-way ANOVA was used to test differences in the state of the bacteria (i.e. percentage of  
168 full granules) among treatments. Normality was judged visually from normal probability plots

169 and homogeneity of variances was verified with the Levene test. A multiple range test using  
170 the Student-Newman-Keuls (SNK) procedure was performed to investigate the difference  
171 between treatments for significant results. All data analyses were carried out using Statistica  
172 v. 6 software.

173

#### 174 **DNA extraction**

175 One *in situ* reference shrimp was dissected under sterile conditions. DNAs from  
176 scaphognathite (SC) and branchiostegite (LB), were extracted using the FastDNA SPIN kit  
177 for soil samples (Bio 101 System, Qiagen) following the kit protocols.

178

#### 179 **PCR and cloning**

180 PCR were performed using the universal primers for Bacteria or Archaea 16S rDNA on both  
181 (SC and LB) extracted DNA samples: E8F (AGA GTT TGA TCA TGG CTC AG) and  
182 U1492R (GTT ACC TTG TTA CGA CTT) for Bacteria and A8F (CGG TGG ATC CTG  
183 CCG GA) and A1492R (GGC TAC CTT GTT ACG ACT T) for Archaea. PCR cycles were  
184 as follows : 1 cycle of 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min 30 at 49°C and 2 min  
185 at 72°C and 1 cycle of 6 min at 72°C.

186 The gene encoding particulate methane monooxygenase subunit A (*pmoA*) was amplified on  
187 the SC DNA using the primers described by Duperron et al. (2007a) A189F ( GGN GAC  
188 TGG GAC TTC TGG ) and MB661R (CG GMG CAA CGT CYT TAC C ). PCR cycles  
189 were as follows : 1 cycle of 4 min at 92°C , 30 cycles of 1 min at 92°C, 1 min 30 at 55°C  
190 and 1 min at 72°C and 1 cycle of 9 min at 72°C.

191 The gene encoding the APS reductase gene was amplified on the SC DNA using the primers  
192 designed before (Blazejak et al., 2006). PCR cycles were as follows : 1 cycle of 4 min at

193 92°C , 30 cycles of 1 min at 92°C, 1 min 30 at 58°C and 1 min at 72°C and 1 cycle of 9 min  
194 at 72°C.

195 Approximately 100 ng of bulk DNA was amplified in a 50 µl reaction mix containing (final  
196 concentration) : 1X Taq DNA polymerase buffer (Q biogen Starsbourg, France), 2 µM of  
197 each dNTP, 20 µM of each primer and 2.5U of Taq DNA polymerase (Q Biogen France).  
198 PCR products were then visualized on an agarose gel containing ethidium bromide before  
199 cloning. The PCR products were cloned with the TOPO TA Cloning kit (Invitrogen Corp.,  
200 San Diego CA USA) following to the manufacturer's protocol. PCR products were purified  
201 using the QIAquick PCR purification kit (Qiagen SA, Grenoble, France) following the  
202 manufacturer's instructions. Clone libraries were constructed by transforming *E. coli*  
203 TOP10F'. Clones were selected on Petri dishes containing ampicilline (50µg/ml) and XGAL  
204 and IPTG for the white – blue selection. White clones were then cultured and treated for  
205 sequencing at the “Ouest Genopole Plateforme” (Roscoff, France, [http://www.sb-](http://www.sb-roscoff.fr/SG/)  
206 [roscoff.fr/SG/](http://www.sb-roscoff.fr/SG/)) on a Abi prism 3100 GA (Applied Biosystem), using the Big-Dye Terminator  
207 V3.1 (Applied Biosystem) following the manufacturer's instructions.

208

### 209 **Phylogenetic analyses**

210 To determine approximate phylogenetic affiliations, sequences were compared to those  
211 available in databases using the BLAST network service (Altschul et al., 1990). Alignments  
212 of 16S rDNA sequences were performed using CLUSTALW (Thompson et al., 1994), further  
213 refined manually using SEAVIEW (Galtier et al., 1996). The trees were constructed by  
214 PHYLO-WIN (Galtier et al., 1996). Only homologous positions were included in the  
215 phylogenetic comparisons. For the 16S rDNA phylogenetic reconstruction, the robustness of  
216 inferred topology was tested by bootstrap resampling (500) (Felsenstein, 1985) of the tree

217 calculated on the basis of evolutionary distance (Neighbor-Joining-algorithm ; Saitou et al.,  
218 1987) with Kimura 2 correction. Sequences displaying more than 97% similarity were  
219 considered to be related, and grouped in the same phylotype. Phylogenies of amino acid  
220 sequences of the *pmoA* (154 aa) and APS (129 aa) were reconstructed using PHYLO-WIN  
221 with Neighbor-Joining-algorithm and PAM distance (according to Dayhoff's PAM model).  
222 The robustness of the inferred topology was tested by bootstrap resampling (500).

223

224 **Nucleotide sequence accession numbers.** Sequences have been deposited at EMBL with  
225 accession numbers: from AM412507 to AM412521 and from AM902724 to AM902731 for  
226 partial 16S rDNA sequences; from AM412502 to AM412506 for partial *pmoA* (particulate  
227 methane monooxygenase subunit A) gene; and from AM902732 to AM902736 for APS  
228 reductase gene.

229

### 230 **3. Results**

#### 231 **Morphology and ultrastructure of the epibionts**

232 A total of 315 pictures was analyzed on which 6567 bacterial cells were counted. On *in situ*  
233 reference shrimps, TEM observations of the scaphognathite bacteria revealed more  
234 morphological diversity (figure 1) than previously described by Scanning Electron  
235 Microscopy (SEM) studies (Segonzac et al., 1993 ; Zbinden et al., 2004).

236 We observed 3 types of filaments (two thin types and one large) and two types of rods.

237 Dimensions are in the range of those previously found (table 1). Two types of rods can be  
238 distinguished based on size, location and aspect of the intracellular contents. The first type

239 (figure 1b) is characterized by short and thick cells, with a dense dark intracellular content.  
240 They are mainly located on the setae. The second type (figure 1b) is longer and thinner, with a  
241 light intracellular content. These rods are mainly located on the barbula that emerge from the  
242 setae. Two types of thin filaments can be distinguished based on the aspect of the intracellular  
243 contents : i) a small number of thin filaments exhibit rectangular cells with no marked  
244 narrowing between two adjacent cells. Cells in these filaments show a homogeneous and  
245 dense content, with few electron light areas and no granules (figure 1d) ; ii) the others, more  
246 numerous, exhibit ovoid-shaped cells, with marked narrowing between two adjacent cells.  
247 Cells of these filaments have a more heterogeneous intracellular content (which seems denser  
248 at the periphery and more diffuse in the center) and contain granules (figure 1e).  
249 Ultrastructural changes are observed between the bacteria of re-pressurised shrimps and those  
250 of reference shrimps (figure 2). No significant morphological differences were noticed  
251 between the bacteria of the shrimps from both pressure experiments. Cells of large and thin  
252 filaments, as well as thick rods, have a less regular shape and exhibit a more heterogeneous  
253 intracellular content than those of reference shrimps (figure 2b-c). Only thin rods keep the  
254 ultrastructural aspect observed in reference shrimps. Some of the bacteria show a globular  
255 intracellular content (figure 2d) or additional membrane folds (figure 2e). These types are  
256 only observed among bacteria of the shrimps maintained at 230 bars. Occasionally, these  
257 morphotypes can have a very degraded aspect, with totally mis-shapen cells (figure 3a),  
258 completely globular cell contents (figure 3c) or cell ghosts (figure 3c). Cell ghosts are also  
259 occasionally observed among bacteria of reference shrimps where they represent 1.5 to 4% of  
260 all the bacteria, and may be due to the usual turn-over of the cells. Cells with irregular shape  
261 and contents account for up to 30% of all cells in the re-pressurised shrimps and ghosts up to

262 15% (intra-individual variation between the five setae is too high to test the significance of  
263 inter-individual variations and the effect of sulfide exposure). Furthermore, very few dividing  
264 cells were observed for re-pressurised shrimps, whereas they were numerous for *in situ*  
265 reference shrimps. Surprisingly we observed, for the first time among *R. exoculata* epibionts  
266 (in reference shrimps, as well as in re-pressurised ones), some bacteria with stacks of  
267 intracytoplasmic membranes typical of methanotrophs (figure 2f) in both reference and re-  
268 pressurised shrimps.

269

#### 270 **Intracellular electron dense granules**

271 Only granules larger than 75 nm in diameter were considered, the largest measuring up to 200  
272 nm. Spots under 75 nm were counted separately, as “spots”. The number of granules and  
273 spots is higher for reference shrimps than for re-pressurised ones (table 2). Granules occurred  
274 only in one type of thin filament, and are absent from thick filaments and rods. A given cell  
275 may contain several granules and spots (up to 7 granules and 10 spots per cell).

276 In the reference shrimps, most of the granules appear full (i.e they are electron dense and  
277 appear black on micrographs, figure 4a), whereas most appear partially or completely empty  
278 for the re-pressurised shrimps (i.e they are electron light and appear, at least partly white on  
279 micrographs, figure 4b). Percentage of full granules for each experiment are illustrated on  
280 figure 5. The percentage of full granules differs significantly between reference and re-  
281 pressurised shrimps (one-way ANOVA test;  $F = 76.942$ ,  $p < 10^{-6}$ ), although no significant  
282 difference was detected between sulfide and non-sulfide shrimps at 230 bars (SNK a  
283 posteriori test,  $p > 0.05$ ).

284

**285 Chemical composition of granules**

286 An EDX microanalysis was performed in order to determine the elemental composition of the  
287 granule content (figure 6). The control spectrum from the cytoplasmic area of the bacteria  
288 showed copper (Cu) peaks due to the support grid, uranyl (U) peaks due to uranyl acetate  
289 staining, and traces of chloride (Cl) due to the epoxy resin. Two types of granules were  
290 analyzed. The first type contains 2 main peaks : phosphorus (P) and iron (Fe), in some cases  
291 associated with small amounts of calcium (Ca) (not shown). The second type of granules  
292 shows a single peak of sulfur (S). Occasionally, traces of iron (Fe) are detected (but it can be  
293 due to the close occurrence of a thick iron oxide layer that surrounds some bacteria).

294

**295 Preliminary screening of bacterial diversity**

296 DNA was successfully extracted from scaphognathite and branchiostegite samples. PCR  
297 amplifications for Archaea failed regardless of the conditions tested, even with nested PCR.  
298 For Bacteria, 69 clones were sequenced for the scaphognathite and 56 for the branchiostegite  
299 of an reference shrimp. Only 53 clones sequences were kept for the scaphognathite sample  
300 and 46 for the branchiostegite sample, the other clone sequences being too short or of bad  
301 quality. No chimera was detected in our study.

302 All the sequences are related to the *Proteobacteria* cluster (figure 7), mainly within the  
303 Epsilon and Gamma groups, the Alpha and *Deltaproteobacteria* being less abundant. One  
304 group of 19 sequences is related to the *R. exoculata* gut clone 15, found in a previous study on  
305 the gut of a specimen from the same vent site (Zbinden and Cambon-Bonavita, 2003). A

306 second group of 13 sequences is related to sequences retrieved from a vent gastropod coming  
307 from Rodriguez Triple junction in the Indian Ocean (Goffredi, 2004). A third group (5 clone  
308 sequences) is related to the *Rimicaris exoculata* epibiont (Polz and Cavanaugh, 1995).  
309 Nineteen clones sequences are related to the *Rimicaris exoculata* gut clone 22 (Zbinden and  
310 Cambon Bonavita, 2003). Six clone sequences are related to the *Deltaproteobacteria*. Twenty  
311 four sequences are affiliated to the *Gammaproteobacteria*. These latter are related to  
312 sequences retrieved on a vent gastropod (Goffredi, 2004) and also to clone sequences  
313 retrieved on carbonate chimney from the Lost City vent field (Brazelton et al., 2006). The last  
314 group comprises eight clones, related to the *Alphaproteobacteria*, close to *Marinosulfomonas*  
315 *methylotropha*, and to a clone isolated from Lost City vent field (Brazelton et al., 2006).

316

### 317 ***pmoA* and APS sequence analyses**

318 We successfully amplified the *pmoA* and APS reductase genes using DNA extracted from the  
319 scaphognathite. Fifteen clones were sequenced for the *pmoA* and 5 for the APS reductase. All  
320 the sequences were kept for the phylogenetic analyses. For the *pmoA* gene (Figure 8), two  
321 clone sequences are affiliated to the *Methylobacter* sp. group, two clones sequences are  
322 affiliated to a *Bathymodiolus* symbiont sequence and 11 clones sequences are affiliated to the  
323 *Methylomonas methanica*. For the APS reductase gene (Figure 9), 5 sequences were related to  
324 the *Deltaproteobacteria*. Ninety sequences were only marginally related to the  
325 *Gammaproteobacteria* APS gene (83% of similarity) and were related to the *Idas* thiotrophic  
326 clone (Duperron et al. 2007b).



327 As no genes, until now, of the iron-oxidation pathway for neutrophilic iron-oxidizing bacteria  
328 are known, this metabolic pathway cannot be investigated by this method.

329

#### 330 4. Discussion

##### 331 Is sulfide oxidation active in the epibiotic community ?

332 Transmission electron microscopy allowed us to refine the morphological descriptions of the  
333 epibionts on the reference shrimps, detecting two types of thin filaments, and two types of  
334 rods, in addition to the thick filaments. These results indicate that the morphological diversity  
335 of bacteria associated with *R. exoculata* is higher than previously reported (Casanova et  
336 al., 1993; Gebruk et al., 1993 ; Zbinden et al., 2004). The molecular survey supports this  
337 result. Even though additional sequence investigations are needed to fully describe the  
338 microbial diversity within the gill chamber, the present study provides a preliminary overview  
339 of the epibiotic community composition. Many Epsilon*proteobacteria* sequences are related  
340 to microbial diversity usually associated with various hydrothermal invertebrates (*Alvinella*  
341 *pompejana*: Alain et al., 2002; *Paralvinella palmiformis*: Alain et al., 2004; gastropods:  
342 Goffredi et al., 2004; Suzuki et al., 2005; and *Rimicaris exoculata* gut: Zbinden and Cambon-  
343 Bonavita, 2003) and to the MAR environment (Lopez-Garcia et al., 2002). Only five  
344 sequences are slightly related to “*Rimicaris exoculata* ecto-epibiont”. The  
345 Deltaproteobacteria diversity is restricted to one cluster, and is related to an uncultured  
346 bacterium colonizing the mineral surfaces of a sulfide-microbial incubator. These  
347 microorganisms are usually thought to play a role in the sulfur cycle. In addition, we obtained  
348 APS reductase gene sequences that are related to those of the *Desulfobulbaceae* (Friedrich,

349 2002) known to be thiotroph. Most of the APS gene sequences obtained were related to the  
350 *Idas* thiotrophic symbiont gene (Duperron et al., 2007b), which is a *Gammaproteobacteria*,  
351 but with a low level of similarity (83%). In our phylogenetic survey, we did not obtain any  
352 16S rDNA gene sequence related to thiotrophic *Gammaproteobacteria*, so it is unlikely that  
353 our APS gene sequences are related to these *Gammaproteobacteria*. As no  
354 *Epsilonproteobacteria* APS gene sequence is available in databanks, our APS gene sequences  
355 are more likely related to the numerous *Epsilonproteobacteria* identified in the phylogenetic  
356 survey. It is noteworthy that the APS gene can be transferred laterally among Bacteria. It is  
357 therefore not a good phylogenetic marker (Friedrich, 2002; Meyer and Kuever, 2007).

358

359 Bacteria associated with re-pressurised shrimps exhibit different ultrastructures compared  
360 to the reference shrimps. A mean of 30% of the epibionts display what we interpret as a  
361 degraded aspect (i.e. heterogeneous or globular cellular content, irregular wall shapes, cell  
362 ghosts). In addition, the number of dividing cells is higher for the reference shrimps,  
363 indicating a better physiological state. These results could indicate that some of the bacteria  
364 cannot withstand the chemical environment of the re-pressurisation experiments, whether or  
365 not sulfides are present.

366 TEM observations of the epibionts reveal the massive occurrence of intracellular granules.  
367 Such granules are often present in prokaryotic organisms (Shively, 1974). They comprise  
368 polyglucoside, polyphosphate granules, crystals or paracrystalline arrays such as  
369 magnetosomes ( $\text{Fe}_3\text{O}_4$ ), poly- $\beta$ -hydroalkanoate (PHA) and sulfur globules. The main roles of  
370 these granules are hypothesized to be storage forms of energy and/or of various compounds

371 such as carbon, sulfur and phosphates. They can also play a part in detoxification processes.  
372 X-ray analyses indicate that there are two type of granules, one type containing phosphorus  
373 (P) and iron (Fe), most probably under polyphosphate form; the other type containing mainly  
374 sulfur (S). Several granules can occur in one bacterial cell, but they are always of the same  
375 type. The maintenance in a pressurized aquarium lead to the emptying of most of the  
376 granules, which suggests a storage role. Addition of sulfide does not affect this emptying  
377 phenomenon. However, the granules were counted as a whole, as it was no longer possible to  
378 morphologically distinguish the polyphosphate from the sulfur granules. It is conceivable that  
379 the slightly higher percentage of full granules, counted in the bacteria that received sulfide  
380 pulses (see figure 5), is due to a better conservation of the sulfur granules. *R. exoculata*  
381 epibionts (from the Snake Pit site) were hypothesized to acquire their metabolic energy from  
382 sulfide oxidation. At the ultrastructural level, sulfur-oxidizing bacteria are characterized by  
383 the accumulation of large granules of elemental sulfur, which is known to dissolve in solvents  
384 like those commonly used for classical TEM preparations (Vetter, 1985). Consequently, these  
385 globules appear empty in thin sections (Lechaire et al., 2006). On our sections, the granule  
386 contents were not removed during preparation steps, which suggests that they are not  
387 elemental sulfur under the form usually found in sulfur-oxidizing bacteria. We can then  
388 hypothesize that these granules are rather formed of another type of more stable cristalline  
389 sulfur or are sulfur-rich organic matter. Nevertheless, sulfur-containing biopolymers are rare :  
390 they are mostly proteins containing methionine and cysteine, or complex polysaccharides that  
391 contain sulfate groups. PTE (polythioester), a new class of sulfur-containing polymer, have  
392 recently been described, (Lütke-Eversloh et al., 2001). It belongs to the  
393 polyhydroxyalkanoates (PHAs), a class of biopolymers known to occur abundantly as storage

394 compounds for energy and carbon, in a large variety of bacteria and archaea (Anderson and  
395 Dawes, 1990).

396 Taken all together, the TEM observations of bacteria associated to re-pressurised shrimps  
397 show a low positive impact of sulfide reexposure. Three hypotheses could thus be put  
398 forward to explain this: 1) the concentration and frequency of the pulses were insufficient to  
399 allow a good maintenance of the epibionts, or 2) these bacteria do not all rely on sulfide for  
400 their growth, or 3) the chemical composition of the fluid in the pressure vessel was not  
401 adapted for epibiont growth that may require more complex substrates as suggested by the  
402 lack of cultures despite many attempts. Considering the results of previous work on the  
403 epibionts of *R. exoculata* (Zbinden et al., 2004) and the chemistry of this peculiar  
404 environment : low sulfide but high iron and methane concentration (Charlou et al., 2002 ;  
405 Douville et al., 2002), it is possible that some bacteria do not rely on sulfide oxidation but  
406 rather on iron or methane oxidation.

407

#### 408 **Occurrence of iron oxidation among the epibiotic community**

409 Genes involved in iron oxidation at neutral pH are still unknown and iron oxidizers show a  
410 broad diversity among the *Proteobacteria* (Edwards et al., 2003). So, iron oxidation  
411 metabolism could not be studied through a molecular approaches. Nevertheless, iron  
412 polyphosphate granules were detected inside the epibiont cells. Polyphosphate granules are  
413 widely distributed in prokaryotes, ranging in diameter from 48 nm to 1µm (Shively, 1974).  
414 Putative roles of polyphosphate are numerous : ATP substitute, energy storage or chelator of  
415 metal ions (Kornberg, 1995). Lechaire et al. (2002) described the occurrence of iron  
416 polyphosphates granules in bacteria associated with the tube of *Riftia pachyptila*, a

417 hydrothermal vent vestimentiferan. Since polyphosphates are known to fluctuate in response  
418 to nutritional and other parameters, these authors suggest that they could act as a reservoir of  
419 oxygen in the case of environmental anoxia. As the occurrence of iron-oxidizers among the  
420 bacteria has been suggested (Zbinden et al., 2004), these granules could be a reservoir for  
421 iron. Alternatively, if these granules occur in non-iron oxidizing bacteria, the chelation of iron  
422 by the polyphosphate granules could reduce its toxicity for the cell.

423 Anyway, the only way to certify the occurrence of iron-oxidizing bacteria among the  
424 epibionts is to successfully cultivate and isolate these strains. Such attempts are under  
425 progress in our lab.

426

427 **A possible alternative metabolism : methanotrophy and methylotrophy**

428 A sixth morphotype, bacteria with stacks of intracytoplasmic membranes typical of type I  
429 methanotrophs, was observed for the first time among *R. exoculata* epibionts. Moreover, our  
430 sequences cluster with known *Gammaproteobacteria* methanotrophic epibionts sequences,  
431 such as *Bathymodiolus* methanotrophic gill symbionts (Duperron et al., 2005). This is also  
432 supported by our three groups of *pmoA* sequences that clearly belong to the methylotrophic  
433 *Gammaproteobacteria* class (*Methylomonas* sp., *Methylobacter* sp. and *Bathymodiolus pmoA*  
434 gene sequences). In addition, some clone sequences are related to *Alphaproteobacteria*  
435 methylotroph species and to *Epsilonproteobacteria* clone sequences retrieved from enriched-  
436 methane environments such as the MAR Lost City and Rainbow sites, or to the Milano mud  
437 volcano (Figure 7).

438

439 **Co-occurrence of different metabolic types in the epibiotic community**

440 Taken all together, our microscopic observations and molecular data seem to indicate that at  
441 least three metabolic types could co-occur among the epibiotic microbial community  
442 associated to *R. exoculata* at Rainbow: iron-oxidation, methanotrophy and thiotrophy.  
443 Desbruyères et al. (2001) tried to correlate biological diversity to the varying composition of  
444 end-member fluids. According to the amount of iron oxide closely associated to the epibionts  
445 (Zbinden et al., 2004), and to the high level of ferrous iron in the pure fluids (Charlou et al.,  
446 2002), we suggest that iron oxidation may be the dominant metabolism for this site. Recently,  
447 Salerno et al. (2005) correlated the relative microbial abundance of epibiont types of two  
448 species of mussels (*Bathymodiolus azoricus* and *B. heckeræ*) with the availability of CH<sub>4</sub> and  
449 dissolved H<sub>2</sub>S in the end-member fluids. They found that when the CH<sub>4</sub>:H<sub>2</sub>S ratio was less  
450 than 1 (as for Snake Pit, Campbell et al., 1988) then thiotrophic epibionts were dominant. If  
451 the ratio was greater than 2 (as for Lost City, Kelley et al., 2001) then methanotrophs were the  
452 dominant epibionts. For Rainbow, the ratio of CH<sub>4</sub>:H<sub>2</sub>S varies from 1.54 to 2.61 in pure fluids  
453 (Charlou et al., 2002). Applying the Salerno et al. (2005) empirical model to *Rimicaris*  
454 epibionts at Rainbow, would suggest that methanotrophy is an important metabolic pathway,  
455 possibly dominating sulfide oxidation. Sampling and *in situ* measurements in shrimp swarms  
456 provide nevertheless a more realistic picture of the environmental conditions experienced by  
457 the shrimps. A recent study on potential electron donors for microbial primary production  
458 within the swarms at Rainbow indicates that ferrous iron is the most favorable energy source  
459 to support epibiotic growth. Methane and sulfide would appear as secondary energy sources  
460 in this environment, where hydrogen could also represent an alternative energy source for the  
461 epibionts (Schmidt et al., in press).

462

463 **Conclusion**

464 Based on TEM observations, and a preliminary molecular survey, the diversity of the  
465 *Rimicaris exoculata* epibionts (in terms of morphology and metabolism) appears to be higher  
466 than previously reported. Based on these results, we propose that the three metabolic types  
467 (iron, sulfur and methane oxidation) co-occur within the epibiont biomass associated with  
468 *Rimicaris exoculata*, and that the relative contribution of each metabolism may differ  
469 according to the local fluid chemical composition. A much wider scale study, with animals  
470 collected from chemically contrasted environments, is needed to better understand the  
471 connections of the epibiotic bacterial communities in response to the chemistry of the  
472 environment.

473

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617 **Figure legends**

618

619 **Figure 1:** Bacteria associated with a scaphognathite seta of a reference shrimp. **a)** General  
620 view of the seta (s) and the associated bacteria. **b to e)** Observed morphotypes: **b)** rods type  
621 (1) attached to the seta (s) and rods type (2) attached to the barbula (ba) ; **c)** large filaments; **e)**  
622 thin filaments without granules inside the cells ; **d)** thin filaments with granules. Scale bars: a  
623 = 5 $\mu$ m, b, c, d, e = 1  $\mu$ m.

624

625 **Figure 2:** Bacteria associated with a scaphognathite seta of the re-pressurised shrimps. **a)**  
626 General view of the seta and the associated bacteria. **b to e)** Observed ultrastructural  
627 modifications: **b)** type 1 rods (type 2 does not seem to be affected); **c)** large filaments with  
628 heterogeneous content; **d)** or with globular content; **e)** thin filaments with heterogeneous  
629 content (**d**), and occasionally occurrence of membrane folds at the boundary of the cell  
630 (arrows). **(f)** methanotrophic bacteria characterized by their stacks of intracytoplasmic  
631 membranes. Scale bars: a = 5 $\mu$ m ; b = 0.5  $\mu$ m ; c, d, e, f = 1 $\mu$ m.

632

633 **Figure 3:** Evolution of the morphotypes observed in the re-pressurised shrimps. Filament  
634 cells exhibit a mis shapen aspect (a), a completely globular content (b) or appear as ghosts (c).  
635 Scale bars: a = 1 $\mu$ m ; b, c = 0.5 $\mu$ m.

636

637 **Figure 4:** Bacterial intracellular granules. Granules are full (arrows) in bacteria associated  
638 with the reference shrimp (**a**) and mostly empty (arrows) in those associated with re-  
639 pressurised shrimps (**b**). Scale bars: a, b = 0.5µm.

640

641 **Figure 5:** Percentage of full granules in bacteria according to treatment. Diagram showing the  
642 percentage of full granules per seta for *in situ* reference shrimp, and re-pressurised shrimps  
643 either in seawater or submitted to sulfide pulses. The mean percentage for each treatment is  
644 also given.

645

646 **Figure 6:** Elemental X-ray microanalyzes of the bacterial intracellular granules. Spectra were  
647 obtained on a) the cytoplasm of the bacteria (as control), b) the first type of granule showing  
648 major Fe and P peaks and traces of Si, c) the second type of granule, showing one major S  
649 peak.

650

651 **Figure 7:** Phylogenetic trees obtained using Neighbor-Joining analysis with bootstrap  
652 resampling (500 replicates). Topologies were confirmed with Maximum Parsimony method.  
653 Bootstrap values are indicated on nodes above 70%. Accession numbers of the sequences  
654 used are indicated on the tree (from AM412507 to AM412521 and from AM902724 to  
655 AM902731).

656

657 **Figure 8:** Neighbor-Joining tree of pmoA amino acid sequences from *Rimicaris exoculata*  
658 gill chamber epibionts based on 154 amino acid positions using PAM distance (according to

659 Dayhoff's PAM model). The robustness of the inferred topology was tested by bootstrap  
660 resampling (500). Accession numbers of the sequences used are indicated on the tree (from  
661 AM412502 to number AM412506).

662

663 **Figure 9:** Neighbor-Joining tree of APS reductase amino acid sequences from *Rimicaris*  
664 *exoculata* gill chamber epibionts based on 129 amino acid positions using PAM distance  
665 (according to Dayhoff's PAM model). The robustness of the inferred topology was tested by  
666 bootstrap resampling (500). Accession numbers of the sequences used are indicated on the  
667 tree (from AM902732 to AM902736).

668



Figure 1  
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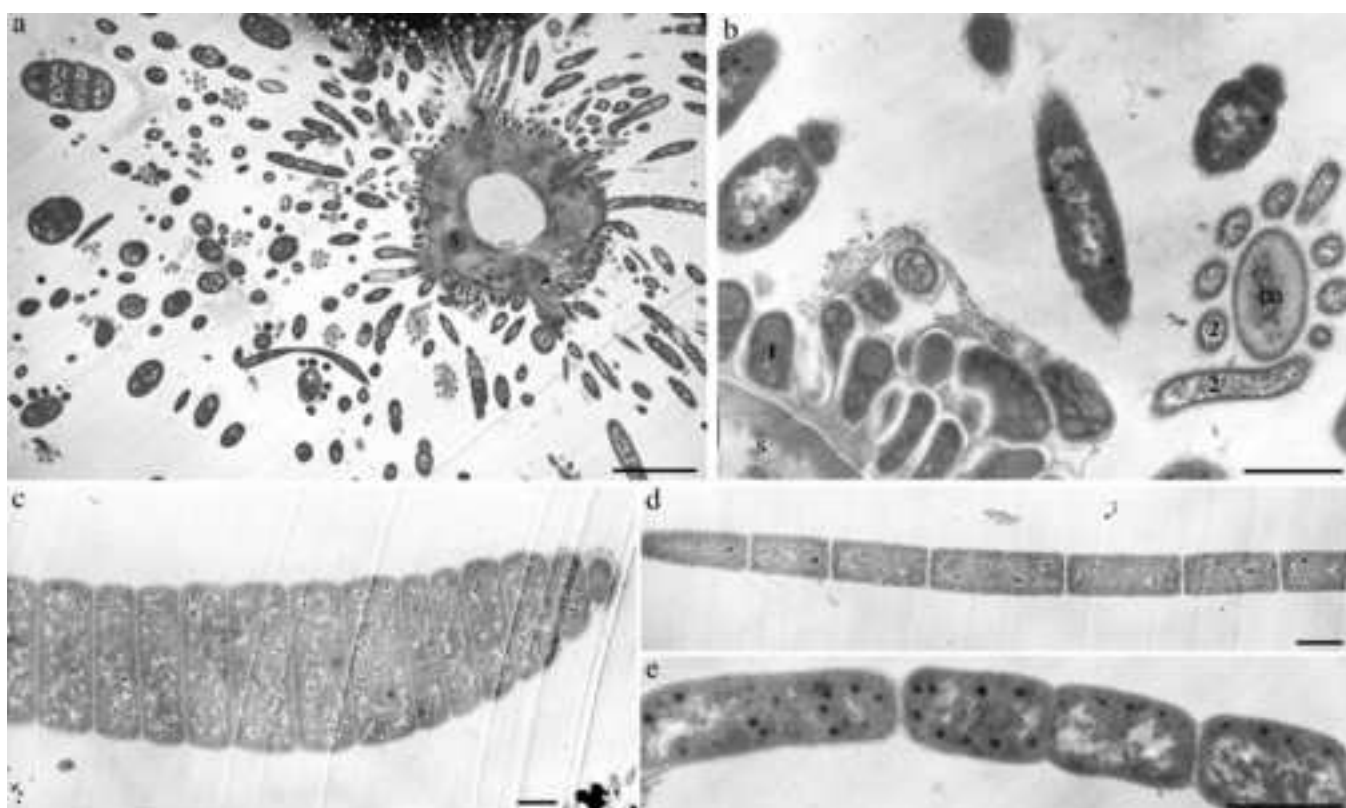
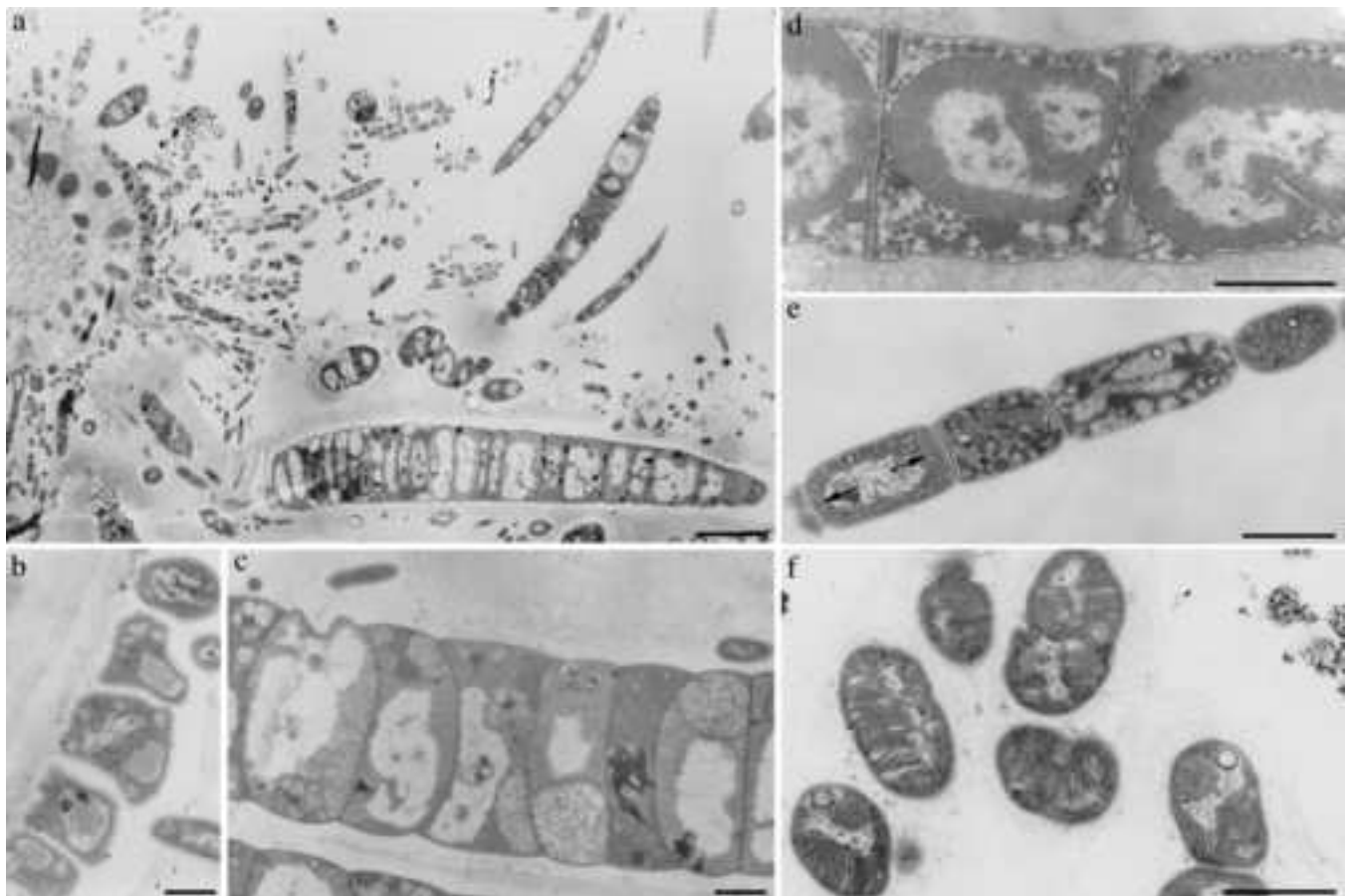


Figure 2  
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**Figure 3**  
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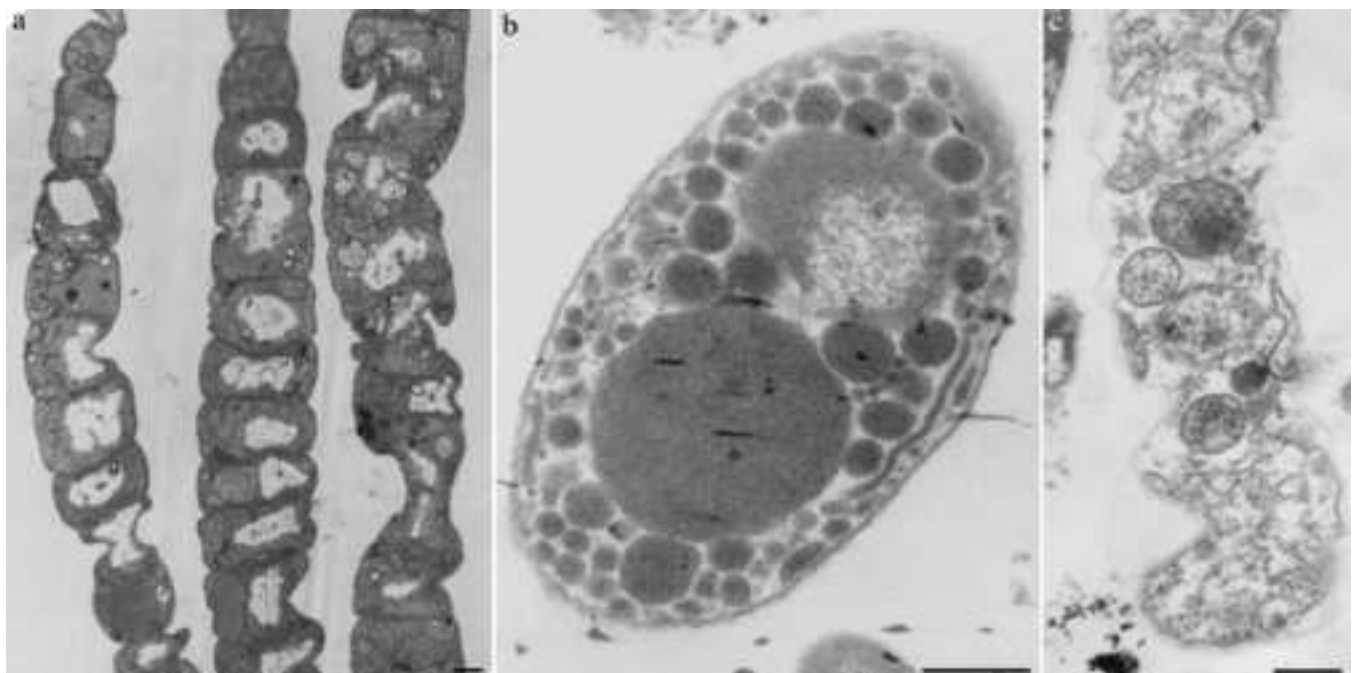


Figure 4  
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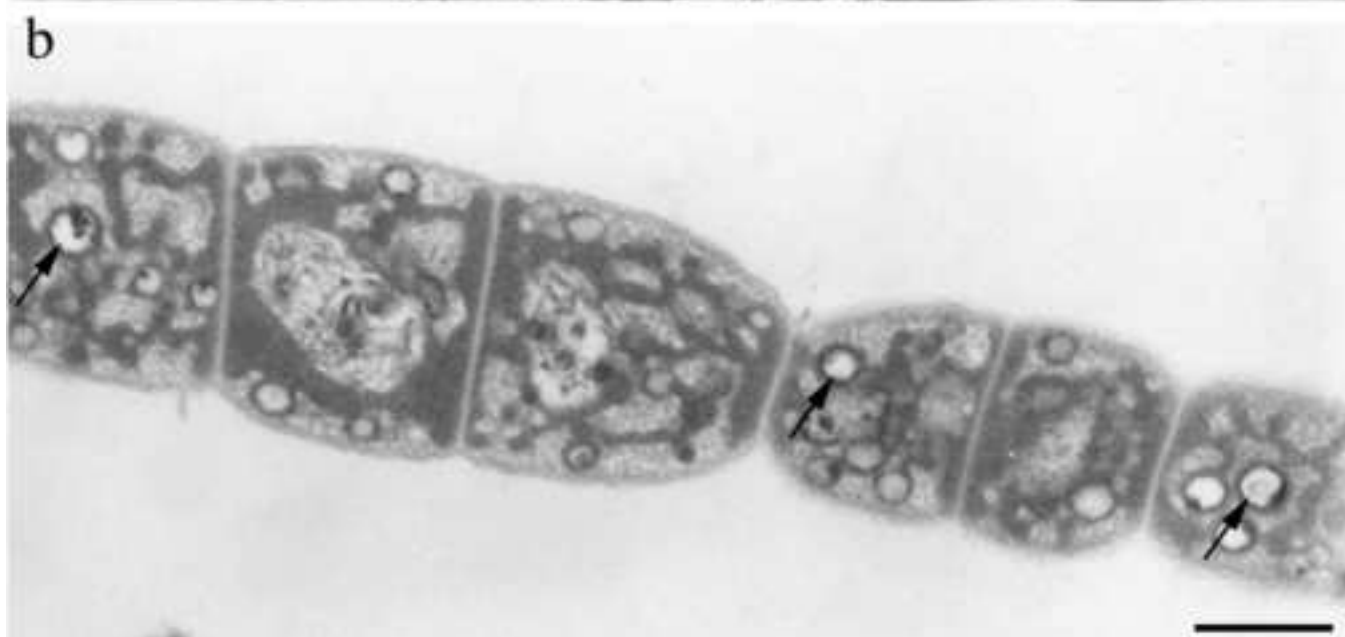


Figure 5

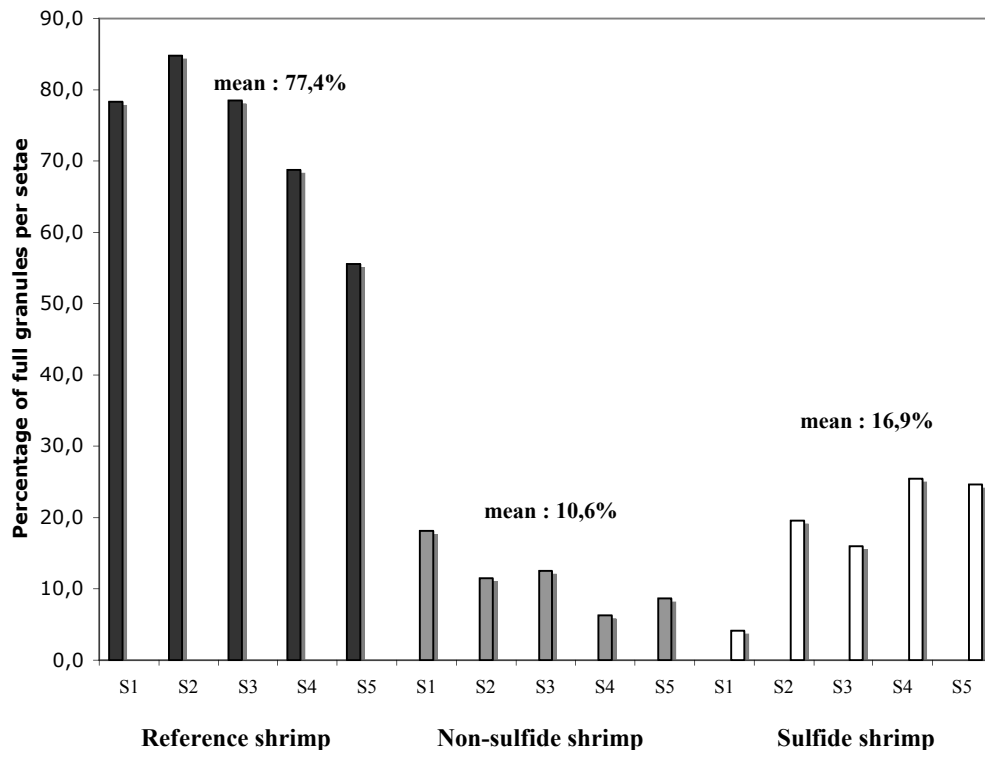


Figure 6  
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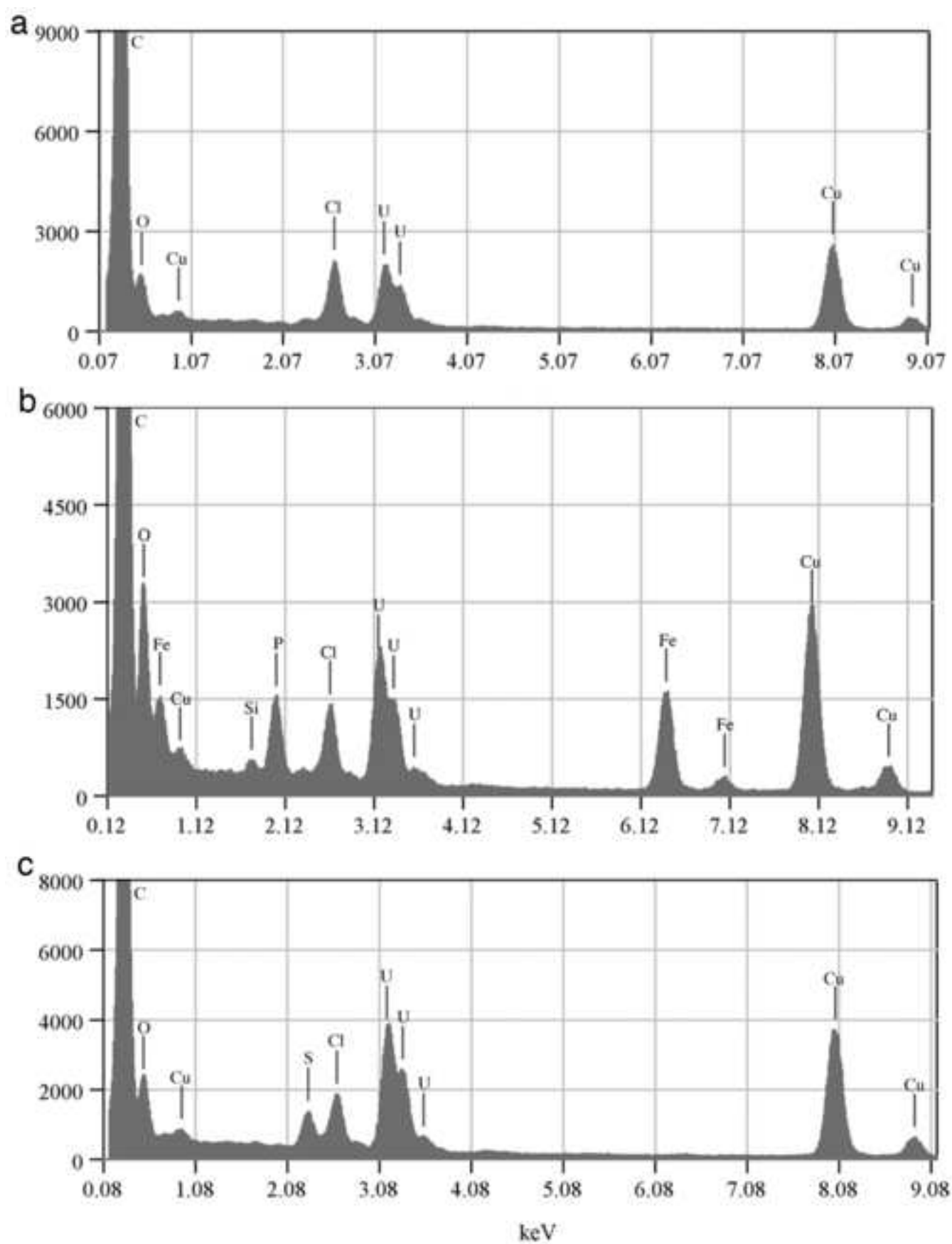


Figure 7

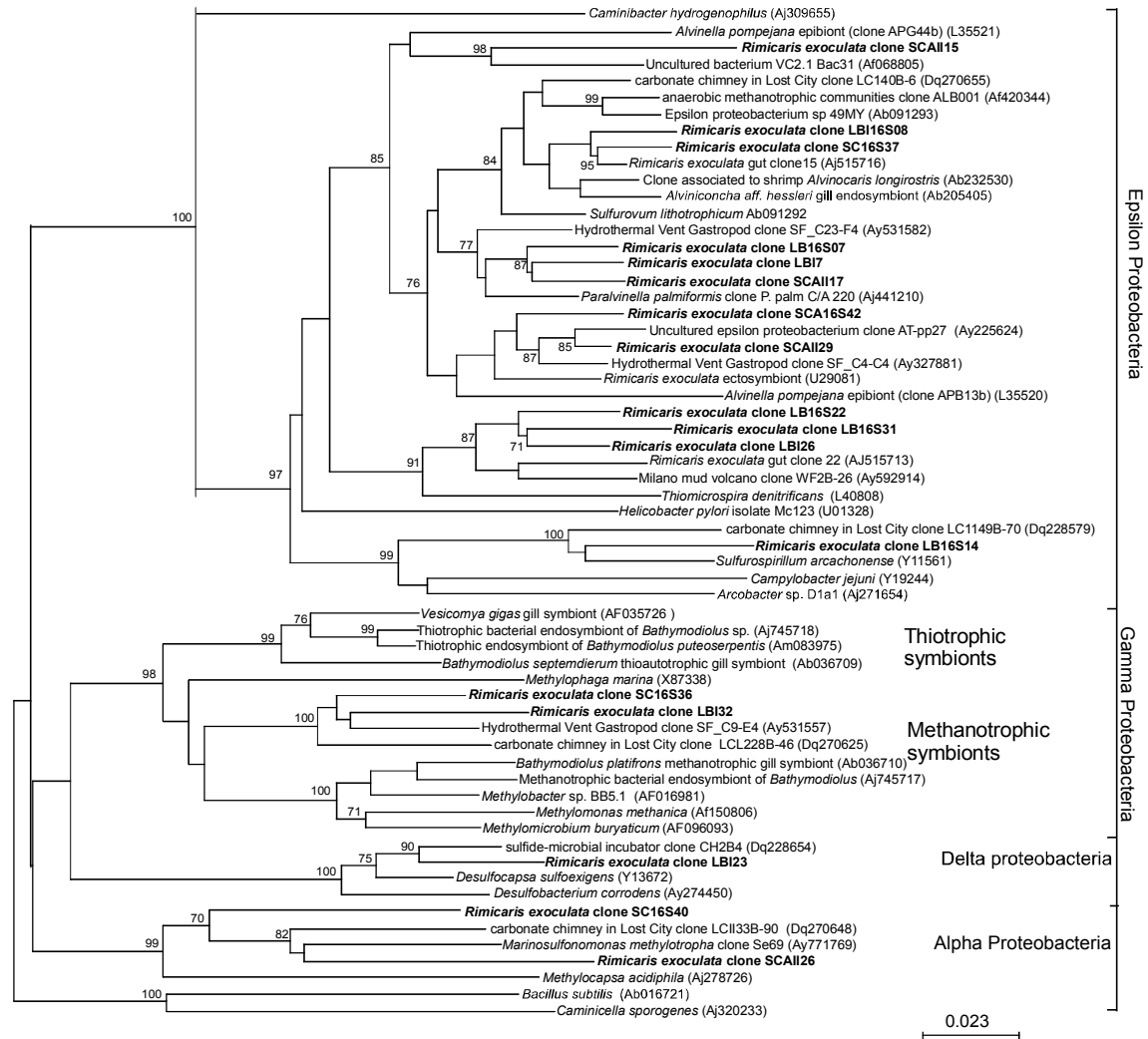


Figure 8

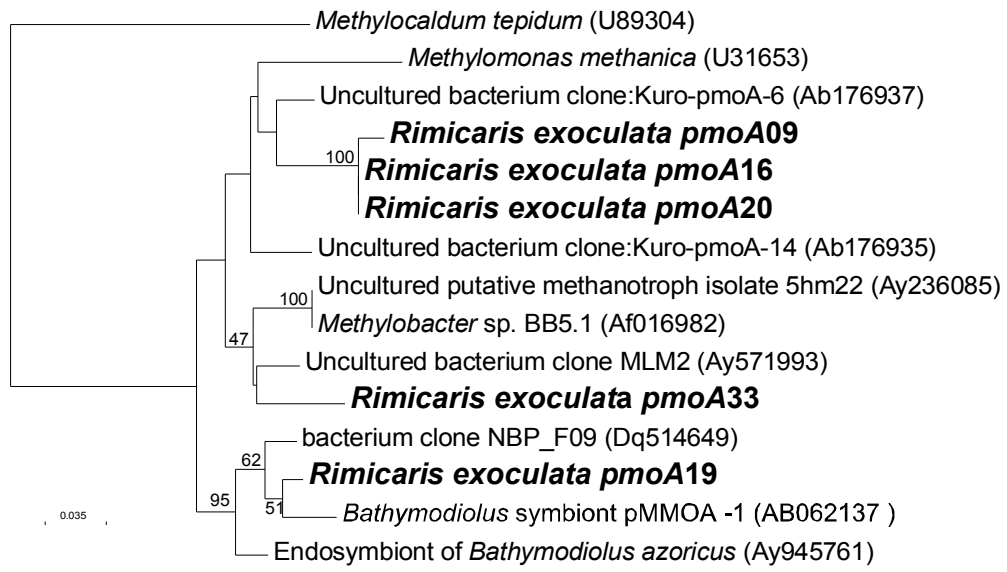
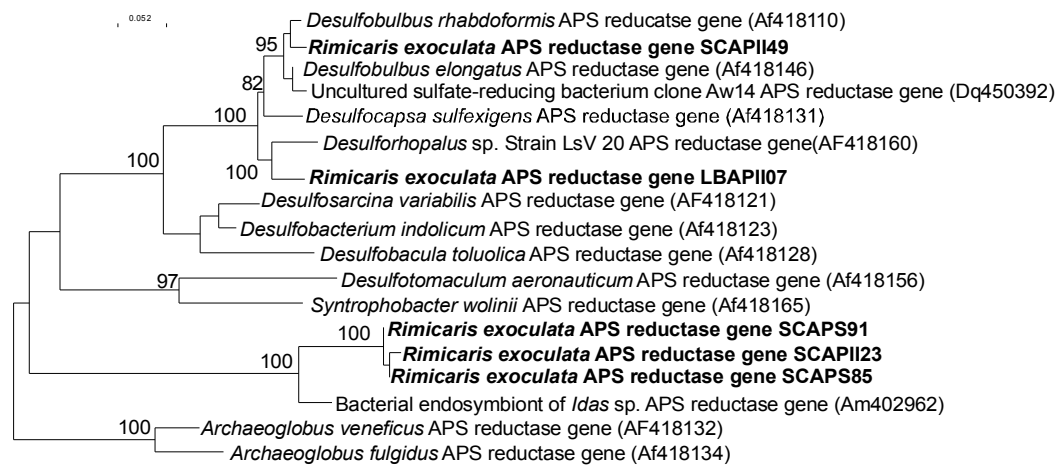




Figure 9



**Table 1** : Cell sizes of the various bacterial morphotype observed (values are given in  $\mu\text{m}$  and correspond to the upper values measured).

	<b>Cell diameter</b>	<b>Cell height</b>
<b>Large filament</b>	5,5	2,45
<b>Thin filament (1)</b>	1,1	2,35
<b>Thin filament (2)</b>	1,5	2,75
<b>Thick rods (type 1)</b>	0,6	1,25
<b>Thin rods (type 2)</b>	0,3	3

**Table 2** : Total numbers of bacteria analyzed, with number of granules and spots for each treatment.

	<b>Bacterial cells</b>	<b>Granules</b>	<b>Spots</b>
<b>Reference shrimps</b>	1574	721	875
<b>Non-sulfide shrimps</b>	3110	911	219
<b>Sulfide shrimps</b>	1883	628	207