Acquisition of epibiotic bacteria along the life cycle of the hydrothermal shrimp *Rimicaris exoculata*

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Abstract:

The caridean shrimp *Rimicaris exoculata* dominates the fauna at several Mid-Atlantic Ridge hydrothermal vent sites. This shrimp has an enlarged gill chamber, harboring a dense ectosymbiotic community of chemoautotrophic bacteria associated with mineral oxide deposits. Until now, their acquisition is not fully understood. At three hydrothermal vent sites, we analyzed the epibionts diversity at different moult stages and also in the first stages of the shrimp life (eggs, hatched eggs (with larvae) and juveniles). Hatched eggs associated with young larvae were collected for the first time directly from gravid females at the Logachev vent site during the Serpentine cruise. An approach using 16S rRNA clone libraries, scanning and transmission electron microscopy, and fluorescent *in situ* hybridization was used. Molecular results and microscope observations indicated a switch in the composition of the bacterial community between early *R. exoculata* life cycle stage (egg libraries dominated by the *Gammaproteobacteria*) and later stages (juvenile/adult libraries dominated by the *Epsilonproteobacteria*). We hypothesized that the epibiotic phylotype composition could vary according to the life stage of the shrimp. Our results confirmed the occurrence of a symbiosis with *Gammaproteobacteria* and *Epsilonproteobacteria*, but more complex than previously assumed. We revealed the presence of active type-I methanotrophic bacteria colonizing the cephalothorax of shrimps from the Rainbow site. They were also present on the eggs from the Logachev site. This could be the first 'epibiotic' association between methanotrophic bacteria and hydrothermal vent crustacean. We discuss possible transmission pathways for epibionts linked to the shrimp life cycle.

Keywords: symbiosis; larvae; methanotrophic symbiont; *Rimicaris exoculata*; transmission pathways

Introduction

Trophic symbioses are common in deep-sea hydrothermal ecosystems. In these environments, symbiosis between chemosynthetic bacteria and invertebrates supports a strikingly diversified fauna and significantly more biomass than in surrounding seawater (Ruehland *et al.*, 2010, Goffredi *et al.*, 2010; Bates *et al.*, 2011). One of these invertebrates is the shrimp *Rimicaris exoculata* (Williams and Rona, 1986). This crustacean, belonging to the family *Alvinocarididae*, is part of the dominant megafauna at several Mid-Atlantic Ridge (MAR) vent sites (Desbruyères *et al.*, 2001), where it forms dense and motile aggregates around the chimney walls (Segonzac, 1992; Gebruk *et al.*, 1993). *R. exoculata* harbours a rich community of epibiotic bacteria on the inner side of its enlarged gill chamber (also called cephalothorax) and on its mouthparts (scaphognathites and exopodites of the first maxillipeds, both covered with abundant bacteriophore setae). These characteristics were encountered in all *R. exoculata* specimens regardless of the site (Van Dover *et al.*, 1988; Casanova *et al.*, 1993; Segonzac *et al.*, 1993; Zbinden *et al.*, 2004), highlighted a possible obligate relationship between the shrimp and its epibionts. A δ^{13}C stable isotope study showed that the predominant source of dietary carbon for the shrimp was the gill chamber epibionts (Rieley *et al.*, 1999), but the bacterial community in the shrimp gut has also been proposed as an

106   alternative nutritional source (Polz *et al.*, 1998; Pond *et al.*, 2000; Zbinden *et al.*,

107   2003; Durand *et al.*, 2010).

108   Recent studies have shown that the diversity of *R. exoculata* epibionts was

109   higher than previously reported (Zbinden *et al.*, 2004, 2008; Petersen *et al.*,

110   2009; Hügler *et al.*, 2011). Based on *in vivo* experiments (IPOCAMP^{TM} (Shillito

111   *et al.*, 2004)), microscopic and molecular analyses, the co-occurrence of three

112   metabolisms (iron, sulfur, and methane oxidation) among gill chamber epibiont

113   communities has been proposed (Zbinden *et al.*, 2008). Moreover the *pmo*A and

114   *aps* genes were amplified. It was also suggested that the relative contribution of

115   each metabolism might differ according to fluid chemical composition (Zbinden

116   *et al.*, 2008; Schmidt *et al.*, 2008). Two filamentous epibiont phylotypes

117   (*Gamma* and *Epsilonproteobacteria*) dominated the *R. exoculata* epibiosis and

118   the sequences clustered spatially across the different vent sites along the MAR

119   (Petersen *et al.*, 2009). Finally, the occurrence of autotrophic carbon fixation

120   (rTCA cycle) via sulfur and hydrogen oxidation and sulfur reduction was

121   suggested on the Snake Pit site (Hügler *et al.*, 2011).

122   Like all arthropods, *R. exoculata* undergoes moults, which regularly

123   eliminate the bacterial community settled on the cuticle. The moult cycle

124   seemed to be shortened (10 days) compared to coastal shrimps (*Penaeus*

125   *japonicas* (21 days), *Macrobrachium rosenbergii* (41 to 98 days)) (Corbari *et*

126   *al.*, 2008). Briefly, the shrimps are white after moulting, turn to grey or light red

in the mid phase and black or red in the late phase according to the sulfur or iron fluid concentration respectively (Corbari *et al.*, 2008). Microscopic observations showed that a new epibiotic community started to form on free-surfaces of the new cuticle within 2 days after exuviations (Corbari *et al.*, 2008).

R. exoculata life cycle is still unknown. It produces lipid-rich orange eggs (Llodra *et al.*, 2000), which suggested the occurrence of planktotrophic larvae. Usually, egg size is about 300 to 400 μm (up to 836 eggs/female) (Tyler and Young, 1999). Gametogenic synchrony has never been observed (Tyler and Young, 1999), but a polymodal population structure for this shrimp suggested periodic recruitment (Copley *et al.*, 1998). Up to now, only very few gravid females have been collected and no larvae have ever been collected around the vent sites. Only juveniles above 1.2 cm were collected at the aggregates periphery and are orange (Komai and Segonzac, 2008). Wax esters, fatty acids, and fatty alcohols found in the juveniles indicated that they might feed for extended periods in the euphotic zone, allowing dispersion (Pond *et al.*, 1997). This was supported by genetic data that suggested high gene flow in *R. exoculata* populations (Teixeira *et al.*, 2011).

In this study, we analyzed the diversity and development of epibionts in *R. exoculata* gill chamber at different moult stages and also in the first stages of shrimp life (eggs, hatched eggs and 2 cm juveniles). An approach using 16S rRNA clone libraries, transmission and scanning electron microscopy

148 (TEM/SEM) and fluorescent *in situ* hybridization (FISH) was performed. Our

149 aims were to examine when the first acquisition of epibionts occurs and to

150 determine whether the epibiont community differs between early life stages and

151 adults, and also between moult stages.

152 <u>Materials and Methods</u>

153 **Collection / Selection / Pretreatment.** Specimens of *R. exoculata* were

154 collected at several hydrothermal vents sites along the MAR: at Logachev

155 (14°45‛N; 44°57‛W; 3037 meters depth) and Ashadze (12°58‛N; 44°51‛W; 4088

156 meters depth) during the Serpentine cruise (March 2007); at Rainbow (36°13‛N;

157 33°54‛W; 2350 meters depth) during the MoMARDREAM-Naut cruise (June

158 2007). Shrimps were collected using the suction sampler of the ROV „Victor

159 6000‟ or the Nautile operated from the R/V „*Pourquoi pas ?*‟. Once on board,

160 living individuals were dissected into body parts (branchiostegites (LB),

161 scaphognathites (Sc), exopodites, gills, stomach and digestive tract). For

162 molecular studies, animal tissues and eggs, hatched eggs (still associated with

163 young larvae) and orange juveniles (2 cm) were directly frozen (-80°C) and

164 DNA extractions were performed in the laboratory. For transmission and

165 scanning electron microscopy, samples were fixed as previously described

166 (Zbinden *et al.*, 2008), as well as for fluorescence *in situ* hybridization (FISH)

167 (Durand *et al.*, 2010). Shrimps were sorted according to moulting stages

168 (corresponding to a colour gradient: white (first stage), light red or grey (middle

169 stage), red or black (last stage)). Only black shrimps were collected at the

170 Ashadze site. Eggs and hatched eggs were only found at the Logachev site.

171 Seawater near shrimp aggregates (pH 7.3 and T°C=13°C) was also sampled at

172 the Rainbow site.

173 **DNA extraction and PCR amplification.** DNA from Rainbow seawater, adult

174 LB / Sc, eggs, hatched eggs and juveniles (Sc) was extracted using the Fast

175 DNA Pro Soil-Direct Kit (Qbiogen, Santa Ana, CA) (Table S2). Extracted DNA

176 was then purified with Quick-Clean Spin Filters (Qbiogen, Santa Ana, CA).

177 Bacterial 16S rRNA gene fragments were PCR-amplified in 30 cycles at an

178 annealing temperature of 49°C with the general bacterial primer set 8F and

179 1492R (Lane, 1991). They were then purified with a QIAquick PCR purification

180 kit (Qiagen, France).

181 **Cloning and sequencing.** The pooled amplified and purified PCR products

182 were cloned using the TOPO XL Cloning kit (Invitrogen, Carlsbad, CA)

183 following the manufacturer's instructions. The plasmid inserts were controlled

184 by amplification with M13F and M13R primers. Positive clones were then

185 cultured and treated for sequencing at the Biogenouest Platform (Roscoff,

186 France, http//www.sb-roscoff.fr/SG/) on an ABI prism 3130 *xl* (Applied

187 Biosystems, Foster City, CA), using the Big-Dye Terminator V3.1 (Applied

188 Biosystems, Foster City, CA).

189 **Phylogenetic analyses.** Sequences (16S rDNA) were compared to those

190 available in databanks using the BLAST online service (Altschul *et al.*, 1990).

191 Unstable (e.g. chimeras) and short sequences were excluded; others were

192 cleaned manually with „EDITSEQ" (DNA STAR, Madison, WI, U.S.A).

193 Alignment of sequences was performed using the CLUSTALW program

194 (Thompson *et al.*, 1994), further refined manually using the SEAVIEW program

195 (Galtier *et al.*, 1996). All trees were built using PHYLO-WIN (Galtier *et al.*,

196 1996). Phylogenetic analyses were performed on the basis of evolutionary

197 distance (Neighbor-Joining, (Saitou and Nei, 1987)) with Kimura two-

198 parameters correction matrix. The robustness of phylogenetic reconstructions

199 was tested by bootstrap resampling (500) (Felsenstein, 1985). Sequences

200 exhibiting more than 97 % similarity were considered to be sufficiently related

201 and grouped in the same phylotype.

202 The rarefaction curves and Simpson indices were performed using DOTUR (at

203 97% similarity) for all libraries (Schloss and Handelsman, 2005). Simpson index

204 was calculated as: $1 - Hsimpson = 1 - \left[\dfrac{\sum_{i=1}^{Sobs} Si(Si-1)}{N(N-1)} \right]$

205 where S_{obs} representing the number of OTUs observed, S_i the number of

206 individuals for one OTUs and N the total number of OTUs. Good's coverage

207 was calculated as a percentage, according to the following relation C = [1 −

208 (*n/N*)] x 100, where *n* represented the number of phylotypes appearing only once

209 in a library and *N* being the library size (Good, 1953, Ravenschlag *et al.*, 1999).

210 **Fluorescence *in situ* hybridization (FISH).** The FISH protocol used was

211 described previously (Durand *et al.*, 2010). Whole LB / Sc (adult and juvenile),

212 eggs and hatched eggs (Table S2) were hybridized with several published probes

213 (Table 2). The hybridization temperature was the same for all sample treated

214 (46°C). Observations and imaging were performed using an Apotome Axio

215 Imager Z_2 with a COLIBRI system (Zeiss, Germany).

216 **Scanning electron microscopy (SEM).** LB / Sc, eggs and hatched eggs were

217 dehydrated in ethanol series (30, 50, 70, 95, 100 % ethanol) and for 5h in a

218 critical point dryer CPD 020 (Balzers union, Balzers, Liechtenstein). Finally,

219 samples were gold-coated with an SCD 040 (Balzers Union). Observations and

220 imaging were performed using a Quanta 200 MK microscope (FEI^{TM}, Hillsboro,

221 OR) and the *SCANDIUM* acquisition program (Soft Imaging System, Munster,

222 Germany).

223 **Transmission electron microscopy (TEM).** Samples were dehydrated in

224 ethanol and propylene oxide series and then embedded in an epoxy resin

225 (Serlabo). Semi–thin and ultra–thin sections were made using a Reichert–Jung

226 Ultramicrotome (Ultracut R) with a diamond knife. Semi-thin sections were

227 stained with toluidine blue for observations by light microscopy (using an

228 Olympus BX61 microscope). Thin sections were laid on copper grids and

229 stained with uranyl acetate and lead citrate. Observations were carried out on a

230 LEO 912 electron microscope (LEO Electron Optics GmbH, Oberkochen,

231 Germany) equipped with a LaB6 source and operated at 80 kV.

232 **Nucleotide sequence accession numbers.** The sequences from this study are

233 available through GenBank under the following accession numbers: FR797908

234 to FR797966 (16S rRNA sequences).

235 Results

236 **Samples description.**

237   Seawater collected inside Rainbow shrimp aggregates was slightly orange.

238 Shrimps collected at the three sites (Rainbow, Logachev and Ashadze) were

239 sorted at different moult stages, according to their colour, from white (no

240 minerals or bacteria) to dark red or black (mineral oxide deposits). At the

241 ultramafic Rainbow vent field, the end-member was characterized by extremely

242 high concentrations of ferrous iron (Charlou *et al.*, 2002, Douville *et al.*, 2002),

243 explaining the reddish colour of majority of shrimps (Zbinden *et al.*, 2004). At

244 the Logachev vent site, there was a majority of grey/black shrimps,

245 corresponding probably to iron sulfate deposits (Gebruk *et al.*, 1993). For

246 Ashadze, only 6 black specimens were retrieved. Surprisingly, Ashadze fauna

247 was dominated by species usually recovered at the periphery of hydrothermal

248 communities (*Maractis rimicarivora* and *Phyllochaetopterus* sp. nov.) (Fabri *et*

249 *al.*, 2010). All the collected shrimps were alive and active when recovered from

250 the slurp gun bowls, but the Ashadze specimens were less active than the other

251 sites. It should be noted that Ashadze is the deepest hydrothermal site (4080 m)

252 where *R. exoculata* has been identified.

253   Orange juveniles (2 cm stage A larvae (Komai and Segonzac, 2008)) were

254   sampled at the Rainbow and the Logachev sites, in the periphery of adults but

255   close to the aggregates (Fig. S3). For the first time, eggs and hatched eggs were

256   collected at Logachev during the Serpentine cruise in March 2007 from females

257   collected among the shrimp aggregates (Fig. 1a, b). The eggs, orange, were at

258   different maturity stages, from small young eggs (around 200 μm) to mature

259   eggs (around 400 μm), with only one stage per female. The eggs were always

260   hatched beneath the female abdomen so that only free larvae would be released

261   in the environment. The collected larvae were just hatched eggs, probably at a

262   zoeal stage (Fig. 1b). The rostrum was absent. Eyes were present, ovoid, and

263   seemed to be borne on short eyestalks. Orange pigmented spots were observed

264   in the eyes. Four pairs of pereiopods were visible, 3 of them were bifid, and bear

265   3 setae at their tip, the 4th was just a bud. Cephalothorax, covered by a loose

266   carapace, contained the same orange lipid droplets observed in eggs. The

267   abdomen was composed of 5 well delimited short segments. A long terminal

268   segment ended with two blades provided with 6 setae. No pleopods were

269   observed.

270   **Microscopic observations.**

271   I). Adult/juvenile gill chamber - SEM observations on cephalothorax

272   pieces (LB: branchiostegite and Sc: scaphognathite) along the moult cycle

273   confirmed the different epibiont morphologies (e.g. rod-shaped, thin and thick

274   long filaments) observed before (Zbinden *et al.*, 2008). Their development

seemed to follow a chronological order along the moult cycle: rod-shaped bacterial mat, followed by long filamentous bacteria, as previously described (Corbari *et al.*, 2008). These morphologies were observed at all the sites studied (Rainbow, Logachev, and Ashadze). FISH observations on LB and Sc along the moult cycle, whatever the hydrothermal site, indicated the predominance of *Epsilonproteobacteria* with thick and thin filamentous morphologies (Fig. 2a, b). This was congruent with molecular studies (Table 1) (Polz and Cavanaugh, 1995; Zbinden *et al.*, 2008; Petersen *et al.*, 2009; Hügler *et al.*, 2011). *Gammaproteobacteria* signals were also detected to a lesser extent, and were related exclusively to some thin filamentous morphologies (Fig. 2a,b) confirming previous results (Petersen *et al.*, 2009; Hügler *et al.*, 2011). Type I methanotrophic *Gammaproteobacteria* morphologies were observed using transmission electron microscopy (Zbinden *et al.*, 2008). For the first time we confirmed it with the typical circular positive FISH signal (Duperron *et al.*, 2005) with both the GAM42a probe and the LBl32/130 probe (Table 2, Fig. 2c). On the Rainbow site, these methanotrophic like bacteria were clearly at the basis of long filaments affiliated to *Epsilonproteobacteria*, directly fixed on the *R. exoculata* tissues (LB and Sc) (Fig. S1). This specific localization seemed to confirm that the type I methanotrophic *Gammaproteobacteria* were not opportunistic. This morphology was observed only associated with Rainbow juveniles and adults, whatever the moult stage. The other phylogenetic groups (Table 1) were not detected in the gill chamber (using FISH analyses, Table 2).

297   II). Eggs – SEM and semi-thin observations showed the presence of a mat

298   of thin rod-shaped bacteria (around 2.5 μm length and 0.3 μm diameter) settled

299   on the egg surface for the majority of eggs observed (Fig. 1c, d, e). This

300   microbial mat hybridized only with the GAM42a probe, but no methanotrophic-

301   like bacteria was revealed by FISH analyses (Fig. 2d). TEM observations

302   confirmed the presence of bacteria embedded in a mucus covering the eggs (Fig.

303   1f) and some had intracytoplasmic membranes like type I methanotroph (Fig.

304   1g). They were smaller (1 μm) than the one observed on the Rainbow adult

305   shrimps (2 μm). No bacteria were observed inside the eggs (TEM and FISH).

306   III). Larvae – larvae used in this study had just hatched (Fig.1b) and were

307   still associated to their egg (Fig. 1a) (Fig. 4). SEM and FISH observations

308   showed no obvious bacterial mats on the larvae itself, but only single cells. No

309   bacteria were observed inside the larvae gill chamber (TEM and FISH).

310   Molecular surveys were therefore not undertaken on larvae alone, but on larvae

311   and its egg (hatched egg).

312   **Bacterial diversity (16S rRNA) along the *R. exoculata* life cycle.**

313   Diversity studies using PCR amplification and cloning are known to

314   underestimate genetic diversity because of faster amplification of some

315   sequences and bias both in amplification and cloning (Qiu *et al.*, 2001).

316   Moreover, sampling methods introduce additional biases (Bent and Forney,

317   2008). Clone libraries obtained in this study can therefore be considered only

318   partially quantitative. As all experiments were performed using the same

319   protocols, they can nevertheless be compared. Moreover FISH analyses

320   confirmed libraries diversity. Phylogenetic diversity along the *R. exoculata* life

321   cycle was completed using rarefaction analyses and diversity indices (Fig. S2,

322   Table S1). A total of 11 bacterial 16S rRNA gene clone libraries were analyzed,

323   corresponding to 817 clone sequences (Table 1). *Epsilonproteobacteria* and

324   *Gammaproteobacteria* dominated all the clone libraries (Table 1). This was

325   consistent with recent studies (Zbinden *et al.*, 2008; Petersen *et al.*, 2009; Hügler

326   *et al.*, 2011). *Deltaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria* and

327   *Bacteroidetes* were poorly represented (Table 1), confirmed by the absence of

328   FISH signal. These sequences might represent opportunistic microorganisms

329   embedded in the mat covering the appendages. Nevertheless, recent Snake Pit

330   site study showing the recovery of one deltaproteobacterial phylotype in high

331   frequency, suggested that it might have a role in the epibiotic community

332   (Hügler *et al.*, 2011). The clone diversity coverage (Good‟s coverage) was high

333   for all clone libraries with an average of 93% (± 5) (Table S1) and the

334   rarefaction curves showed that the clone libraries correctly described the

335   epibiotic communities, excepted for hatched eggs library (Fig. S2).

336   In this study, *Epsilonproteobacteria* sequences were overwhelmingly

337   related to sequences usually retrieved from hydrothermal invertebrates (e.g.

338   *Crysomallon squamiferum* (Goffredi *et al.*, 2004); *Alvinocaris longirostris*

339   (Tokuda *et al.*, 2008); *Shinkaia crosnieri* (unpublished) ; *Rimicaris exoculata*

340 gut (Zbinden *et al.*, 2003) and gill chamber (Polz and Cavanaugh, 1995;

341 Zbinden *et al.*, 2008; Petersen *et al.*, 2009) and also to the MAR environment

342 (Lost City (Brazelton *et al.*, 2006); Rainbow (Lopez-Garcia *et al.*, 2003); Snake

343 Pit (unpublished)) (Fig. 3A, B, Table S3). The main nine *Epsilonproteobacteria*

344 clusters fell within the "hydrothermal invertebrates associated epibionts" group

345 (Marine Group 1) (Fig. 3A). The closest cultivated relative was *Sulfurovum*

346 *lithotrophicum*, a sulfur-oxidizing chemolithoautotroph isolated from a

347 hydrothermal vent in the mid-Okinawa Trough (Inagaki *et al.*, 2004) (94%

348 similarity with *R. exoculata* RBR (AM412509)), Fig. 3B). Other

349 *Epsilonproteobacteria* sequences were affiliated to known genera:

350 *Thiomicrospira, Campylobacter, Arcobacter* and *Sulfospirillum* (Fig. 3A). The

351 three latter genera belong to the *Campylobacteraceae* family are known to

352 exhibit important metabolic diversity (including sulfur-oxidizing and reducing

353 bacteria). The closest *Thiomicrospira* species *T. denitrificans* (Fig. 3A, 89%

354 similarity with *R. exoculata* RBR (AM412516)) is an obligate chemolithotroph

355 oxidizing sulfide and thiosulfate, and is also a denitrifier (Muyzer *et al.*, 1995).

356 The Logachev and the Ashadze *Epsilonproteobacteria* related sequences

357 clustered together (Fig. 3B, cluster 3 and 6) but not with the Rainbow sequences

358 (Fig.3B, cluster 1, 2, 5, 7 and 8). Phylogenetic analyses showed that the same

359 epibiont sequences were retrieved all along the shrimp life cycle, from eggs to

360 adult on Logachev (Fig.3B, cluster 6 and 9). At the Rainbow site, some seawater

361 sequences (*R. exoculata* RBF (FR797932)) were almost identical (99.9%

362 similarity) to shrimp epibiont sequences (*R. exoculata* RBR (AM412509)) (Fig.

363 3B, cluster 7).

364   The *Gammaproteobacteria* were mostly affiliated to bacteria associated

365 with hydrothermal vent invertebrates (e.g. *C. squamiferum* and *K. hirsuta*

366 (Goffredi *et al.*, 2004); *Shinkaia crosnieri* (unpublished); *R. exoculata* (Zbinden

367 *et al.*, 2008)) (Fig. 3C, Table S3). The closest cultured relative to the cluster 1

368 *Gammaproteobacteria* epibionts (90.6% similarity) was *Leucothrix mucor*

369 (Grabovich *et al.*, 1999), a filamentous sulfur-oxidizer (Fig. 3C). The closest

370 cultivated relative to the cluster 2 *Gammaproteobacteria* epibionts (92.5%

371 similarity) was *Methylomonas methanica* (Costello and Lidstrom, 1999), a rod-

372 shaped methanotrophic bacterium (Fig. 3C).

373   All adult, juvenile and seawater libraries were dominated by the

374 *Epsilonproteobacteria* related sequences (Table 1). *Epsilonproteobacteria*

375 sequences dominated Logachev grey, Rainbow light red and Ashadze black

376 moult stages libraries compared to others (Table 1). The *Gammaproteobacteria*

377 were more represented in the Rainbow red moult library (Table 1).

378   Eggs and hatched eggs clone libraries distribution at Logachev were

379 clearly different compared to the adult, juvenile and seawater libraries (Table 1).

380 They were dominated by sequences related to the *Gammaproteobacteria* (Table

381 1), confirmed by FISH observations (Fig. 2d). For cluster 1, most of eggs and

382 hatched eggs sequences were closely related (99% similarity) to a *Shinkaia*

383   *crosnieri* epibiont (Fig.3C). For cluster 2, eggs and hatched eggs sequences were

384   closely related to *Methylomonas methanica* (Fig. 4).

385   <u>Discussion</u>

386   **Female behavior and life cycle.**

387   Until now, there was no report of *R. exoculata* females carrying eggs

388   inside the shrimp aggregates close to the hydrothermal chimney walls at the

389   MAR vent sites. One assumption was that gravid females were not inside the

390   aggregates to avoid damaging the eggs (Vereshchaka *et al.*, 1998), but only few

391   gravid shrimp have been observed around the MAR vent sites (Tyler and

392   Young, 1999). During the Serpentine cruise, gravid *R. exoculata* females were

393   observed and collected from aggregates at the Logachev vent chimney Irina II.

394   For the first time hatched eggs with larvae were collected, improving the

395   knowledge about the shrimp life cycle (Fig. 4). This cruise was held earlier in

396   the season (March) than others did (from May to November). The small size and

397   the composition (rich in lipids) of *R. exoculata* eggs could indicate short

398   embryonic development with larvae hatching at an early stage and undergoing a

399   relatively long planktotrophic period (Llodra *et al.*, 2000). *R. exoculata* could

400   thus exhibit seasonal reproduction, in which larvae hatch in early spring and

401   undertake an as yet unspecified period of planktotrophic development in the

402   water column. The lack of year-round data (absence of specimen between larvae

403   and 1.2 cm juvenile) made it difficult to conclude on the full life cycle of this

404   shrimp (Fig. 4). All eggs on a given female were at the same maturity stage, but

405   the stage differed from one female to another. This indicated that they were not

406   sexually mature at the same time, and that reproductive period would be longer

407   than the egg development duration. Eggs were still associated with the gravid

408   females when the hatching occurred so only mature larvae would be released.

409   To evaluate the egg development duration, pressured incubator (IPOCAMP^{TM})

410   maintenance of gravid females would be necessary.

411   **Epibiont diversity and acquisition.**

412   Some epibiont sequences were retrieved all along the shrimp life cycle

413   (Fig. 3B, cluster 6 and 9; Fig. 3C, cluster 1 and 2). This result suggested a high

414   specificity and the occurrence of an acute recognition mechanism such as in

415   nematode ectosymbioses (Nussbaumer *et al.*, 2004). Moreover, molecular

416   surveys indicated a bacterial community switch occurring between the first

417   stages of the *R. exoculata* life cycle (egg and hatched egg libraries dominated by

418   the *Gammaproteobacteria*) and latter stages (juvenile / adult libraries dominated

419   by the *Epsilonproteobacteria*) (Table 1), confirmed by FISH observations (Fig.

420   2a, b versus d). These results reinforced the occurrence of a complex stable

421   symbiosis in *R. exoculata* with the same *Gamma* and *Epsilonproteobacteria*

422   related   sequences   and   further   showed   that   symbiont   phylotypes

423   representativeness could vary according to the life stage of the host.

424   Observations highlighted the presence of colorless mucus-like material

425   surrounding the eggs. Mucus could be a „scaffolding" that provides anchorage

426   and protection for the eggs (Davies and Viney, 1998), while epibionts embedded

in the mucus could have a protective role in detoxication and also against potential pathogens (e.g. bacteria and fungi). This was the case for epibiotic bacteria associated with the *Homarus americanus* embryo which produce substances inhibiting pathogenic fungi growth (Gilturnes and Fenical, 1992). Bacteria within the gill chamber could have roles, such as detoxication or nutrition for the host (Zbinden *et al.*, 2004, 2008).

Ashadze and Logachev sequences clustered together (Fig. 3B, cluster 3 and 6) which might be explained by the very close proximity between these two sites (Fabri *et al.*, 2010). A recent study showed a significant correlation between genetic (16S rRNA) and geographic distances for *R. exoculata* epibionts along the MAR (Petersen *et al.*, 2009). The depth could also explain the clustering with the possible depth limit of 3000 m previously proposed (Priede *et al.*, 2006). Some *Epsilon* and *Gammaproteobacteria* sequences retrieved from the Rainbow seawater sample were closely related (99% similarity) to epibiont sequences from the gill chamber of shrimps from the same site (Fig. 3B cluster 1, 5 and 7; Fig. 3C cluster 1). All of these results would indicate the existence of horizontal (environmental) transmission for the shrimp cephalothorax epibionts. Epibionts associated to egg mucus could also be a result of vertical transmission (from mother to offspring) through mucus secretion (Mira and Moran, 2002). Vertical transmission usually implies internalization of symbionts inside the egg or in oviducts (Bright and Bulgheresi, 2010). But, microscopic observations (SEM, TEM and FISH)

449   showed that (I) there were no active bacteria inside the eggs, but only associated

450   with their outer surface (Fig. 1c), and (II) no bacterial mat was observed

451   associated with the young larvae just after hatching. The egg mucus interface

452   probably facilitated attraction, accumulation and host recognition of epibionts

453   for horizontal transmission. This epibiont transmission pathway is in adequacy

454   with the large colonization of the MAR by *R. exoculata* because horizontal

455   transmission is supposed to promote dispersal compared to vertical transmission

456   (Chaston and Goodrich-Blair, 2010). In terms of evolution, it was suggested that

457   episymbiosis represents a more primitive stage than endosymbiosis (Dubilier *et*

458   *al.*, 2008). The internalization of symbionts would then represent the final step

459   of the association. Nevertheless, a recent study based on 16S rRNA analyses

460   demonstrated that bathymodiolin epibionts were not ancestral to bathymodiolin

461   endosymbionts (Duperron *et al.*, 2009). These authors suggested that the

462   location of symbionts was not always a conserved trait and that both the host

463   and the symbiotic bacteria were more versatile in their ability to establish

464   associations than previously assumed.

465   It should be noted that only three gravid females were used for

466   phylogenetic studies (Table S2). More specimens of the first stages of the

467   shrimp life cycle (notably free larvae at each developmental stage) are necessary

468   for complementary analyses.

469

470

The methanotrophic metabolism hypothesis

Methanotrophic symbionts use methane as both an electron donor and a carbon source, with oxygen as the final electron acceptor. These symbionts have been described in deep-sea hydrothermal vents and cold seeps, where methane co-occurs with oxygen (Petersen and Dubilier, 2009). *In situ* observations showed that *R. exoculata* lives in the mixing zone between reduced hydrothermal fluid (containing methane at Rainbow and Logachev) and oxidized ambient seawater. Methane oxidation metabolism was previously suspected (Zbinden *et al.*, 2008). To our knowledge, all 16S rRNA sequences of methanotrophic symbionts from marine invertebrates belong to a single monophyletic lineage within the *Gammaproteobacteria* phyla related to type I methanotrophs. These bacteria are coccoid and have a concentric stacking of intracytoplasmic membranes, where the methanotrophic enzymes are located (Hanson and Hanson, 1996). These membranes probably push back the cellular material (including ribosomes) to the cell periphery, explaining the characteristic circular FISH hybridization signal (Fig. 2c). In this study, we have shown using molecular and microscopic approaches, the presence of active type I methanotrophic bacteria occurring in the cephalothorax of the Rainbow specimens (Fig. 2c and Fig. 3C, cluster 2) and located at the base of the filamentous bacteria (Fig. S1). This result was congruent with the fluid composition of this site, which is highly enriched in methane (Charlou *et al.*,

492 2002) and confirmed a previous study (Zbinden *et al*., 2008). Regarding the

493 eggs, TEM observations revealed methanotrophs shaped bacteria associated

494 with their membrane (Fig. 1g) and sequences affiliated to the methanotrophic

495 cluster were retrieved (Fig. 3C, cluster 2). According to their small size, these

496 cells might then be dormant, which could explain the absence of FISH signal.

497 Logachev, like Rainbow, is enriched in methane (Schmidt *et al.*, 2007) (Table

498 S2). Therefore, methanotrophy might also occur at this site, but at a lower

499 activity level. No methanotrophic related sequence has been retrieved in the

500 Rainbow seawater sample. This could be due to a PCR bias, the low number of

501 sequences treated or could indicate they were poorly present as free living

502 forms. Taken altogether, our results indicated the presence of methanotrophic

503 bacteria associated with *R. exoculata* (eggs and adults) in two sites, reinforcing

504 the symbiosis hypothesis. This could therefore be the first description of an

505 epibiotic association between methanotrophic bacteria and hydrothermal vent

506 crustaceans.

507 **Conclusion**

508 By describing the young larva just after hatching (Fig. 4), we improved

509 the knowledge of the *R. exoculata* life cycle. Nevertheless, the dispersion and

510 recruitment of *R. exoculata* along the MAR vent sites still unknown (Fig. 4).

511 Like larval dispersion, symbiont transmission is obviously an integral factor

512 influencing colonization efficiency (Teixeira *et al.*, 2011). Our results indicated

a possible horizontal transmission for the gill chamber epibionts of *R. exoculata* that could explain colonization along the MAR.

We have also described for the first time epibiotic communities associated with eggs and different stages from adults, and highlighted a community switch between *Gamma* and *Epsilonproteobacteria*. By coupling molecular biology and microscopic approaches we have demonstrated the occurrence of type I methanotrophic *Gammaproteobacteria*, one of the three metabolisms (iron, sulfur, and methane oxidation) expected to occur in the gill chamber (Zbinden *et al.*, 2008). Our results indicated that the epibiotic community was globally conserved along the MAR. We suggest that the phylotype relative abundance and the activity of the epibionts could vary according to the shrimp life stage and to the geochemical environment, reinforcing the symbiotic hypothesis. Future investigations will focus on identification (by PCR and RT-PCR) of functional genes implied in these different metabolisms. Deeper sequencing using high throughput sequencing technologies would be useful to exhaust the diversity. Finally, more sampling in the aggregates and in the water column will be necessary to complete the shrimp life cycle, as well as incubation experiments using gravid females.

540 **Supplementary information is available at ISME's website.**

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Table and Figure legends.

Table 1: Clone library results (based on partial 16S rRNA sequences). The main phylogenetic group per sample is shown in bold (*by Zbinden et al., 2008).

Phylogenetics groups	Logachev						Rainbow				Ashadze	Total
	Eggs	Hatched eggs	Juvenile	White moult	Grey moult	Black moult	White moult	Light Red moult	Red moult*	Hydrothermal fluid	Black moult	
Alphaproteobacteria	3	2					2	1	3			11
Betaproteobacteria							4	1				5
Gammaproteobacteria	**53**	**30**	1			1	10	1	25	5		126
Deltaproteobacteria	1	2	3	15		21			3	1		46
Epsilonproteobacteria	6	3	**50**	**71**	**84**	**83**	**38**	**67**	**45**	**54**	**95**	596
Bacteroidetes	4	7	7	1		3	5	1		4	1	33
Total	67	44	61	87	84	108	59	71	76	64	96	817

Table 2: Fluorescent probes (The probes sequences have been compared using BLAST to our sequences to check their specificity and determined their mismatches).

Specificity	Probe name	Sequence (5'-3')	Fluorescent dye	% Formamide	References
Archaea	Arch915	GTGCTCCCCCGCCAATTCCT	Cy3	10-20-30	Stahl and Amann, 1991
Eubacteria	Eub338	GCTGCCTCCCGTAGGAGT	Cy3 or Cy5 or ATTO488	10-20-30-40	Amann *et al.*, 1990
Alphaproteobacteria	ALF968	GGTAAGGTTCTGCGCGTT	Cy3	10-20-30-40	Manz *et al.*, 1992
Betaproteobacteria	BET42a	GCCTTCCCACTTCGTTT	Cy3	10-20-30-40	Manz *et al.*, 1992
Deltaproteobacteria	DELTA495b	AGTTAGCCGGCGCTTCCT	Cy3	10-20-30-40	Loy *et al.*, 2002
Gammaproteobacteria	GAM42a	GCCTTCCCACATCGTTT	Cy3	10-20-30-40	Manz *et al.*, 1992
Epsilonproteobacteria	EPSY549	CAGTGATTCCGAGTAACG	Cy3	20-30	Lin *et al.*, 2006
Bacteroidetes	CF319	TGGTCCGTGTCTGAGTAC	ATTO488	10-20-30-40	Manz *et al.*, 1996
Gammaproteobacteria R. exoculata cephalothoracic clones	LBl32/130	TCCTGGCTATCCCCCACTAC	ATTO488	10-20-30	Durand *et al.*, 2009

844 Figure 1: Microscopic observations of eggs and larvae (SEM : c, e ; TEM : f and

845 g).

a. *R. exoculata* eggs from Logachev. Scale bar = 500 μm.

b. *R. exoculata* larvae which just hatched from Logachev (manually

separated from egg). Scale bar = 200 μm.

c. Egg surface from Logachev covered by thin rod-shaped bacterial mat.

d. Egg thin-section from Logachev, showing bacterial mat (indicated with

dark arrows) on *R. exoculata* egg membrane. Scale bar = 10 μm.

e. Focus on picture c, showing thin rod-shaped bacterial mat.

f. Egg thin-section, showing thin rod-shaped bacteria (indicated with dark

arrows) on the egg membrane. Scale bar = 2 μm.

g. Methanotrophic like bacteria (with intracytoplasmic membranes, indicated

with a dark arrow) retrieved in the thin rod-shaped bacterial mat

associated with the egg membrane. Scale bar = 500 nm.

Figure 2: Fluorescence *in situ* hybridization.

a. Longitudinal view of Scaphognathite setae from Logachev black moult

shrimp with epibionts. *Gammaproteobacteria* (red) were hybridized with

GAM42a probe, *Epsilonproteobacteria* (green) were hybridized by

EPSY549 probe.

b. Transversal view of Scaphognathite *setae* from Logachev black moult

shrimp with epibionts. *Gammaproteobacteria* (red) were hybridized with

GAM42a probe, *Epsilonproteobacteria* (green) were hybridized by

EPSY549 probe.

c. Longitudinal view of Scaphognathite *setae* from Rainbow red moult shrimp with epibionts. The methanotrophic Gamma symbionts (orange) were hybridized with both LBl32/130 and GAM42a probes. The DAPI stained are in blue.

d. Egg membrane (mb) with epibionts, (in) eggs content, (out) outer environment. *Gammaproteobacteria* (red) were hybridized with GAM42a probe. The DAPI stained are in blue, showing eukaryotic nucleus of the egg.

Figure 3:16S rRNA phylogeny of the *Epsilonproteobacteria* (A and B, calculated on 817 bp) and *Gammaproteobacteria* (C, calculated on 804 bp) associated with the *R. exoculata* gill chamber. The robustness was tested using 500 bootstraps resampling of the tree calcuted using the Neighbor-Joining algorithm with Kimura two-parameters correction matrix (only bootstrap values over 70 are shown). Sequences names have been resumed as: AD, LG or RB for Ashadze, Logachev or Rainbow specimens, respectively, and E, HE, J, W, G, B, LR, R and F for Eggs, Hatched Eggs, Juvenile, White moult, Grey moult, Black moult, Light Red moult, Red moult and Fluid, respectively, and finally the numbers in brackets refer to the number assigned to each individual. Our clones are shown in color.

886   A. Global tree representing the *R. exoculata* epsilon symbiont and their close

887   relatives.

888   B. Secondary tree showing the "Hydrothermal invertebrates associated

889   epibionts" (Marine Group 1) (see Fig. 3A). Black arrow indicates the first *R.*

890   *exoculata* epibiont sequence discovered in the Snake Pit site.

891   C: 16S rRNA phylogeny of the *Gammaproteobacteria* associated with *R.*

892   *exoculata* gill chamber

893   Figure 4: Partial *R. exoculata* life cycle completed by this study.

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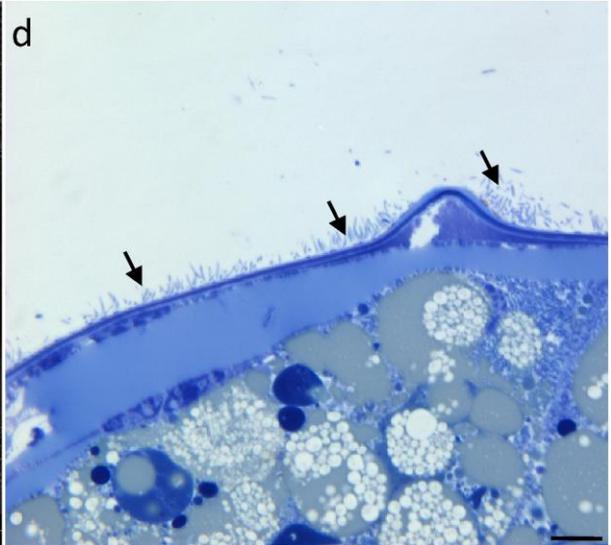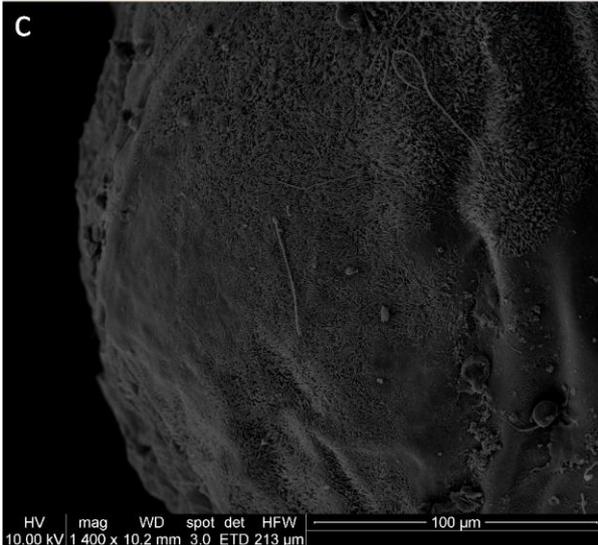
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Hydrothermal invertebrates associated epibionts

R. exoculata LGW (FR797962)

Uncultured epsilon *proteobacterium* Lost City (DQ228579)

R. exoculata RBF (2) (FR797934)

Sulfurospirillum arcachonense (microaerophilic / sulfur-reducing) (NR026408)

80

R. exoculata RBR (FR797921)

97

R. exoculata ADB (2) (FR797940)

76

Dehalospirillum multivorans (strictly anaerobic) (X82931)

74

R. exoculata RBR (3) (AM412514)

83

Sulfospirillum

R. exoculata LGJ (4) (FR797946)

95

R. exoculata LGE (2) (FR797949)

100

Uncultured epsilon *proteobacterium* Rainbow (AY225610)

99

Vestimentiferan trophosome endosymbiont (D83061)

99

Arcobacter sp. Black Sea shelf sediments (AJ271654)

Arcobacter

R. exoculata LGJ (3) (FR797941)

99

R. exoculata LGG (FR797958)

72

Osedax mucofloris endosymbiont North Atlantic (FN773271)

92

R. exoculata LGB (2) (FR797953)

100

C. squamiferum epibiont Indian Ocean (AY531580)

C. squamiferum epibiont Indian Ocean (AY531590)

100

R. exoculata RBW (FR797927)

70

Campylobacter jejuni (Y19244)

Campylobacter

R. exoculata RBR (13) (AM412516)

95

R. exoculata RBF (8) (FR797931)

100

R. exoculata gut clone 22 (AJ515713)

100

Milano Mud Volcano associated bacteria Eastern Mediterranean (AY592914)

94

Thiomicrospira denitrificans intertidal mud flat of Dutch Wadden Sea (L40808)

Thiomicrospira

Bacillus subtilis (AB016721)

0.05

1027 1026 1025 1024 1023 1022 1021 1020 1019 1018 1017 1016 1015 1014 1013 1012 1011 1010 1009 1008 1007 1006 1005 1004

96 *R. exoculata* RBR (10) (AM412518)
70 *R. exoculata* RBF (5) (FR797911)
R. exoculata LGJ (FR797912)
R. exoculata LGB (FR797920)
99 *R. exoculata* LGE (11) (FR797917)
R. exoculata LGHE (7) (FR797915)
88 *R. exoculata* LGHE (12) (FR797916)
100 *R. exoculata* LGE (34) (FR797919)
Shinkaia crosnieri epibiont in Okinawa Trough (EU107481)
70 *R. exoculata* RBW (9) (FR797909)
88 **R. exoculata RBR (15) (AM412520)**
R. exoculata RBLR (FR797910)
C. squamiferum epibiont Indian Ocean (AY531557)
100 *Kiwa hirsuta* epibiont Pacific-Antartic Ridge (EU265784)

Cluster 1
(106 sequences)

95 *Leucothrix mucor* (X87277)
99 *Calyptogena phaseoliformis* gill symbiont (AF035724)
Vesicomya gigas gill symbiont (AF0355726)
100 78 Thiotrophic endosymbiont of *Bathymodiolus puteoserpentis* (Logachev) (AM083975)
95 Thiotrophic endosymbiont of *Bathymodiolus sp.* (Golf of Guinea, Regab vent) (AJ745718)
Mytilidae sp. gill symbiont associated with wood fall (Bohol sea) (AM503921)

**Thiotrophic
symbionts**

66 99 *R. exoculata* LGE (8) (FR797918)
100 *R. exoculata* LGHE (10) (FR797914)
100 *R. exoculata* RBW (FR797908)
R. exoculata LGHE (FR797913)

Cluster 2
(20 sequences)

92 *Methylomonas methanica* (AF150806)
93 90 Methanotrophic gill symbiont of mussel in Lousiana Slope (U05595)
Methanotrophic endosymbiont of *Bathymodiolus* (AJ745717)
79 *Methylophaga marina* (X95459)

**Methanotrophic
symbionts**

Caminibacter hydrogenophilus (AJ309655)

0.02

Water column, planktotrophic life ?

Dispersion ?

Recruitment ?

Hydrothermal vent, chimiosynthetic life

Hatched egg   Eggs   Adult shrimp   Juveniles
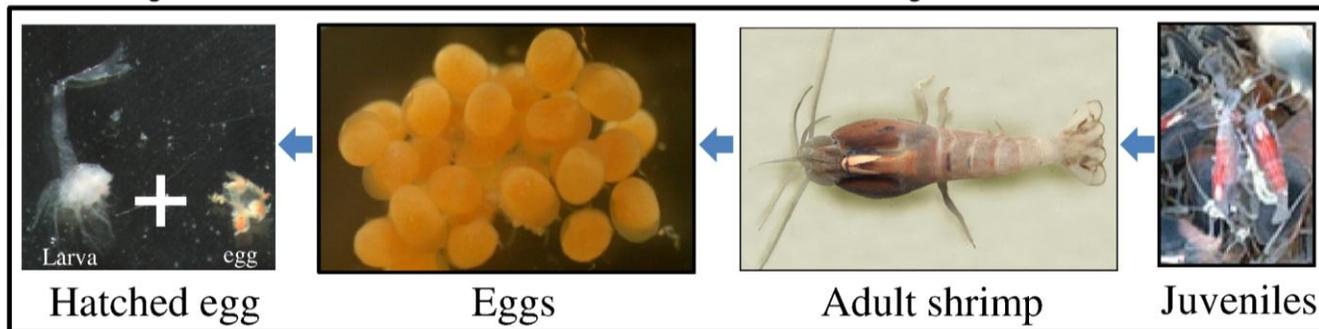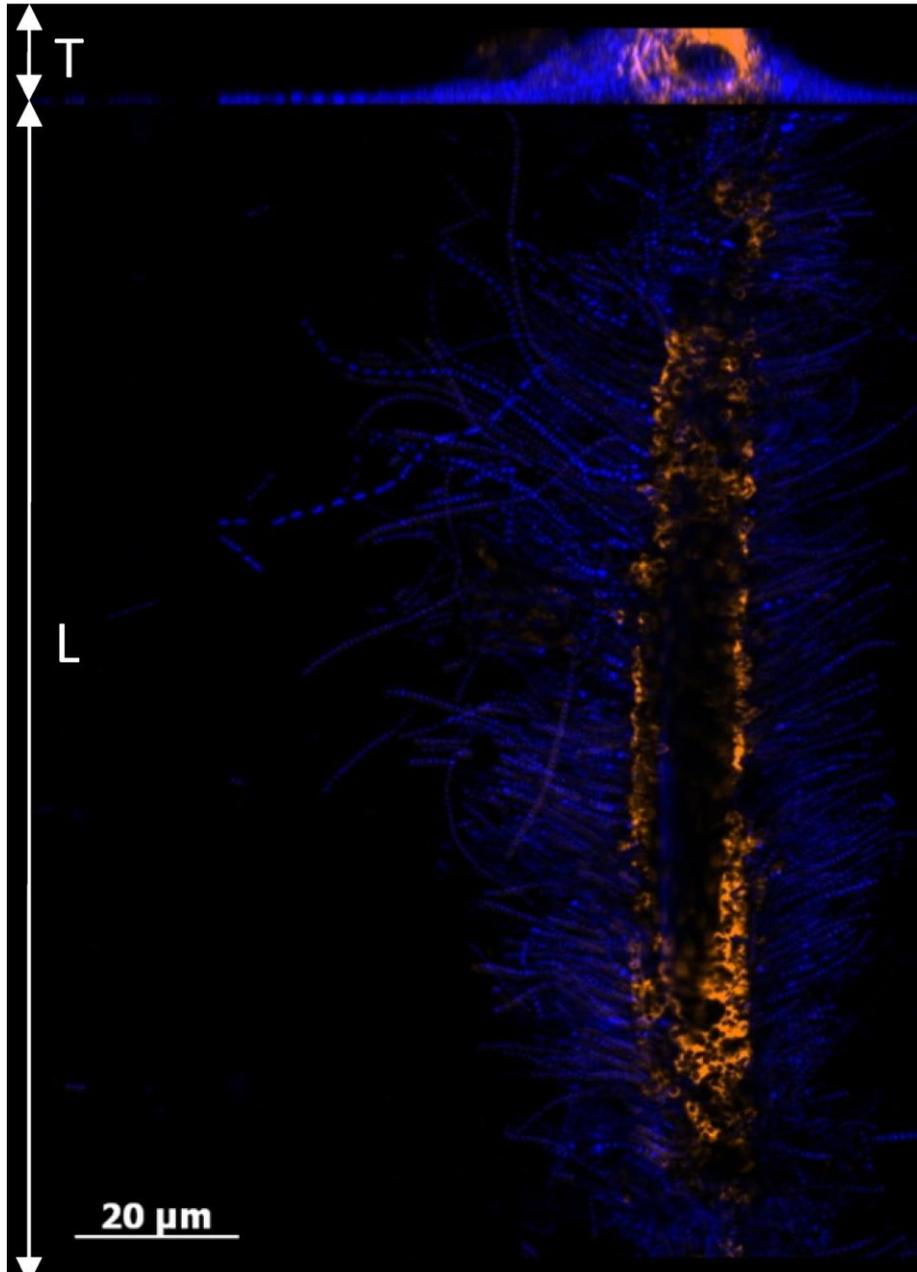
Figure S1: Longitudinal (L) and transversal (T) views of Scaphognathite setae from Rainbow shrimp with epibionts. The methanotrophic Gamma symbiont (orange) was hybridized with both LBl32/130 and GAM42a probes. The DAPI stained are in blue.
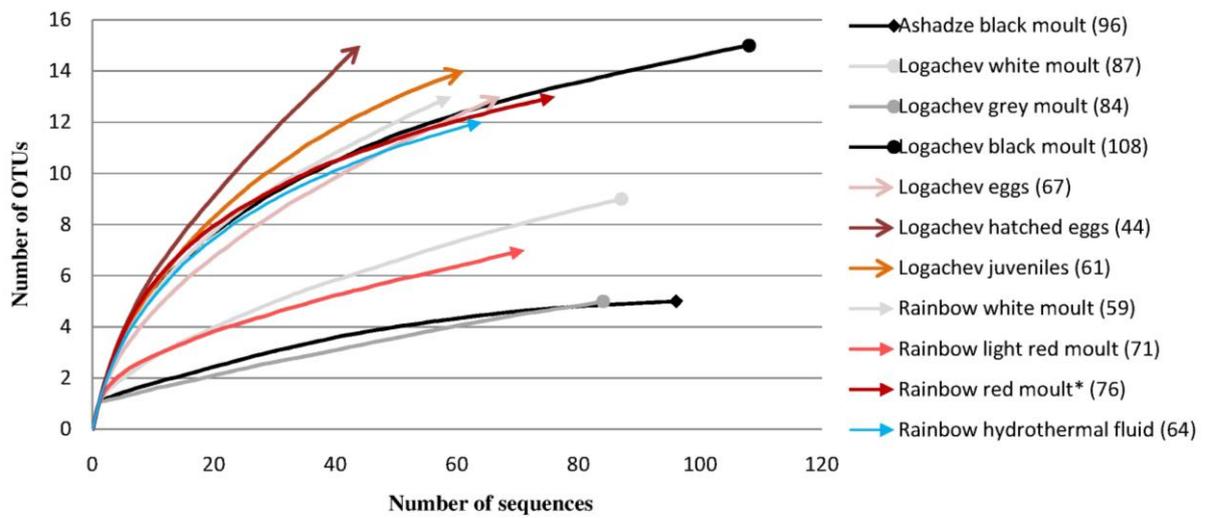
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1076 **Figure S2**: Rarefaction curves (*by Zbinden *et al.* 2008).

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1085 **Figure S3**: Picture showing the presence of *R. exoculata* orange juvenile nursery

1086 (on the bottom right hand corner) in shrimp aggregates.

1087 **Table S1**: Analysis of bacterial diversity associated with *R. exoculata* (gill

1088 chamber and eggs). [1]Simpson indices values are presented as 1-H for best readability, the

1089 diversity increasing from 0 (one species) to 1 (maximal diversity). [2] by Zbinden *et al.* 2008.

Library	Number of clones	OTUs number (97% similarity)	Good's coverge	1-H $_{simspon}$[1]
Ashadze black moult	96	5	98	0,16
Logachev eggs	67	13	82	0,71
Logachev hatched eggs	44	15	87	0,86
Logachev juvenile	61	14	93	0,82
Logachev white moult	87	9	92	0,38
Logachev grey moult	84	5	97	0,12
Logachev black moult	108	15	92	0,80
Rainbow white moult	59	13	92	0,77
Rainbow light red moult	71	7	95	0,48
Rainbow red moult[2]	76	13	97	0,84
Rainbow hydrothermal fluid	64	12	95	0,77

1096 **Table S2**: List of specimens and treatment.

1097 [*] Zbinden *et al.*, 2008.
1098 [1]Values for Rainbow are from Charlou *et al.*, 2002, for Logachev from Schmidt *et al.*, 2007, and for
1099 Ashadze from Charlou *et al.*, 2010.
1100 This table shows the number of shrimps used by analyses.

Ultramafic Hydrothermal site	Pure fluid chemistry[1]				Stage / Sampling	Number of sample per Analyzed				
	Depth (m)	H$_2$S (mM)	H$_2$ (mM)	CH$_4$ (mM)		16SrRNA	FISH	SEM	TEM	Total
Rainbow (36°13'N; 33°54'W)	2350	1.2	16	2.5	*R. exoculata* orange juvenile (2 cm, stage A)	-	2	-	-	2
					R. exoculata adult white moult	2	3	2	-	7
					R. exoculata adult light red moult	2	3	2	-	7
					R. exoculata adult red moult	2*	3	2	1	9
					Hydrothermal fluid inside shrimp aggregates	+	-	-	-	-
Logachev (14°45'N; 44°57'W)	3037	2.5	19	3.5	*R. exoculata* gravid female (number of eggs treated)	1(20+20) + 1(20+20)	1 (20)	1 (20)	4 (120)	
					R. exoculata gravid female with hatched eggs + larvae (number of hatched eggs + larvae)	1(20+20+20)			-	1 (60)
					R. exoculata orange juvenile (2 cm, stage A)	2	3	2	-	7
					R. exoculata adult white moult	2	3	2	-	7
					R. exoculata adult grey moult	2	3	2	-	7
					R. exoculata adult black moult	2	3	2	-	7
Ashadze (12°58'N; 44°51'W)	4080	1	8/19	0,5/1,2	*R. exoculata* adult black moult	2	3	2	-	7

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Tables S3: Closest match of representative 16S rRNA gene clone sequences

(*by Zbinden et al., 2008).

Phylogenetic group	Representative clone sequences	Hit of BLAST (accession no.)	Similarity (%)
Epsilonproteobacteria	RBR (AM412516)*, RBF (FR797931)	R. exoculata gut clone 22 (AJ515713)	99
	RBW (FR797927)	C. squamiferum epibiont Indian Ocean clone SFC23D7 (AY531590)	97
	LGB (FR797953), LGG (FR797958), LGJ (FR797941)	Osedax mucofloris endosymbiont North Atlantic clone Omu3c42 (FN773271)	97-99
	LGJ (FR797946), LGE (FR797949)	Epsilonproteobacteria Rainbow clone ATpp13 (AY225610)	98
	RBR (AM412514)*	Uncultured bacterium clone FS1402B02 (AY704396)	93
	ADB (FR797940)	Uncultured bacterium clone SGYF448 (FJ792213)	96
	RBR (FR797921)*	Sulfospirillum carboxydovorans strain MV (AY740528)	94
	RBF (FR797934)	Epsilon-proteobacterium clone ATOS Iris 7 Rainbow 26 (AJ969489)	98
	LGW (FR797962)	Uncultured bacterium clone SGYF450 (FJ792215)	96
	RBW (FR797925)	Ectosymbiont of R. exoculata clone Rc20eps3 (FM203377)	99
	RBLR (FR797929), RBF (FR797935)	Ectosymbiont of R. exoculata clone Rc18eps2 (FM203406)	99
	LGG (FR797959), LGJ (FR797943)	Epsilon-proteobacterium clone aH8 (FN562827)	99
	RBLR (FR797930)	Epsilon-proteobacterium Rainbow clone ATpp27 (AY225624)	99
	RBW (FR797923)	Ectosymbiont of R. exoculata clone Rc18E7 (FM393026)	98
	LGB (FR797955), LGG (FR797966), LGJ (FR797945), ADB (FR797938)	Ectosymbiont of R. exoculata clone LOG283/74F1 (FM203395)	99
	LGW (FR797963)	Ectosymbiont of R. exoculata clone LOG272/69P2C1 (FM203396)	99
	LGJ (FR797944), LGB (FR797956)	Epsilon-proteobacterium clone aE8 (FN562833)	98
	ADB (FR797936), ADB (FR797939)	Uncultured bacterium (microcolonizers) Snake Pit clone 3 (AY766315)	92-97
	RBW (FR797926), RBF (FR797933)	R. exoculata gill clone SCAII15 (AM412513)	98-99
	RBR (AM412513)*	C. squamiferum epibiont Indian Ocean clone SFC23F5 (AY531605)	92
	LGG (FR797961), LGJ (FR797942), LGE (FR797951), LGB (FR797954), ADB (FR797937), LGW (FR797964)	Ventiella sulfuris associated bacteria clone e34 (FN429840)	99
	RBF (FR797932)	R. exoculata gill clone LBI7 (AM412509)	98
	LBI7 (AM412509)*	C. squamiferum epibiont Indian Ocean clone SFC23F4 (AY531582)	96
	RBLR (FR797928), RBW (FR797924)	R. exoculata gill clone LBI16 (AM412507)	97-98
	RBR (AM412507)*	Epsilon-proteobacterium clone bC9 (FN562855)	96
	RBR (FR797922)	Uncultured bacterium clone 102B111 (EF687148)	94
	LGW (FR797965), LGHE (FR797948), LGJ (FR797947), LGG (FR797960)	R. exoculata gut clone R28R (FM863740)	98
	LGB (FR797957), LGE (FR797950), LGB (FR797952)	Epsilon-proteobacterium clone bH8 (FN562857)	96-100
	RBR (AM902731)*	R. exoculata gut clone 15 (AJ515716)	99
Gammaproteobacteria	RBR (AM412518)*	Ectosymbiont of R. exoculata clone Rc20gam1 (FM203378)	98
	RBF (FR797911), LGJ (FR797912)	R. exoculata gill clone LBI32 (AM412518)	99
	LGB (FR797920), LGE (FR797917)	Ectosymbiont of R. exoculata clone LOG272/69D1 (FN393024)	99
	LGHE (FR797915)	R. exoculata gill clone LBI32 (AM412518)	98
	LGHE (FR797916), LGE (FR797919)	Uncultured bacterium clone Ba9 (FJ640825)	99
	RBW (FR797909), RBLR (FR797910)	R. exoculata gill clone SCAII16 (AM412520)	99
	RBR (AM412520)*	Ectosymbiont of R. exoculata clone Rc16gam3 (FM203375)	97
	LGE (FR797918), LGHE (FR797914)	Gamma-proteobacterium (methane oxidizer) clone HMMVCen4 (AJ704661)	99
	RBW (FR797908), LGHE (FR797913)	Gamma-proteobacterium clone IBNC12 (AB175550)	98