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ORIGINAL ARTICLE

Inorganic carbon fixation by chemosynthetic ectosymbionts and nutritional transfers to the hydrothermal vent host-shrimp *Rimicaris exoculata*

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The shrimp *Rimicaris exoculata* dominates several hydrothermal vent ecosystems of the Mid-Atlantic Ridge and is thought to be a primary consumer harbouring a chemoautotrophic bacterial community in its gill chamber. The aim of the present study was to test current hypotheses concerning the epibiont's chemoautotrophy, and the mutualistic character of this association. *In-vivo* experiments were carried out in a pressurised aquarium with isotope-labelled inorganic carbon ($NaH^{13}CO_3$ and $NaH^{14}CO_3$) in the presence of two different electron donors ($Na_2S_2O_3$ and Fe^{2+}) and with radiolabelled organic compounds (^{14}C-acetate and ^{3}H-lysine) chosen as potential bacterial substrates and/or metabolic by-products in experiments mimicking transfer of small biomolecules from epibionts to host. The bacterial epibionts were found to assimilate inorganic carbon by chemoautotrophy, but many of them (thick filaments of *epsilonproteobacteria*) appeared versatile and able to switch between electron donors, including organic compounds (heterotrophic acetate and lysine uptake). At least some of them (thin filamentous *gammaproteobacteria*) also seem capable of internal energy storage that could supply chemosynthetic metabolism for hours under conditions of electron donor deprivation. As direct nutritional transfer from bacteria to host was detected, the association appears as true mutualism. Import of soluble bacterial products occurs by permeation across the gill chamber integument, rather than via the digestive tract. This first demonstration of such capabilities in a decapod crustacean supports the previously discarded hypothesis of transtegumental absorption of dissolved organic matter or carbon as a common nutritional pathway.
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Introduction

Mostly described as endosymbioses (Dubilier *et al.*, 2008), associations between invertebrates and chemoautotrophic bacteria are regularly encountered in reducing marine habitats (Cavanaugh *et al.*, 2006). Among the Crustacea, chemosynthetic epibioses are reported in only a few hydrothermal decapods: the shrimp *Rimicaris exoculata* (Segonzac *et al.*, 1993), the galatheid crabs *Kiwa hirsuta* (Macpherson *et al.*, 2005; Goffredi *et al.*, 2008), *Kiwa puravida* (Thurber *et al.*, 2011), and *Shinkaia crosnieri* (Miyake *et al.*, 2007), and two amphipods from littoral sediments (Gillan and Dubilier, 2004; Gillan *et al.*, 2004) and sulphide-rich caves (Dattagupta *et al.*, 2009). Most of these associations are regarded as nutritional ectosymbioses, but to date, there is little direct evidence of bacterial autotrophy and no direct demonstration of mutualistic nutritional bacteria–host interactions.

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The shrimp *R. exoculata* (Williams and Rona, 1986) dominates the megafauna of several deep-sea hydrothermal vent sites of the Mid-Atlantic Ridge, forms dense aggregates around active chimneys (Segonzac, 1992) and harbours a luxuriant bacterial community in its enlarged gill chamber (Van Dover *et al*., 1988; Casanova *et al*., 1993; Segonzac *et al*., 1993; Polz and Cavanaugh, 1995; Zbinden *et al*., 2004) (Figure 1). The integument surfaces are periodically re-colonised during the moult cycle

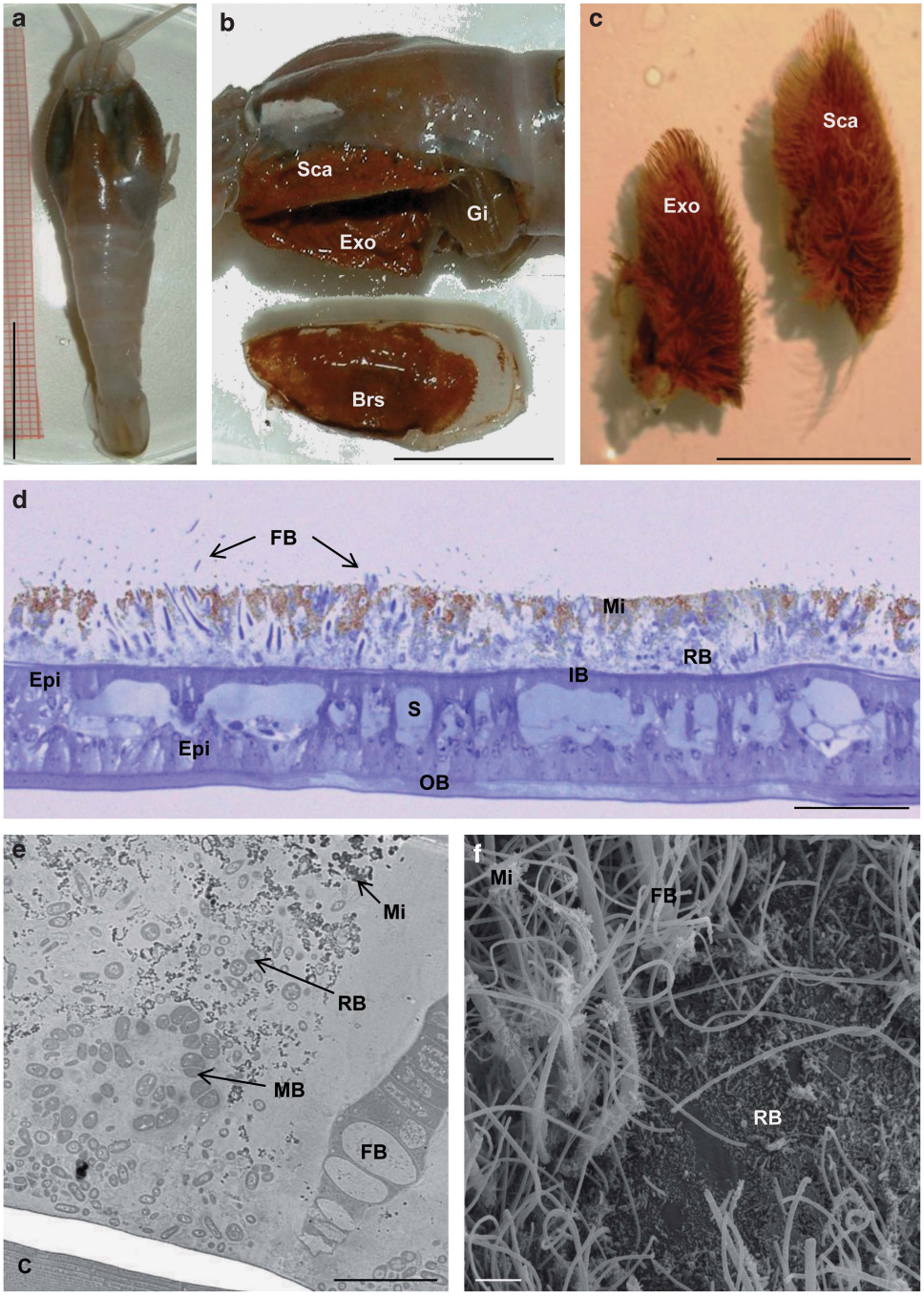

Figure 1 Illustration of the shrimp *Rimicaris exoculata* gill chamber with the associated bacterial community. (**a**) Whole shrimp with dilated gill chamber visible on both sides of the cephalothorax. (**b**) Left gill chamber opened by removal of the branchiostegite (lying below, *Brs*) and showing the very large mouthparts (scaphognathite, *Sca*, and exopodite, *Exo*) as well as the posterior gill set (*Gi*)—both the mouthparts and the inner side the branchiostegite are colonised by bacterial mats encrusted with particles of red iron oxide. (**c**) Isolated mouthparts bearing bacteriophore setae sheathed by a thick iron-oxide-encrusted biofilm. (**d**) Semi-thin cross-section (toluidine blue stained) of the branchiostegite (anterior part), showing the inner and outer integument leaflets (*IB* and *OB*) and the bacterial biofilm growing on the inner side. (**e**, **f**) Transmission electron microscopy and scanning electron microscopy images of the branchiostegite biofilm, showing several bacterial morphotypes including rods, nested methanotrophic-like bacteria, thick and thin filaments (pictures taken by Dr L Corbari). Scale bars: (**a**) 1 cm; (**b**) and (**c**) 500 mm; (**d**) 100 μm; (**e**) 5 μm; (**f**) 10 μm. *C*, cuticle; *Epi*, epidermis; *FB*, filamentous bacteria; *IB*, inner branchiostegite; *MB*, methanotrophic-like bacteria; *Mi*, mineral particles; *RB*, rod-shaped bacteria; *S*, blood sinuses.

(Corbari *et al.*, 2008b), each ecdysis causing shedding of the old cuticle with its bacterial mats. The shrimp *R. exoculata*, listed as a model organism of an extreme deep-sea environment (CAREX, 2010), is exceptional among crustaceans for its association with bacteria. Recent studies suggest that *R. exoculata* gill chamber epibionts constitute a diversified community with various morphotypes, phylotypes and metabolisms (Zbinden *et al.*, 2004, 2008; Petersen *et al.*, 2009; Hügler *et al.*, 2011; Guri *et al.*, 2012), but no study has directly demonstrated which substrate(s) the bacteria oxidise to fuel their 'chemosynthetic' metabolism(s). This shrimp also harbours a digestive bacterial community (Zbinden and Cambon-Bonavita, 2003; Durand *et al.*, 2010) whose role remains hypothetical (detoxification, nutrition and/or pathogen control).

There is strong evidence, based on stable isotope signatures and essential fatty acid composition, suggesting that the shrimp gets organic matter mainly from its epibionts, rather than from grazing free-living bacteria associated with chimney walls (Gebruk *et al.*, 1993; Pond *et al.*, 1997a, 2000; Rieley *et al.*, 1999; Colaço *et al.*, 2007). Yet this evidence of symbiont–host nutritional relationships is indirect, and how carbon is transferred from bacteria to shrimp remains an open question. The hypothesis of direct transfer of dissolved molecules across the shrimp integument, especially the gill chamber lining, has been proposed on the basis of morphological observations (features dedicated to bacteria farming, absence of scrape marks on the biofilm) (Zbinden *et al.*, 2004; Corbari *et al.*, 2008b), but the nature of the transferred molecules and the integument areas involved remain to be discovered.

The major aims of this study were to test (1) inorganic carbon fixation by active bacterial chemosynthetic metabolisms and (2) the hypothesis of bacterial organic carbon transfer (soluble molecules) to the shrimp tissues. For this, we incubated live shrimps in a pressurised vessel with different isotope-labelled molecules and electron donors, to trace carbon assimilation by the epibionts and shrimp tissues.

Materials and methods

Shrimp collection

R. exoculata specimens were collected with the slurp-gun operated from the manned submersible Nautile at the Rainbow hydrothermal vent site (36°14′N–33°54′W, 2320 m depth) during the 'MoMARDREAM-Naut' cruise, July 2007. Healthy specimens were maintained alive in a pressurised IPOCAMP incubator (Shillito *et al.*, 2001) at *in-situ* pressure (230 bars) and temperature (15 °C) for *in-vivo* experiments (Zbinden *et al.*, 2008). Control specimens were directly frozen at −80 °C after collection.

Experiments

Experiments were carried out only on red-coloured pre-ecdysial specimens (that is, having an established and still-growing bacterial community) (Corbari *et al.*, 2008b). In the IPOCAMP, incubations with isotopic tracers were carried out in flasks filled till to the top with seawater (SW), whose volume (50 or 250 ml) was chosen according to the treatment duration and number of shrimps to ensure shrimp survival in good physiological condition at the end of the experiment (Shillito *et al.*, 2001; Ravaux *et al.*, 2003). Incorporation of ^{13}C- and ^{14}C-bicarbonate by the chemoautotrophic bacteria was tested with different chemical energy sources. ^{14}C-acetate and ^{3}H-lysine were chosen as representative microbial substrates and/or metabolic by-products liable to mimic transfer of small biomolecules from epibionts to host.

After each experiment, tracer excess was washed off in 0.22-μm-filtered SW: 15 min for ^{13}C and 1, 5 and 30 s, 5 and 15 min for the other experiments. The shrimps were then either directly frozen at −80 °C or dissected into body parts and tissues of interest: (1) regions colonised by the bacteria: the branchiostegites (integument folds enclosing the gill chamber), later separated into the inner bacterial biofilm (*BB*) and the outer branchiostegite (*OB*) or shrimp tissues, and the mouthparts (*MP*, scaphognathites and exopodites); (2) exchange or absorption organs: gills (*Gi*), digestive tract (*DT*) and hepatopancreas (*HP*); (3) abdominal muscles (*Mu*). In some specimens, the old abdominal cuticle (*AC*) was separated from the inner tissues (new cuticle and epidermis). In the laboratory, the samples were rinsed once again (see below) and treated to remove unfixed tracers and to preserve the labelled organic molecules before quantification.

^{13}C-bicarbonate incorporation. Four and two shrimps were incubated for 4 and 10 h, respectively, in 250-ml flasks containing 1 g/L $NaH^{13}CO_3$ (98%; Sigma 372382; St Louis, MO, USA) in filtered SW enriched or not (control) with iron (100 μM $FeCl_2$; Sigma 372870) or thiosulphate (100 μM $Na_2S_2O_3$; Sigma 72049) as chemical energy source. Before use, the dissected tissue samples were rinsed three times in distilled water before dehydration overnight at 60 °C. Dried samples were finely ground to obtain a powder to vaporise in the mass spectrometer.

^{14}C-bicarbonate incorporation. Two specimens were incubated for 6 h in 50-ml flasks containing 0.25 g/L $NaH^{12}CO_3$ labelled with 80 μM $NaH^{14}CO_3$ (4 μCi/ml; GE Healthcare CFA3-5MCI; Buckinghamshire, UK) as inorganic carbon source and dissolved in SW enriched or not with an electron donor (Fe^{2+} or $Na_2S_2O_3$) at the same concentration as above. The first shrimp for each condition was directly frozen at −80 °C for autoradiography and scintillation analysis. The second shrimp was dissected into body parts as described above before storage in

cryotubes at −80 °C. In unlabelled shrimps (control), the radioactivity level was undetectable to very low in both bacterial mats and shrimp tissues.

In the laboratory, the samples were rinsed four times in distilled water and their fresh weight was determined. After overnight digestion at 50–55 °C in glass scintillation vials containing 1 ml quaternary ammonium hydroxide (0.5 N) in toluene (BTS-450 Tissue Solubilizer; Beckman Coulter 580691; Fullerton, CA, USA), they were acidified for 2 h at room temperature by adding 250 µl acetic acid to remove any remaining ^{14}C-bicarbonate and the calcium carbonate present in the cuticle or *HP*. Scintillation liquid (Ready Organic; Beckman Coulter 586600) (10 ml) was added to all samples just before reading.

^{14}C-acetate and ^{3}H-lysine incorporation. Two sets of three to four shrimps were incubated for 1 h in 50 ml SW containing either 20 µCi/ml ^{3}H-lysine (GE Healthcare TRK520-1MCI) (that is, 0.2 µM) or 10 µCi/ml sodium ^{14}C-acetate (GE Healthcare CFA14-1MCI) (that is, 0.2 mM). In both experiments, one entire specimen was immediately frozen after rinsing (for autoradiography and analysis). The others were dissected and the pieces stored at −80 °C. Samples were treated as in the ^{14}C-bicarbonate experiment.

Stable isotope and elemental analysis
The atomic percentage of stable isotope ($\%_{at}\,^{13}C$) of each sample and its carbon content expressed as a percentage of dry weight ($\%C_{dw}$) were obtained with an isotope ratio spectrometer (Optima, Isoprime, UK) coupled to an elemental analyser (Carlo Erba, Milan, Italy) (Lepoint *et al.*, 2004).

The percentage of natural ^{13}C in control shrimps was first subtracted from the measured ^{13}C percentage in each sample to obtain the percentage of ^{13}C (or total carbon) incorporated during the experiment ($\%_{at}\,^{13}C_{inc}$). Carbon incorporation rates (C_{inc}), expressed in moles of carbon incorporated per time unit and weight unit of total carbon in the sample ($\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$), were calculated according to the formulas:

$$\%_{at}{}^{13}C_{inc} \cdot (DW_{sam} \cdot \ \%C_{dw}) = W^{13}C_{inc}$$

$$\frac{W^{13}C_{inc}/MM_C}{(DW_{sam} \cdot \ \%C_{dw}) \cdot t} = C_{inc}$$

where $\%_{at}\,^{13}C_{inc}$ is atomic percentage of incorporated ^{13}C; DW_{sam} is the dry weight of the sample, $\%C_{dw}$ is the carbon content expressed as a percentage of dry weight, $DW_{sam}.\%C_{dw}$ is the weight of carbon in the sample, $W^{13}C_{inc}$ is the weight of ^{13}C incorporated into the sample during the experiment, MM_C is the molar mass of carbon, t is the duration of the experiment, and C_{inc} is the carbon incorporation rate expressed in moles of incorporated carbon per time unit and weight unit of total carbon in the sample.

Measurement of incorporated radioactivity
The beta-radioactivity of the samples was measured for 15 min in liquid phase in a Beckman LS 6500 Scintillation Counter (effective resolution: 0.06 keV).

Moles of incorporated radioelement are known from the measured number of disintegrations per second (disintegrations per second × 2 half-life in seconds/Avogadro's number). The total incorporation rate, expressed in moles of incorporated molecules per time unit and weight unit of total carbon in the sample ($\mu molR_{inc} \times gC_{sam}^{-1} \times h^{-1}$), was calculated according to the formula:

$$\frac{({}^{x}E_{inc}/f)}{[(FW_{sam} \cdot p) \cdot \ \%C_{dw}] \cdot t} = R_{inc}$$

where ${}^{x}E_{inc}$ is the number of moles of incorporated radioelement, f is the ratio of radioactive to cold molecules in the incubation medium, FW_{sam} is the fresh weight of the sample, p is the dry weight/fresh weight ratio measured on control shrimps, $\%C_{dw}$ is the carbon content expressed as a percentage of sample dry weight, and R_{inc} is the total incorporation rate expressed in moles of incorporated molecules per time unit and weight of total carbon in the sample.

Autoradiography
Histological localisation of radiotracers was done by autoradiography on frozen shrimps incubated with a radiotracer. To isolate shrimp segments, each frozen shrimp was embedded in Tissue-Tek gel (Sakura 4583; Torrance, CA, USA), directly frozen in liquid nitrogen, and cut into segments 1 cm long with a hacksaw in a cryostat at −20 °C. Segments of the cephalothorax (including *MP* and gills) and the abdomen were directly thawed overnight in fixative solution (2.5% glutaraldehyde), decalcified in EDTA (0.2 M, pH 8), post-fixed in 1% OsO_4 for 2 h, rinsed in distilled water (4 × 10 min), dehydrated with ethanol, and embedded in Steedman's wax (polyethylene glycol distearate; Sigma 305413—hexadecanol 9:1; 99%; Sigma 258741). Twelve-micrometre cross-sections were obtained with a microtome (Reichert, Austria) at three levels (*MP*, gills and abdominal muscles) and deposited on gelatinised glass slides. They were rehydrated, washed in distilled water and then coated with a photographic emulsion film (Hypercoat LM-1; GE Healthcare RPN40) in the dark according to the classical wet method (Ullberg *et al.*, 1982). The slides were developed with KODAK products (KODAK D19-Revelator and KODAK-Fixator; Rochester, NY, USA) after an exposure time of 6–9 months for ^{14}C-bicarbonate or 6 h to 3 months for ^{14}C-acetate and ^{3}H-lysine. The slides were mounted in epoxy resin before being observed and photographed with an Olympus AX70 light microscope (Shinjuku, Tokyo, Japan) equipped with a Visicam 5.0 camera (Hilden, Germany).

Statistical analysis
All data are reported as mean values ± s.d. Outliers (possibly due to experimental contamination or to the absence of labelling) were eliminated with Dixon's *Q* test ($P<0.05$). One-way analysis of variance (Kruskall–Wallis) was used and all pairwise multiple comparison procedures (Dunn's Method) performed with Sigma Plot 11.0 software (Systat Software Inc., San Jose, CA, USA) were used for sample comparisons. $P<0.05$ was taken as fiducial limit for statistical significance.

Dunn's multiple comparisons vs control group method were used to test the real incorporation of ^{14}C.

Results

^{13}C-bicarbonate experiments
The data for ^{13}C-bicarbonate incorporation are shown in Figure 2a. Bacterial mats of branchiostegites and *MP* displayed a high rate of inorganic carbon incorporation from ^{13}C-bicarbonate. Other body parts (*OB*, *DT* and *Mu*) showed lower rates. All the results appeared to be statistically significant.

After a 10-h incubation, the ^{13}C uptake measured in the bacterial mats was approximately twice as high as after 4 h (Table 1). In the *BB*, it was higher in thiosulphate-incubated shrimps (78.8 $\mu molC_{inc} \times gC_{sam}^{-1}$) than in SW-incubated specimens (52.4 $\mu molC_{inc} \times gC_{sam}^{-1}$).

The incorporation rate was higher in the *MP* (12.1–16.7 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$) than in the *BB* (3.9–7.3 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$). In shrimp tissues, it was higher in the *OB* (1.1 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$, in the presence of thiosulphate) than in the *DT* (0.3 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$) or the *Mu* (0.2 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$). The incorporation rates for *MP* and *BB* were significantly different from those of shrimp tissues ($P<0.05$).

^{14}C-bicarbonate experiments
The radioactivity levels measured in the bacteria and tissues of control shrimps appeared negligible as compared with radiotracer uptake ($P\leqslant0.001$) (Figure 2b).

High ^{14}C incorporation rates were observed in the bacterial mats of the *MP* and *BB* (Figure 2b), of the same order of magnitude as the corresponding ^{13}C-bicarbonate incorporation rates. The rate was much higher in the presence of iron or thiosulphate. In the *MP* and *BB*, respectively, it reached 10.4 and 5.4 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$ in the presence of thiosulphate and only 3.9 and 2.8 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$ for shrimps incubated in SW alone.

Most of the shrimp tissues analysed showed significant ^{14}C incorporation, whatever the incubation conditions (Figure 2b). The highest carbon incorporation rates were recorded in the tegumental tissues of the gill chambers, that is, the *OB* (2.7 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$) and the *Gi* (0.2 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$). The rates recorded for the internal tissues of all shrimps (*Mu*: 0.01; *DT*: 0.04; *HP*: 0.01 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$) were low to very low, but nevertheless significant ($P\leqslant0.001$), the digestive gland displaying the lowest values. The *AC* showed a surprisingly high incorporation rate (0.6 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$ in the presence of iron), to be explained below.

^{14}C-acetate experiments
The results for ^{14}C incorporation from acetate are shown in Figure 3a. Bacterial mats (*MP* and *BB*) showed high rates, indicating that acetate is a suitable carbon source for these bacteria. The rate recorded in SW (61.3 $\mu molAc_{inc} \times gC_{sam}^{-1} \times h^{-1}$) was 15 times the rate of carbon incorporation from ^{14}C-bicarbonate (3.9 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$ in *MP*).

High rates of ^{14}C incorporation were measured in the integument, especially in areas lining the gill

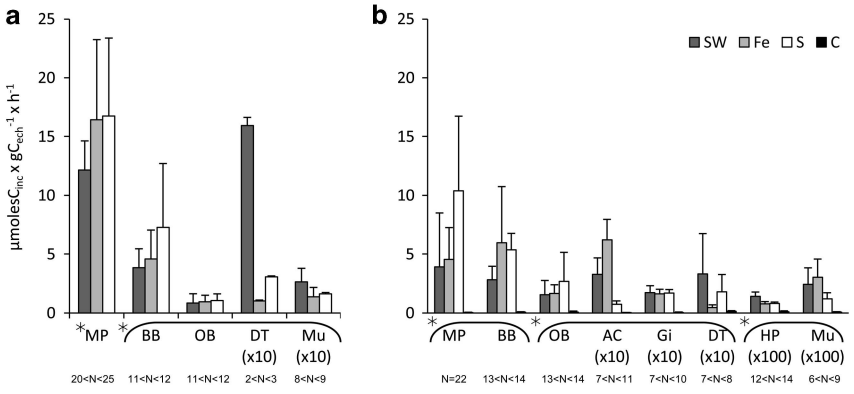

Figure 2 Rates of inorganic carbon incorporation into the bacterial mats of the mouthparts and branchiostegite and into shrimp tissues, as calculated from ^{13}C fixation (**a**) or ^{14}C fixation (**b**) data. The shrimps were incubated: (**a**) for 4 or 10 h in $NaH^{13}CO_3$ in pure seawater (dark grey), supplemented with reduced iron (light grey) or thiosulphate (white); (**b**) for 6 h in pure SW with $NaH^{14}CO_3$ (dark grey), supplemented with iron (light grey) or thiosulphate (white). The radioactivity measured in control shrimp tissues is shown (black) for comparison. Stars indicate significant differences between groups of tissues; ($\times10$, $\times100$) indicates tissues for which the values were multiplied by 10 or 100 for graph presentation; '*N*' indicates the number of samples for each data bar.

Table 1 Total amounts of inorganic carbon ($\mu molC_{inc} \times gC_{sam}^{-1} \pm$ s.d.) incorporated after 4 and 10 h into the body parts of shrimps incubated with ^{13}C-bicarbonate in the presence of seawater, or seawater supplemented with iron or thiosulphate

	Incorporated inorganic ^{13}C per g of organic C in the sample ($\mu molC_{inc} \times gC_{sam}^{-1}$) after 4 and 10 h incubations with $NaH^{13}CO_3$ in SW, SW+Fe and SW+S	
	$NaH^{13}CO_3$–4 h	*$NaH^{13}CO_3$–10 h*
Shrimp tissues		
SW		
MP	50.4 ± 10.3	103.0 ± 8.2
BB	13.3 ± 6.3	52.4 ± 3.4
OB	4.3 ± 3.3	3.1 ± 0.5
DT	/	15.9 ± 0.7
Mu	1.1 ± 0.6	2.6 ± 0.0
Fe		
MP	65.7 ± 27.3	/
BB	22.8 ± 9.8	24.0 ± 2.1
OB	4.8 ± 3.5	8.4 ± 0.3
DT	/	1.3 ± 0.5
Mu	0.8 ± 0.1	0.5 ± 0.0
S		
MP	67.9 ± 29.9	158.5 ± 7.0
BB	28.4 ± 25.0	78.8 ± 3.7
OB	5.0 ± 2.1	4.8 ± 0.4
DT	/	3.1 ± 0.1
Mu	0.6 ± 0.0	1.7 ± 0.14

Abbreviations: BB, branchiostegite biofilm; DT, digestive tract; Fe, iron condition; MP, mouthparts; Mu, muscles; OB, outer branchiostegite; S, thiosulphate condition; SW, seawater.

chamber, that is, *OB* and *Gi* (7.9 and 7.3 $\mu molAc_{inc} \times gC_{sam}^{-1} \times h^{-1}$, respectively). The level remained low but measurable in the internal tissues. In the *Mu* and *HP*, the rate did not exceed 0.1 $\mu molAc_{inc} \times gC_{sam}^{-1} \times h^{-1}$. It was slightly higher in the *DT* (0.4 $\mu molAc_{inc} \times gC_{sam}^{-1} \times h^{-1}$). In the *AC*, a distinction was made between the old, outer cuticle (AC_o) and the inner tissues (AC_n). The ^{14}C incorporation rate for the whole *AC* was mainly due to high ^{14}C uptake into the inner, living tissues (AC_n, 4.8 $\mu molAc_{inc} \times gC_{sam}^{-1} \times h^{-1}$), that is, the epidermis secreting the new cuticle, while the inert old cuticle showed low labelling (AC_o, 0.7 $\mu molAc_{inc} \times gC_{sam}^{-1} \times h^{-1}$).

^{3}H-lysine experiments

^{3}H-lysine incorporation rates were about 100 times lower than the ^{14}C-acetate incorporation rates (0.7 $\mu molLys_{inc} \times gC_{sam}^{-1} \times h^{-1}$ vs 61.7 $\mu molAc_{inc} \times gC_{sam}^{-1} \times h^{-1}$ in the *BB*) (Figure 3b). The highest rates were again found in the bacterial mats (*MP* and *BB*), suggesting that these bacteria can use lysine, probably for protein synthesis. Significant rates were measured in the gill chamber integument (*OB* and *Gi*) (34.1 and 16.1 $nmolLys_{inc} \times gC_{sam}^{-1} \times h^{-1}$, respectively), while the internal tissues (except the *Mu*: 0.8 $nmolLys_{inc} \times gC_{sam}^{-1} \times h^{-1}$) showed low labelling.

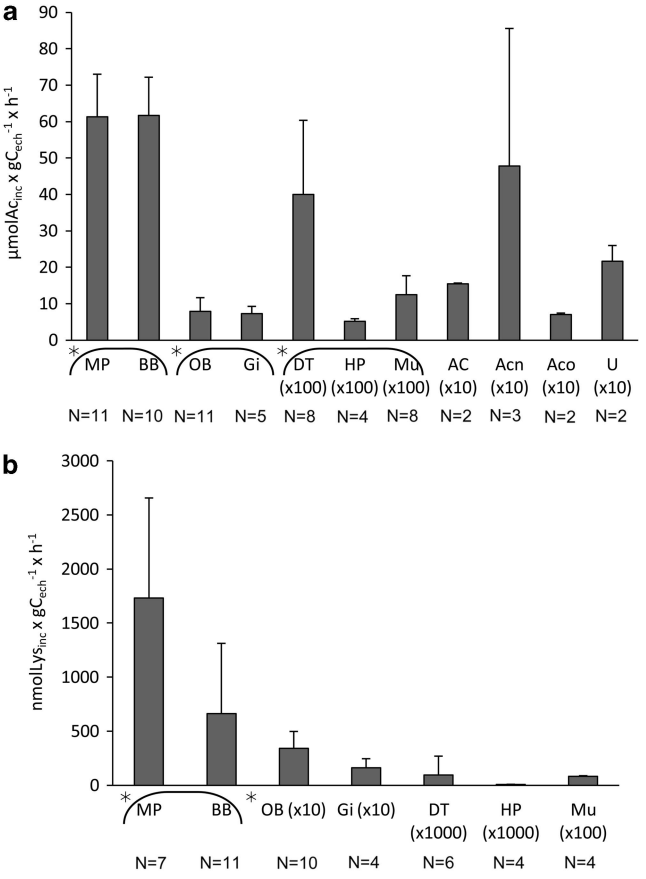

Figure 3 Rates of incorporation of ^{14}C (**a**) and ^{3}H (**b**) into the bacterial mats and inner tissues of *R. exoculata* shrimps incubated for 1 h with ^{14}C-acetate or ^{3}H-lysine in pure SW. Stars indicate significantly different groups of tissues; ($\times 10$, $\times 100$, $\times 1000$) indicates tissues for which the values were multiplied by 10, 100 or 1000 for graph presentation; '*N*' indicates the number of samples for each data bar. *U*, uropods.

Autoradiography

Our autoradiographic observations were in good agreement with the analytical data, confirming the distribution of the radiolabelled molecules among the bacterial mats and shrimp tissues (see Supplementary Information 2—Figures 7a and b for negative controls).

In the bacterial mats associated with the *MP* and branchiostegites, results for the three radiotracer incubations were quite similar. The labelling intensity depended on the specific radioactivity of the isotope, the exposure time and the amount of radiotracer incorporated. ^{3}H-lysine gave the best result with the shortest exposure time, followed by ^{14}C-acetate and ^{14}C-bicarbonate. Silver grain precipitation (in the photographic emulsion) always appeared first along the bacterial filaments (Figures 4a and c), eventually covering the whole length of the filaments as a dense black deposit (Figures 4b and d). By comparing the pictures obtained after short and long exposure times we were able to distinguish the labelled filamentous bacteria from the mineral crust of iron oxides: the

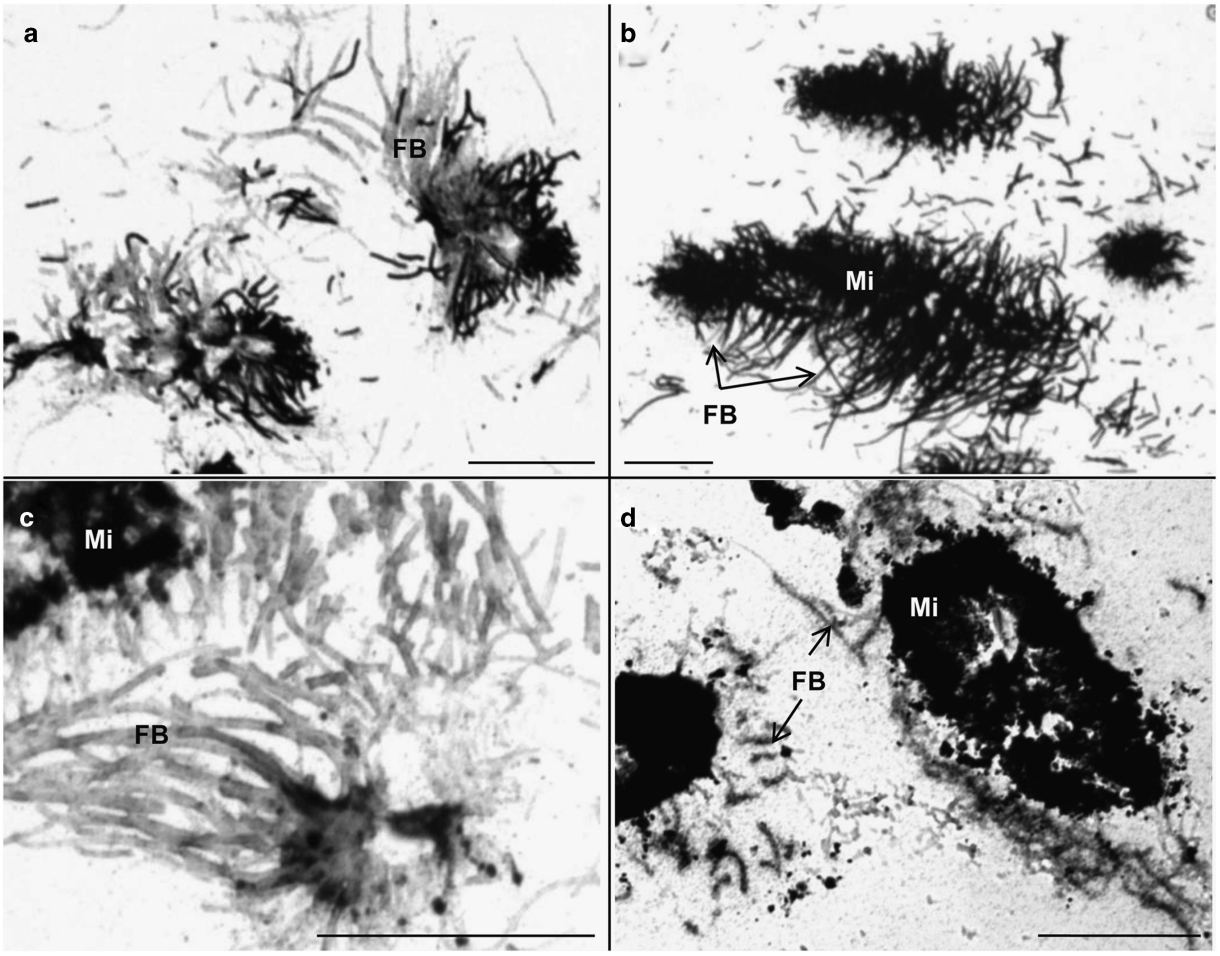

Figure 4 Autoradiographs of histological sections: cross-sectioned bacteriophore setae of the mouthparts of *R. exoculata* shrimps incubated for 1 h with ^{3}H-lysine (**a**, **b**) or for 6 h with ^{14}C-bicarbonate (**c**, **d**). (**a**) Filamentous bacteria are distinguished from mineral deposits and labelled on short segments only. (**b**) The filamentous bacteria are labelled over their entire length. (**c**) Filamentous bacteria are poorly or not labelled. (**d**) Discontinuous labelling appears on the distal part of the filamentous bacteria but does not stain them close to the setae. Exposure times: (**a**) 12 h, (**b**) 11 days, (**c**) 10 weeks and (**d**) 34 weeks. Scale bars: 100 μm. *FB*, filamentous bacteria; *Mi*, mineral.

latter also appeared as dark masses, but they were not labelled, whereas the bacteria located inside or below the mineral crust were moderately labelled (Figure 5a).

The shrimp tegumental tissues appeared densely labelled after incubation with ^{3}H-lysine or ^{14}C-acetate, but sections of shrimps incubated with ^{14}C-bicarbonate required very long exposure times before the label appeared. After 20–30 days of exposure, gills and muscles also appeared moderately labelled (Figures 5b and c), but neither the *DT* nor the *HP* showed any measurable labelling. Detailed views of the branchiostegite (Figure 5a) confirmed and explained the analytical data obtained for the abdominal integument: living tissues synthesising new cuticle and the new cuticle itself showed the most intense labelling. In contrast, the overlying old cuticle remained free of silver grains except in its lower part, which was probably infused with moulting fluid enzymes secreted by the epidermis (Compère *et al*., 2004).

Discussion

The *in-vivo* experiments presented here provide direct evidence that *R. exoculata* epibionts are autotrophic. These are the first experiments to have been carried out on live shrimps together with their epibionts, and the first to demonstrate a mutualistic relationship in this bacteria–shrimp symbiosis under *in-situ* pressure conditions.

Carbon fixation by bacterial chemosynthesis

Once thought to be a monoculture of a single, pleomorphic phylotype of *epsilonproteobacteria*, the gill chamber epibiont population of *R. exoculata* is now regarded as a functional consortium or syntrophic community (Stams and Plugge, 2009) dominated by *gamma*- and *epsilonproteobacteria*. By combining 16S rRNA and functional gene analysis with *in-situ* hybridisation and transmission electron microscopy observations on *R. exoculata*

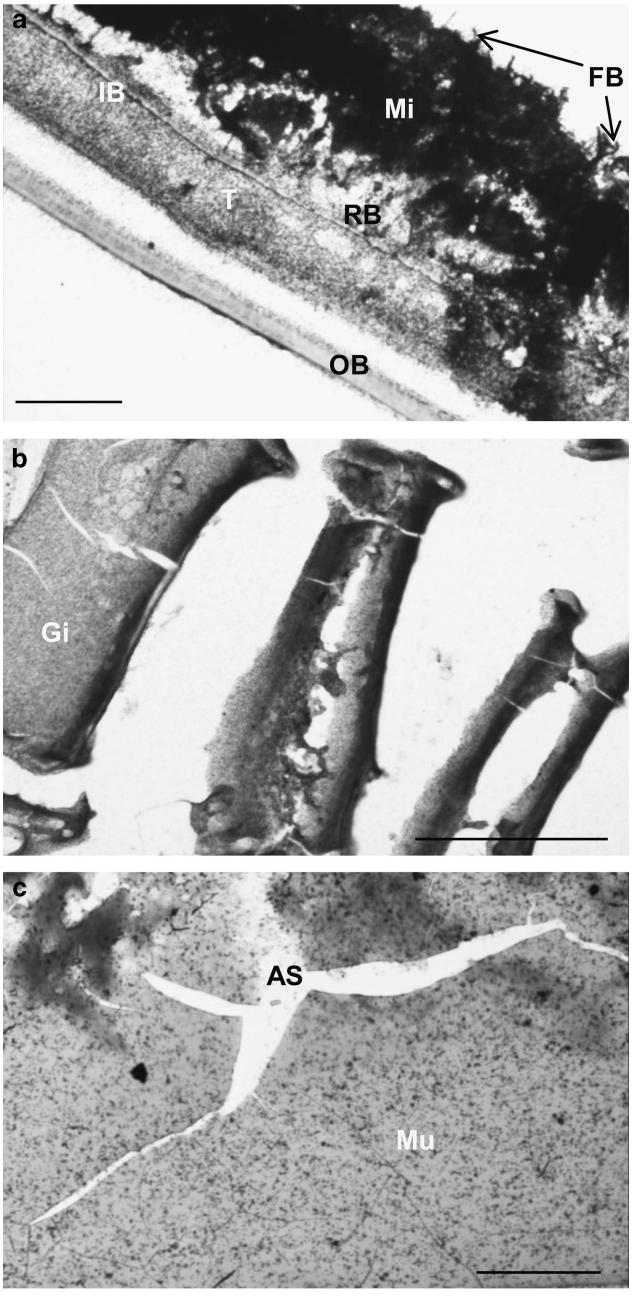

Figure 5 Autoradiographs of different cross-sectioned body parts of *R. exoculata* shrimps incubated for 1 h with ^{14}C-acetate (**a**, **c**) or ^{3}H-lysine (**b**): (**a**) branchiostegite (3-day exposure) showing intense labelling of emerging filamentous bacteria and much weaker labelling below the mineral crust, a dense silver deposit covering the living tissue, that is, both the inner and the outer epidermis and their respective new cuticles, while the old outer cuticle remains almost free of silver particles; (**b**) gills (20-days exposure); (**c**) abdominal muscles (29-days exposure) labelled moderately but more intensely than the background. Scale bar: 100 μm. *AS*, sectioning artificial space; *FB*, filamentous bacteria; *Mi*, mineral crust; *Mu*, muscles; *OB*, outer branchiostegite (old cuticle separated from the new one); *RB*, rod-shaped bacteria; *T*, tissues including the epidermis bordered by the new cuticle being deposited.

epibionts and their free-living or symbiotic relatives from vent habitats, investigators (Campbell *et al.*, 2006; Zbinden *et al.*, 2008; Petersen *et al.*, 2009; Goffredi, 2010; Hügler *et al.*, 2010, 2011; Guri *et al.*, 2012) have evidenced *epsilonproteobacteria* of Marine Groups 1 (the new family Thiovulgaceae) and 2, as thick (3-μm wide) and thin (1-μm wide) filaments, respectively. Both should oxidise sulphur compounds through the *Sox* pathway and fix carbon through the reverse tricarboxylic acid cycle (*SoxB* and *acl*A gene sequences). Authors have also identified *gamma-proteobacterial* epibionts of the Thiotrichaceae family (*Leucothrix* group), together with some free-living members and some other invertebrate ectosymbionts, mainly of crustaceans (Goffredi, 2010). These *gammaproteobacteria* appearing at least as filamentous and rod-shaped morphotypes can probably grow as sulphide oxidisers, most likely using the APS pathway for energy generation and the Calvin-Benson-Bassham cycle for carbon fixation (*apr*A and *cbb*M gene sequences, respectively) (Zbinden *et al.*, 2008; Hügler *et al.*, 2010, 2011; Guri *et al.*, 2012). In addition, coccoid-shaped cells with intracytoplasmic membrane stacks have been identified as type-I methanotrophic *gammaproteobacteria* (*pmo*A gene sequence) (Corbari *et al.*, 2008a; Zbinden *et al.*, 2008; Guri *et al.*, 2012), and *deltaproteobacteria* related to *Desulfocapsa* have been identified thanks to their *apr*A and hydrogenase gene sequences (Hügler *et al.*, 2011), suggesting they can grow lithotrophically using hydrogen as electron donor for sulphate reduction. On this basis, the authors hypothesise that an internal sulphur cycle between sulphur-oxidising *epsilon-* and *gammaproteobacteria* and sulphate-reducing *deltaproteobacteria* could take place in the shrimp gill chamber, as proposed for oligochete symbiosis (Dubilier *et al.*, 2001).

The following results of the present study support the view that shrimp-associated bacteria carry out chemosynthetic inorganic carbon fixation: (1) the carbon incorporation rates for ^{13}C- and ^{14}C-bicarbonate are in good agreement, (2) they increase in the presence of electron donors ($S_2O_3^{2-}$, Fe^{2+}), (3) they are stable over time, as confirmed by the time-related increase in ^{13}C incorporation and (4) autoradiographs of histological sections show labelling of bacterial mats.

The carbon fixation rates observed here are similar to but somewhat higher than those observed by Polz *et al.* (1998): 3.9 vs 2 $\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$ for the *MP* of shrimp incubated in SW. This comparison is somewhat uncertain, as we had to convert Polz's data, expressed per weight unit of protein, into values given per weight unit of total carbon, the carbon content of proteins being estimated at 45% (Rouwenhorst *et al.*, 1991). Yet, the consistently higher rates found here might be due to the incubation of live shrimps under *in-situ* pressure (230 bars in this study), as opposed to the use, in previous experiments, of pieces of dead shrimp under atmospheric pressure. Active chemoautotrophy might be promoted by preserving shrimps and

bacteria in good physiological condition (Ravaux *et al.*, 2003). Accordingly, Zbinden *et al.* (2008) observed <30% degraded epibionts in re-pressurised shrimps. The carbon fixation rates determined here are much lower, however, than those obtained for other chemosynthetic symbionts (bacterial homogenates or endobacteria of bivalve gills), for example, those of *Bathymodiolus thermophilus*: $150\,\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$ in SW (Nelson *et al.*, 1995), or *Solemya velum*: $2500\,\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$ in the presence of thiosulphate (Scott and Cavanaugh, 2007). Moreover, owing to the relatively low energy level of thiosulphate as compared with sulphide, the latter authors observed threefold higher carbon fixation rates with 0.2 mM Na_2S ($70\,\mu molC \times g_{prot}^{-1} \times min^{-1}$) than with 1 mM $Na_2S_2O_3$ ($20\,\mu molC \times g_{prot}^{-1} \times min^{-1}$).

That the presence of an electron donor increases the carbon fixation rate indicates that $Na_2S_2O_3$, and Fe^{2+} at least contribute to fuelling *R. exoculata* epibionts, and our autoradiography data suggest that chemosynthesis occurs at least in the large filamentous bacteria (*epsilonproteobacteria* of Marine Group 1), which appeared more intensely labelled than the thin filamentous and rod-shaped ones (*epsilonproteobacteria* of Marine Group 2 and *gammaproteobacteria*). The view that *R. exoculata* epibionts have sulphide-oxidising activity is consistent, moreover, with the fact *epsilon-* and *gammaproteobacteria* are assumed to have such activity, on the basis of the group affiliation of the former (Petersen *et al.*, 2009; Goffredi, 2010; Guri *et al.*, 2012) and because the latter possess functional *SoxB* and *aprA* genes (Hügler *et al.*, 2010, 2011). We cannot say, however, what the epibiotic bacteria of *R. exoculata* use as predominant energy source. Methane and hydrogen, found in hydrothermal fluids from the Rainbow vent field along with sulphide and iron (Charlou *et al.*, 2002; Douville *et al.*, 2002; Schmidt *et al.*, 2008), are also potential drivers of chemoautotrophy. In particular, the type-I methanotrophic *gammaproteobacteria* may use methane as both electron and carbon source (Zbinden *et al.*, 2008; Guri *et al.*, 2012), *deltaproteobacteria* are believed to use H_2 for sulphate reduction (Hügler *et al.*, 2011), and *epsilonproteobacteria* (of Marine Groups I and II) may use H_2 instead of sulphide for chemosynthesis, as proposed for *Bathymodiolus* gill endosymbionts (Petersen *et al.*, 2011). The possibility that *R. exoculata* epibionts might use H_2 is strongly supported by the identification of a *hupL* gene sequence from Group-2 *epsilonproteobacteria* (Hügler *et al.*, 2011) among the *R. exoculata* epibionts and by the metabolic capabilities reported for Campylobacterales, especially of the family Thiovulgaceae (Campbell *et al.*, 2006; Goffredi, 2010). We have also tested methane and hydrogen (see Supplementary Information 1), but we observed rapid death of the incubated shrimps, so they were discarded. Furthermore, whether CH_4- or H_2-metabolic genes are actively expressed or not has not been determined. Biotic iron oxidation by *R. exoculata* epibionts has been proposed (Zbinden *et al.*, 2004, 2008; Corbari *et al.*, 2008a) on the basis of the close association of these bacteria with heavy iron-oxyhydroxide deposits. Our results support this hypothesis, but evidence is lacking as to the ability of the bacterial phylotypes identified so far to oxidise iron. Furthermore, abiotic iron-oxide precipitation can compete strongly with biotic iron oxidation (Schmidt *et al.*, 2009), bacterial cell walls can induce it (Corbari *et al.*, 2008a).

We also show here that *R. exoculata* epibionts can incorporate ^{14}C-acetate and ^{3}H-lysine much faster than they incorporate ^{14}C from bicarbonate. In the branchiostegite bacterial mats, the rate of organic carbon incorporation from acetate ($61.7\,\mu molAc_{inc} \times gC_{sam}^{-1} \times h^{-1}$) was 10–20 times the rate observed with ^{14}C-bicarbonate, depending on whether thiosulphate was present ($5.4\,\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$) or not ($2.8\,\mu molC_{inc} \times gC_{sam}^{-1} \times h^{-1}$). This may be due to the presence of heterotrophic bacteria, but to date, this has not been investigated. Our observation that the thick bacterial filaments were intensely labelled with all three radiotracers, and that the thin ones were also labelled to a lesser extent, suggests that at least the *epsilonproteobacteria* might switch to a mixotrophic metabolism and use small organic molecules, when available, as energy and/or carbon sources. Acetate and amino-acid assimilation is thus unsurprising and might reflect a capacity of certain chemolithoautotrophic bacterial epibionts to adjust their metabolism to changes in their geochemical environment. A versatile (autotrophic/mixotrophic/heterotrophic) energy metabolism using several electron donors (including hydrogen and organic compounds) and acceptors (including oxygen and nitrate) has indeed been reported for free and symbiotic *epsilonproteobacteria* of the Thiovulgaceae family (Campbell *et al.*, 2006; Goffredi, 2010) and suggested for *R. exoculata* ectosymbionts (Hügler *et al.*, 2011). Mixotrophy/heterotrophy is also commonly accepted as a possible feature of *gammaproteobacteria* of the Thiotrichaceae family (Goffredi, 2010), as most *Beggiatoa* species can grow on acetate as sole added carbon source (Larkin and Strohl, 1983). It has been proposed but not investigated for *R. exoculata* epibionts (Hügler *et al.*, 2011). In support of this hypothesis, a metagenomic study has revealed in *Riftia pachyptila* endosymbionts the presence of all genes required for heterotrophic metabolism (Robidart *et al.*, 2008), and a metaproteomic study has pinpointed acetate as a substrate for bacterial endosymbionts of the gutless marine worm *Olavius algarvensis* (Kleiner *et al.*, 2012). Schulz and Jørgensen (2001) interpret the metabolic flexibility of filamentous sulphur-oxidising bacteria, especially those of the *Thiothrix* group, and their ability to grow heterotrophically or autotrophically on different sulphur species, as a need to overcome disadvantages of their sessile life and to adapt their activity to changes in substrate availability (oxygen,

sulphide). In *R. exoculata*, the obvious variability of bacterial assemblages according to the gill chamber area, the individual and the vent site (Zbinden *et al.*, 2008; Petersen *et al.*, 2009; Hügler *et al.*, 2011) suggests a consortium composition plasticity as a response to varying vent fluid composition (Charlou *et al.*, 2002; Schmidt *et al.*, 2008) and to fluctuating geochemical or micro-environmental conditions in the zone of mixing with deep water.

The relatively high carbon fixation rate observed in SW alone suggests that *R. exoculata* epibionts have an internal energy store that supplies autotrophic metabolisms in the absence of an external electron donor, for example, when the shrimps swim from reduced-compound-rich hydrothermal fluid to oxic (reduced-compound-poor) deep water. Such carbon fixation has previously been evidenced in *Bathymodiolus thermophilius* and *R. exoculata* symbionts (Nelson *et al.*, 1995; Polz *et al.*, 1998). Energy could be stored as iron polyphosphate and sulphur globules like those observed ultrastructurally by Wirsen *et al.* (1993) and Zbinden *et al.* (2008) in thin filamentous bacteria. These could be identified as *Leucothrix*-like *gamma-ectosymbionts* because their APS pathway allows production of intermediate sulphur compounds and because the storage role of similar globules is well documented in their shallow-water relatives (Larkin and Strohl, 1983; Schulz and Jørgensen, 2001). Furthermore, these globules were shown to be empty in specimens maintained in a pressurised aquarium without electron donors (Zbinden *et al.*, 2008). This suggests that the enhanced carbon fixation observed in the presence of thiosulphate as compared with pure SW might reflect a switch of *gamma-* and/or *epsilon-epibionts* to an external energy source supporting autotrophic metabolism.

Bacteria–host transfers

Several authors propose that organic nutrients might be transferred from epibionts to *R. exoculata* (Casanova *et al.*, 1993; Zbinden *et al.*, 2004; Corbari *et al.*, 2008b). Others, arguing that the cuticle is impermeable, reject this hypothesis (Segonzac *et al.*, 1993; Gebruk *et al.*, 2000). The latter authors refer to an experiment (Anderson and Stephens, 1969) or to subsequent review papers (Stephens, 1988; Preston, 1993; Gebruk *et al.*, 2000) suggesting that crustaceans, in contrast to many other marine invertebrates, cannot take up dissolved organic matter or dissolved organic carbon. According to Anderson and Stephens (1969), the apparent incorporation of radiotracers by crustaceans was due to uptake by epibionts alone and not by the crustaceans themselves. Yet these authors worked with whole organisms, and never separated the internal tissues from the integument and the epibionts as we have done here.

The present results demonstrate the transfer of carbon fixed by bacteria to the host shrimp tissues and the ability of shrimps to take up dissolved organic molecules such as acetate or lysine across their integument. Together with our autoradiographs, the higher radiotracer concentrations in the shrimp tissues than in the incubation media (Table 2) and the incorporation of ^{3}H-lysine and ^{14}C-acetate into the *Mu* (proteins) and the *HP* (storage lipids) confirm their active uptake and their accumulation in shrimp insoluble organic matter. Our separate analysis of the old and new cuticles of the abdominal integument and the corresponding autoradiographs also suggest that the new cuticle and the secreting epithelium incorporate more labelled molecules, probably because of the intense biosynthetic activity at this level in pre-ecdysial crustaceans.

These results thus constitute *in-vivo* experimental evidence of nutritional transfer of bacteria-fixed

Table 2 Initial and final concentrations (in μM) of labelled compounds (^{13}C, ^{14}C-bicarbonate, ^{14}C-acetate, ^{3}H-lysine) in the incubation media and the shrimp body parts

Labelled compounds in each incubation medium	*Initial concentration (μM) of labelled compounds in the incubation medium*	*Final concentration (μM) obtained after 1 h in bacteria and shrimps tissues*	
$NaH^{13}CO_3$	11 764	MP	157.17 ± 52.92
		DT	49.94 ± 0.29
		BB	42.04 ± 13.84
		Mu	20.42 ± 9.13
		OB	8.30 ± 7.33
$NaH^{14}CO_3$	3000	MP	33.01 ± 16.31
		OB	19.88 ± 15.13
		DT	15.84 ± 16.30
		BB	12.20 ± 4.89
		AC	7.02 ± 2.95
		Gi	5.97 ± 2.04
		HP	2.13 ± 0.55
		Mu	1.75 ± 1.01
^{14}C-acetate	200	**MP**	**524.24 ± 100.53**
		BB	**281.48 ± 47.86**
		Gi	**245.47 ± 66.43**
		OB	111.52 ± 53.91
		AC_n	101.97 ± 80.37
		U	46.19 ± 9.14
		AC	33.01 ± 0.35
		DT	18.79 ± 9.61
		AC_o	14.87 ± 0.91
		Mu	9.01 ± 3.76
		HP	7.84 ± 0.99
^{3}H-lysine	0.2	**MP**	**14.82 ± 7.92**
		BB	**3.02 ± 2.97**
		Gi	**0.54 ± 0.28**
		OB	**0.48 ± 0.22**
		Mu	0.06 ± 0.00
		DT	0.00 ± 0.01
		HP	0.00 ± 0.00

Abbreviations: AC, abdominal cuticle; ACn, new abdominal cuticle; AC_o, old abdominal cuticle; BB, branchiostegite biofilm; DT, digestive tract; Gi, gills; HP, hepatopancreas; MP, mouthpart; Mu, muscles; OB, outer branchiostegite; U, uropods.
In bold are the data for compartments that accumulate the radiotracers (in > out). For the comparative estimation of tissue concentrations, 1 g fresh weight was considered equal to 1 ml liquid. All values are taken from experiments in pure seawater.

carbon to shrimps. They are in good agreement with previous stable isotope studies (Gebruk *et al.*, 1993; Pond *et al.*, 1997a, b, 2000; Rieley *et al.*, 1999; Colaço *et al.*, 2007) and confirm that *R. exoculata* depends on its epibionts for its nutrition. They thus strengthen the view that the relationship between bacteria and shrimp is mutualistic: the epibionts supply carbon compounds to the shrimp and the shrimp, thanks to its gill chamber flow and its swimming behaviour, offers them protection and a supply of chemical compounds, maintaining their position at the oxic/anoxic interface around active chimneys (Casanova *et al.*, 1993; Segonzac *et al.*, 1993). The *R. exoculata* bacterial epibiosis can thus be regarded as a true mutualistic trophic ectosymbiosis, similar to what is known about the chemosynthetic endosymbiosis of organisms such as *R. pachyptila* (Felbeck, 1981; Fisher *et al.*, 1989), *Calyptogena magnifica* (Childress *et al.*, 1991) and *S. velum* (Stewart and Cavanaugh, 2006; Scott and Cavanaugh, 2007).

Finally, the view that bacterial products are assimilated across the shrimp integument rather than *via* the *DT* is strongly supported by: (1) the significant incorporation of both ^{14}C-acetate and ^{3}H-lysine after an incubation time as short as 1 h. This is insufficient for uptake by ingestion assimilation, which commonly takes several hours (ingestion assimilation of carbon from bicarbonate, for example, requires prior incorporation by the bacteria, followed by grazing of bacteria from the *MP*) (Chipps, 1998; Hoyt *et al.*, 2000); (2) the high incorporation levels recorded in the gill chamber integument lining (*OB*, *Gi*), as opposed to (3) the much lower levels recorded in the *DT* (Figure 6). These results are in agreement with the view that the shrimps do not graze on their epibionts (Zbinden *et al.*, 2004; Corbari *et al.*, 2008b), but rather farm them (Segonzac *et al.*, 1993; Polz and Cavanaugh, 1996), although they might also ingest some chimney bacteria along with sulphides (Van Dover *et al.*, 1988; Segonzac, 1992). The low but nevertheless significant incorporation of ^{14}C from bicarbonate in the *DT* corroborates the data of Polz *et al.* (1998), although they found higher rates than ours (upon incubating isolated gut segments). This incorporation might be due to chemosynthetic activity of the midgut microbial community (Polz *et al.*, 1998; Zbinden and Cambon-Bonavita, 2003; Durand *et al.*, 2010), but this community would be less active than the gill chamber community.

The view that soluble carbon-containing compounds are transferred from bacteria to shrimp by crossing the gill chamber integument tallies with the ultrastructural characteristics of this integument, which resemble those of permeable and/or transporting tissues (Martinez *et al.*, 2005): a very thin cuticle (30 μm), reduced blood-water distance, mitochondria-rich cells with apical infolding in the branchiostegite epithelium. In *R. exoculata*, the uptake of bacterial nutrients across the integument

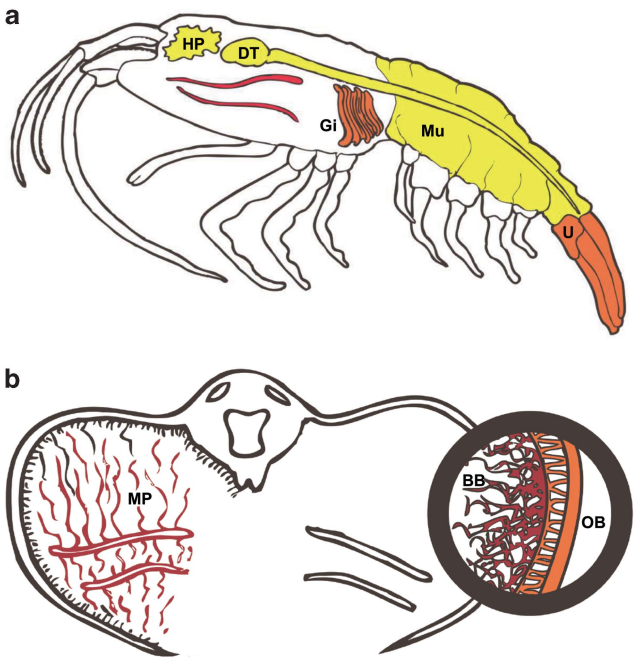

Figure 6 Schematic view of *R. exoculata* (**a**) and its gill chamber—transversal view (**b**) showing the level of ^{14}C-tracers incorporation in the internal organs by colour range from the highest levels in red to the lowest in yellow. *U*, uropods.

could be the major source of nourishment, along with complementary sources such as grazing on chimney minerals (Van Dover *et al.*, 1988; Segonzac *et al.*, 1993), ingestion of bacteria-colonised exuviae, and midgut microflora activities (Zbinden and Cambon-Bonavita, 2003; Zbinden *et al.*, 2004; Corbari *et al.*, 2008b; Durand *et al.*, 2010). It could be regarded as a peculiar adaptation of the shrimp. Although some authors have discarded this hypothesis, the present results may bring it back into favour. By extension, they suggest that this process might exist in many crustacean species, which would derive their nourishment at least partly from dissolved organic matter or dissolved organic carbon, as is commonly accepted for many marine invertebrates: annelids (Richards and Arme, 1982; Ahearn *et al.*, 2000; Peppler and Ahearn, 2003), molluscs (Wright and Secomb, 1986; Wright, 1988; Wright and Pajor, 1989; De Eguileor *et al.*, 2000) and cuticle-lined parasitic organisms such as nematodes (Fleming and Fetterer, 1984; Gordon and Burford, 1984; Masood, 1984). Arbitrarily rejected for crustaceans (Stephens, 1988) and especially decapods, this idea is surprisingly accepted without demonstration for parasitic rhizocephalan barnacles such as *Sacculina carcini* (Bresciani and Høeg, 2001) and for parasitic copepods (Kannupandi, 1976; Poquet *et al.*, 1994) with a modified cuticle. It is well known, furthermore, that in pre-ecdysis, crustaceans (and other arthropods) absorb soluble molecules (for example, sugars and amino acids) from their degrading old cuticle through the new cuticle and re-utilise them for its synthesis (Compère *et al.*,

2004). These conflicting views regarding the capacity of marine crustaceans to assimilate dissolved organic carbon will be tested in further studies.

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