RESEARCH ARTICLE

Lack of endosymbiont release by two *Lucinidae* (*Bivalvia*) of the genus *Codakia*: consequences for symbiotic relationships

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Abstract

Associations between marine invertebrates and chemoautotrophic bacteria constitute a wide field for the study of symbiotic associations. In these interactions, symbiont transmission must represent the cornerstone allowing the persistence of the association throughout generations. Within *Bivalvia*, in families such as *Solemyidae* or *Vesicomyidae*, symbiont transmission is undoubtedly vertical. However, in *Lucinidae*, symbiont transmission is described in the literature as 'environmental', symbionts being acquired from the environment by the new host generations. Hence, if there is transmission, symbionts should be transmitted from adults to juveniles via the environment. Consequently, we should observe a release of the symbiont by adults. We attempted to detect such a release within two *Lucinidae* species of the genus *Codakia*. We sampled 10 *Codakia orbicularis* and 20 *Codakia orbiculata* distributed in 10 crystallizing dishes containing filtered seawater. During 1 month of investigation, we analyzed water of the dishes in order to detect any release of a symbiont using catalyzed report deposition-FISH techniques. For 140 observations realized during this period, we did not observe any release of symbionts. This suggests that the idea of host-to-host passage in *Lucinidae* is inaccurate. We could therefore consider that the transmission mode from generation to generation does not occur within *Lucinidae*, symbiosis appearing to be advantageous in this case only for the host, and constitutes an evolutionary dead-end for the bacteria.

Introduction

Since their discovery in hydrothermal vents (Felbeck *et al.*, 1981), associations between marine invertebrates and chemoautotrophic bacteria have been described in more accessible environments such as shallow water sea-grass beds (Berg & Alatalo, 1984), mangrove swamps (Schweimanns & Felbeck, 1985), cold seeps (Juniper & Sibuet, 1987), whale bones (Deming *et al.*, 1997) and sunken woods (Gros & Gaill, 2007; Duperron *et al.*, 2008). These associations are present all over the world in many host taxa as an endo- or an ecto-symbiosis (Gill & Vogel, 1962; Polz *et al.*, 1992; Polz & Cavanaugh, 1995; Haygood & Davidson, 1997; Zbinden *et al.*, 2004). According to 16S rRNA gene sequences, the majority of marine symbionts known to date belong to the *Gammaproteobacteria* subdivision (Distel *et al.*, 1994).

One of these associations involves *Lucinidae* (*Mollusca*: *Bivalvia*) and sulfur-oxidizing (thioautotrophic) intracellular symbionts (Distel *et al.*, 1988, 1994; Durand & Gros, 1996; Williams *et al.*, 2003). *Lucinidae* are the most diverse bivalve family, live in a wide variety of marine habitats and are geographically very widespread (Taylor & Glover, 2005). All *Lucinidae* genera studied to date harbor endosymbiotic thioautotrophic bacteria localized in specialized cells of the gill called bacteriocytes (Fisher, 1990).

As in all endosymbioses, symbiont transmission must constitute the most important factor enabling symbiotic associations to continue. Such transmission can be (1) vertical (from parents to offspring), which implies the integration of the symbiont into the gametes, (2) horizontal (spread of symbionts between contemporary hosts) or (3) environmental. The latter term has been proposed to define

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the reinfection of the new generation by a stock of microorganisms present in the environment without, however, discussion of the origin of this environmental stock (Le Pennec *et al.*, 1988; Cary & Giovannoni, 1993).

In *Lucinidae*, no symbionts in ovaries, eggs, embryos and larvae of *Codakia orbicularis* have been described (Gros *et al.*, 1996), which eliminates the vertical transmission hypothesis. However, aposymbiotic juveniles of *C. orbicularis* can be infected in the laboratory with crude sand from natural habitats of *C. orbicularis* (Gros *et al.*, 1996). Later, free-living forms of the *C. orbicularis* gill endosymbiont were detected in *Thalassia testudinum* sea-grass bed sediments (Gros *et al.*, 2003). These observations led the authors to conclude that *Lucinidae* sulfur-oxidizing endosymbionts were environmentally transmitted.

However, environmental transmission requires the passage of the symbiont from an infected host (i.e. adults) to a noninfected host (i.e. aposymbiotic juveniles) via the environment (Fig. 1), a phenomenon that has not yet been described in *Lucinidae*. Consequently, if transmission occurs, a release of the symbiont by adults in the environment must exist. In this study, we attempted to find it.

Materials and methods

Specimen collection

Approximately 40 mm shell length individuals of *C. orbicularis* (Linné, 1758) and 15 mm shell length *Codakia orbiculata* (Montagu, 1808) individuals were collected by hand in March 2007 from *T. testudinum* sea-grass sediments on îlet Cochon (16°12′53.76″N; 61°32′05.74″W) in Guadeloupe (French West Indies).

Rearing conditions

Clams were divided into 10 crystallizing dishes containing 500 mL of 0.2 μm filtered seawater according to the following distribution: two crystallizing dishes contained a single *C. orbicularis*; four crystallizing dishes contained two *C. orbicularis* each; and four crystallizing dishes contained five to six *C. orbiculata* each.

To avoid symbiont reacquisition due to bivalve filtration, individuals were kept raised halfway up the dishes. No sulfur, food or oxygen was added to the dishes. Every day, 'old' seawater was discarded and replaced by 'fresh' 0.2 μm filtered seawater. A positive control (0.2 μm filtered seawater containing *C. orbicularis* gill extract) was also realized in order to check whether symbionts could be detected by our analysis technique.

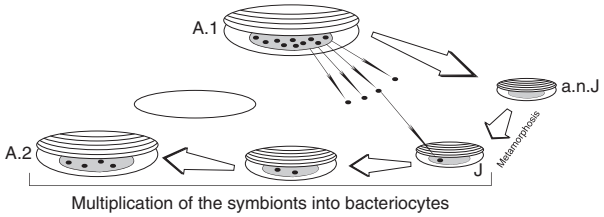

Fig. 1. Schematic representation of the environmental transmission. Symbionts acquired by juveniles derive from symbionts released by adults of the contemporary generation after multiplication in the gills of these last ones. A.1: adult of the parental generation, A.2: adult of the following generation, a.n.J: aposymbiotic-naive juvenile, J: juvenile, full ellipses: endosymbiotic bacteria.

Probes

Various studies have used fluorescent probes to detect environmental bacteria or bacterial symbionts in the environment (Christensen *et al.*, 1999; DeLong *et al.*, 1999; Manz *et al.*, 2000; Gros *et al.*, 2003). Here, as a positive control, we used the universal probe EUB338, which targets 16S rRNA gene of most eubacteria (Amann *et al.*, 1990; Daims *et al.*, 1999), and the specific probe Symco2A, which hybridizes in a specific manner the 16S rRNA gene of the symbiont of *C. orbicularis* (Gros *et al.*, 1996) and of *C. orbiculata*, which harbor a symbiont with the same 16S rRNA gene sequence (Durand & Gros, 1996).

Catalyzed report deposition (CARD)-FISH

Histological sections or pieces of filters were used for hybridization using the following probes: EUB338 to target all eubacteria and Symco2A for *C. orbicularis* or *C. orbiculata* gill endosymbiont. This protocol follows Pernthaler *et al.* (2002) up to the washing buffer step. Sections were then washed twice in 1 × phosphate-buffered solution (PBS) [15 min at room temperature (RT)] and incubated in a solution of amplification buffer (1/10 [w/v] dextrane sulfate, 1 × PBS, 2 M NaCl and 1/100 [v/v] 10% blocking reagent), 0.5% diluted H_2O_2 in 1 × PBS and 2 μL of fluorescent-labeled tyramide for 15 min at 37 °C in the dark. Sections were then washed in 1 × PBS (5 min at RT in the dark), in sterilized water and twice in 50 mL of absolute ethanol. Sections were air-dried, 4′,6-diamino-2-phenylindole (DAPI) counterstained and embedded in DAKO medium before observation under an epi80i fluorescent microscope (NIKON, France).

CARD-FISH detection threshold

We purified a gill extract on percoll, following Caro *et al.* (2007), and quantified its bacterial concentration by counting on a Malassez cell. We then used this inoculum to constitute samples containing 0, 10, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 bacteria mL^{-1}. These samples were treated and analyzed in a manner similar to those used for Symbiont release detection experiments.

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Symbiont release detection

The experiment was conducted over 30 days. To investigate the release of symbionts, 50 mL of seawater from the 500-mL experimental batches were sampled and analyzed at T_0, every 4 h the first day, once a day until day 5 and then every 5 days (for a total of 140 observations). Every 50-mL sample was fixed in 4% PFA for 3 h at 4 °C and filtered on GTTP filters (Millipore, France). Each section of the filter (1/4 of filter) was analyzed directly or stored at −80 °C before analysis.

Monitoring of the symbiotic population in the gills by CARD-FISH on slides

To follow the evolution of the symbiotic population in the gills, individuals were sacrificed at T_0, T_0+10 days, T_0+20 days and T_0+30 days, and gill sections were CARD-FISH analyzed. Slides were prepared following Dubilier *et al.* (1995), with some modifications: after embedding in paraffin, 4 µm sections were realized and pasted on SuperFrost® slides (Menzel-Glaser®). Slides were used immediately or stored at −20 °C. Sections were then deparaffinized in toluene and rehydrated (100%, 95%, 70% ethanol and distilled water). Slides were washed in 0.2 M HCl (12 min at RT), Tris/HCl 20 mM (10 min at RT), Proteinase K (0.5 µg mL^{-1} of Tris/HCl 20 mM; 5 min at 37 °C) and finally in Tris/HCl 20 mM for 10 min at RT. Slides were then directly used for CARD-FISH.

Monitoring of the symbiotic population in the gills by transmission electron microscopy (TEM)

After dissection, gill samples were prefixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.2 isosmotic to seawater by addition of sodium chloride and $CaCl_2$) for 2 h, and washed three times in the same isosmotic buffer. Pieces of gill were fixed for a further hour at RT in 1% osmium tetroxide in the same buffer, and then rinsed in distilled water before postfixation, using 2% aqueous uranyl acetate (1 h), dehydration through an ethanol series and propylene oxide before embedding in an Epon–Araldite mixture. Thin sections, 60 nm thick, were cut for TEM observation. They were processed and stained by uranyl acetate and lead citrate using standard TEM methods and observed under a Leo 912 TEM microscope.

Results

The purpose of this study was to check whether sulfur-oxidizing gill endosymbionts of two tropical lucinids belonging to the genus *Codakia* (*C. orbicularis* and *C. orbiculata*) are released by their hosts in the environment. To answer this question, we attempted to detect these non cultivable thioautotrophic bacteria using a specific fluorescent probe targeting 16S rRNA gene sequences from a controlled environment.

CARD-FISH detection level

To ensure that the lack of symbiont detection was not due to a lack of sensitivity of the technique used, we determined the minimal number of bacteria necessary to observe a signal using 0.2 µm filtered seawater containing a purified fraction of *C. orbicularis* gill endosymbionts extracted from a freshly collected individual of *C. orbicularis* (Caro *et al.*, 2007) as a positive control. The EUB338 (Fig. 2b) and Symco2A (Fig. 2c) probes were sufficiently effective in detecting at least 10 bacteria mL^{-1} (minimal density analyzed); the negative control (0.2 µm filtered seawater without a gill extract) remained negative for the two probes (data not shown).

Symbiont release

Filters of the different sampling points were hybridized with EUB338 and Symco2A probes. For all the 140 samples analyzed, no positive hybridization with Symco2A was found, whether early [every 4 h without seawater change

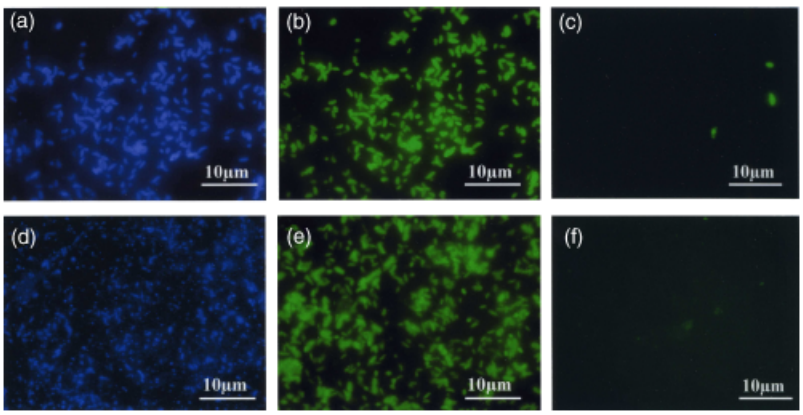

Fig. 2. Symbiont release detection experiment. (a) DAPI staining for nucleic acid coloration (positive control). (b) Hybridization with the universal EUB338 probe for detection of majority of eubacteria (positive control). (c) Hybridization with a Symco2A probe specific to *Codakia orbicularis* and the *C. orbiculata* gill symbiont (positive control, 10 bacteria mL^{-1}). (d) DAPI T_0+1 day. (e) EUB338 T_0+1 day. (f) Symco2A T_0+1 day.

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(T_0 to T_0+16 h)] or late in the experiment [seawater change every day (T_0 to T_0+30 days)] (Fig. 2f). In all cases, the internal positive control of hybridization (EUB338) was positive, confirming that bacteria could be detected using our technique (Fig. 2e). A DAPI counterstaining was used to confirm that positive hybridizations represented bacteria, and to eliminate auto fluorescent particles from the sediments (Fig. 2a and d). No host mortality was observed during the experiment.

Evolution of the symbiotic population inside gills

CARD-FISH

Gill sections of *C. orbicularis* and *C. orbiculata* at 0, 10, 20 or 30 days of starvation were hybridized with EUB338 and Symco2A. In both cases, probes hybridized only in the lateral zone of the gill where bacteriocytes are localized. Based on microscopic observations, sections of T_0 (Fig. 3a), T_0+10 days and T_0+20 days (data not shown) revealed no hybridization differences (Fig. 3a). However, on the T_0+30 days (Fig. 3b), the sample presented a weaker hybridization signal.

TEM

Through the lateral zone of each gill filament, bacteriocytes correspond to large cells (up to 35 μm length) characterized by a rounded apical pole in contact with circulating seawater and a cytoplasm filled with individually enclosed bacteria (Fig. 4a). Bacterial endosymbionts, which could reach 5 μm in length (Fig. 4a), are characterized by numerous sulfur granules (appearing as electron-lucent vesicles after conventional TEM preparation) located in the periplasmic space as described recently using cryo-EFTEM (Lechaire *et al.*, 2008). After 1 month of starvation, modifications could be noted in the gill filaments observed, with a decrease of intracellular bacteria and the appearance of numerous large secondary lysosomes (Fig. 4b), indicating an increase of intracellular digestion of the symbionts.

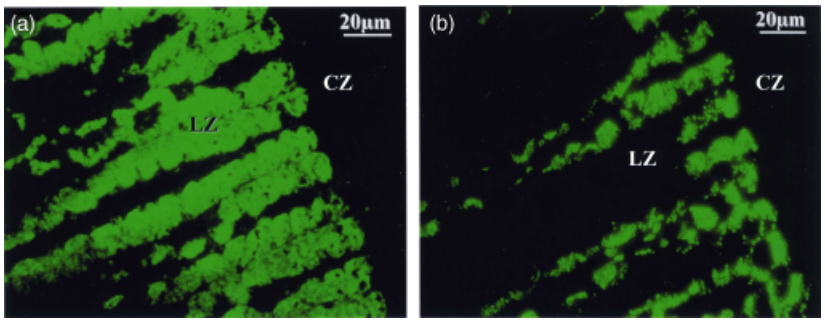

Fig. 3. *In situ* hybridization (CARD-FISH) with a Symco2A probe on *Codakia orbicularis* gill sections. (a) Gill section of *C. orbicularis* at 0 day (T_0) of starvation. (b) Gill section of *C. orbicularis* at 30 days of starvation (T_0+30). LZ: Lateral zone, CZ: ciliated zone.

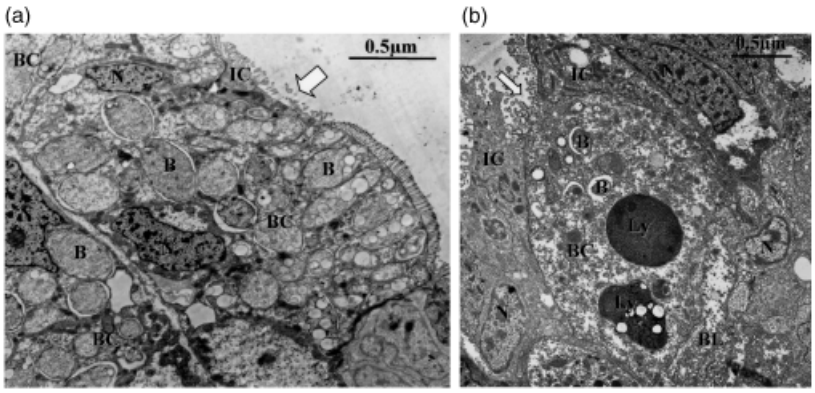

Fig. 4. Ultrastructural modifications in the *Codakia orbicularis* gills during starvation according to ultrathin sections (TEM analysis). (a) Bacteriocytes (BC), which are the most prevalent cells in the gill filament, have a basal nucleus (N) and a rounded apical pole developing a broad contact with pallial seawater (white arrow). The cytoplasm is crowded by envacuolated bacteria (B), which are individually enclosed inside the bacteriocyte vacuole. Intercalary cells (IC), characterized by a trumpet shape and an apical nucleus, are regularly interspersed among bacteriocytes. (b) In 1-month starved specimens, residual bacteriocytes (BC) can be observed with scarce bacteria (B) and large secondary lysosomes (Ly) in their cytoplasm. The larger symbionts have disappeared and only small symbionts, mostly located at the periphery of the cell, can be observed. BL, blood lacuna; IC, intercalary cells; N: nucleus.

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Discussion

Symbiont transmission must constitute the most important factor permitting the persistence of symbiotic associations. Within *Lucinidae* (*Mollusca*: *Bivalvia*), associated with the sulfur-oxidizing gill endosymbiont, previous studies (1) revealed an absence of symbionts in the ovaries, eggs, embryos and larvae of various lucinids, (2) showed that juveniles can be infected with crude sand from *T. testudinum* sea-grass beds and (3) detected the free-living form of the symbiont in these sea-grass beds (Gros *et al.*, 1996, 1998, 1999, 2003). Accordingly, the authors of these studies excluded the possibility of a vertical transmission of symbionts in *Lucinidae* and concluded that transmission was environmental. Nevertheless, environmental transmission being a passage from an infected host (here adults) to a noninfected host (here juveniles) via the environment, it would require a release of the symbiosis-competent bacteria by the adult clams (Fig. 1). In the present study, using CARD-FISH techniques, we attempted to detect the release of thioautotrophic symbionts by the lucinids *C. orbicularis* and *C. orbiculata* and we did not find any evidence of such a release over 1 month of experiment.

No evidence of symbiont release

What hypotheses could explain this result? (1) Our experimental conditions might not be optimal enough to allow symbiont release to occur, although rearing conditions kept the hosts alive in an apparently good condition for at least a month (not a single host died during the whole experiment). (2) The natural release of symbionts could be restricted to a defined period at the end of the larval development after metamorphosis (July–September), to permit a more efficient transmission. The failure to detect a release could then be because our experiment was conducted in April. However, we can infect juveniles with crude sand in the laboratory all the year round and whatever the age of the juvenile (O. Gros, personal observations). These observations suggest that the release of symbionts, if it does take place, could occur efficiently all throughout the year (in sea-grass beds), which makes the 'period-restricted release' hypothesis unlikely. (3) The release took place, but was not detected. Considering the sensitivity of the method we used, this is very unlikely because some batches included several individuals and sampling was conducted 24 h after filtered seawater exchange. Moreover, the 30 days of the experiment provided ample opportunities for release to take place, if it really existed. (4) Release could occur at the death of the host. Nevertheless, a transmission strategy based on the death of the host can be successful only in the case of parasitic microorganisms (Ewald, 1987). Now, sulfur-oxidizing endosymbionts associated with marine invertebrates were not considered as deleterious (Stewart *et al.*, 2005). Finally (5), the symbiont release phenomenon does not exist and consequently the environmental transmission hypothesis proposed by Gros *et al.* (1996) could be invalid.

Evolution of the symbiotic population into the gills

The diminution of the hybridization signal observed on gill sections must be considered because it could reveal a release undetected by CARD-FISH. However, this hypothesis is unlikely because the diminution of the hybridization signal seems large enough to correspond to a release we should have been able to detect. Considering that bivalve gills could harbor *c.* 3×10^9 bacteria g^{-1} of gills, and considering that the symbiont population decreases up to 30% per month (A. Caro *et al.*, unpublished data), it may have a release of 3×10^7 bacteria per clam day^{-1}. Considering our detection limit of 10 bacteria mL^{-1} (i.e. 5000 bacteria per crystallizing dish), this release could not have gone undetected. Alternative explanations for the decrease of the hybridization signal are (1) decreasing metabolic activity due to experimental conditions (related with rRNA copy) or (2) lysosomal degradation of the symbionts into the gills. We favor the latter hypothesis because our TEM observations show a reduced number of bacteria in the bacteriocytes and an increased number of secondary lysosome-like structures (Fig. 4b) similar to those described in the literature (Frenkiel & Mouëza, 1995; Frenkiel *et al.*, 1996; Liberge *et al.*, 2001).

Consequences for symbiotic relationships

Thus, the lack of release-detection in our experimental conditions, although not refuted completely, does not support the environmental transmission mode. However, the absence of vertical transmission, the ability to infect with crude sand, the detection of the free-living form and the absence of symbiont release all agree with the hypothesis that host-to-host transmission does not take place within *Lucinidae*. If this is so, what consequence is there for the symbiotic relationship?

In chemoautotrophic symbioses, it is commonly supposed that both partners benefit from the symbiosis. On the host side, harboring a symbiont is very important considering the clearly established feeding role of bacteria in this type of symbiosis (Berg & Alatalo, 1984; Fisher, 1990). Indeed, symbiont carbon fixation covers an important part of the host's nutritional needs in chemoautotrophic endosymbioses (Stewart *et al.*, 2005). On the symbiont side, gills would constitute a more favorable and more stable environment than seawater or sediments, and would provide a better access to metabolic substrates (e.g. sulfide, CO_2) (Stewart *et al.*, 2005). This pattern leads us to think that the relation is a mutualism. However, in *Lucinidae*, if the symbiont does not multiply in his host (Caro *et al.*, 2007)

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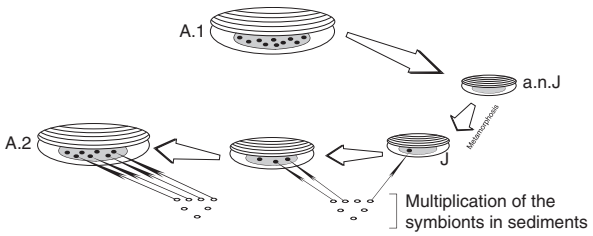

Fig. 5. Schematic representation of the capture of the bacteria. Symbionts acquired by juveniles come from an environmental stock constituted by the multiplication of free-living bacteria in the environment. A.1, adult of the parental generation; A.2, adult of the following generation; a.n.J, aposymbiotic-naive juvenile; J, juvenile; full ellipses, endosymbiotic bacteria; empty ellipses, free-living bacteria.

and if release does not occur (as suggested by our results), juveniles acquire the bacteria only from a free-living form of the symbiont that multiplies only in the surroundings (Fig. 5). This 'capture' enables the host to benefit from the bacteria as described above. On the contrary, for the bacteria enclosed and exploited inside the gills, this relation constitutes an evolutionary dead-end.

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References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microb* **56**: 1919–1925.

Berg CJJ & Alatalo P (1984) Potential of chemosynthesis in molluscan mariculture. *Aquaculture* **39**: 165–179.

Caro A, Gros O, Got P, De Wit R & Troussellier M (2007) Characterization of the population of the sulfur-oxidizing symbiont of *Codakia orbicularis* (*Bivalvia*, *Lucinidae*) by single-cell analyses. *Appl Environ Microb* **73**: 2101–2109.

Cary SC & Giovannoni SJ (1993) Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps. *P Natl Acad Sci USA* **90**: 5695–5699.

Christensen H, Hansen M & Sorensen J (1999) Counting and size classification of active soil bacteria by fluorescence *in situ* hybridization with an rRNA oligonucleotide probe. *Appl Environ Microb* **65**: 1753–1761.

Daims H, Bruhl A, Amann R, Schleifer KH & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.

DeLong EF, Taylor LT, Marsh TL & Preston CM (1999) Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent *in situ* hybridization. *Appl Environ Microb* **65**: 5554–5563.

Deming JW, Reysenbach AL, Macko SA & Smith CR (1997) Evidence for the microbial basis of a chemoautotrophic invertebrate community at a whale fall on the deep seafloor: bone-colonizing bacteria and invertebrate endosymbionts. *Microsc Res Techiq* **37**: 162–170.

Distel DL, Lane DJ, Olsen GJ, Giovannoni SJ, Pace B, Pace NR, Stahl DA & Felbeck H (1988) Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences. *J Bacteriol* **170**: 2506–2510.

Distel DL, Felbeck H & Cavanaugh CM (1994) Evidence for phylogenetic congruence among sulfur-oxidizing chemoautotrophic becterial endosymbiont and their Bivalve hosts. *J Mol Evol* **38**: 533–542.

Dubilier N, Giere O, Distel DL & Cavanaugh CM (1995) Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (*Oligochaeta*: *Annelida*) by phylogenetic 16S rRNA sequence analysis and *in situ* hybridization. *Appl Environ Microb* **61**: 2346–2350.

Duperron S, Laurent MC, Gaill F & Gros O (2008) Sulphur-oxidizing extracellular bacteria in the gills of Mytilidae associated with wood falls. *FEMS Microbiol Ecol* **63**: 338–349.

Durand P & Gros O (1996) Bacterial host specificity of *Lucinacea* endosymbionts: interspecific variation in 16S rRNA sequences. *FEMS Microbiol Lett* **140**: 193–198.

Ewald PW (1987) Transmission modes and evolution of the parasitism-mutualism continuum. *Ann NY Acad Sci* **503**: 295–306.

Felbeck H, Childress JJ & Somero GN (1981) Calvin–Benson cycle and sulphide oxidation enzymes in animals from sulphide-rich habitats. *Nature* **293**: 291–293.

Fisher CR (1990) Chemoautotrophic and methanotrophic symbioses in marine invertebrates. *Rev Aquat Sci* **2**: 399–613.

Frenkiel L & Mouëza M (1995) Gill ultrastructure and symbiotic bacteria in *Codakia orbicularis* (*Bivalvia*: *Lucinidae*). *Zoomorphology* **115**: 51–61.

Frenkiel L, Gros O & Mouëza M (1996) Gill structure in *Lucina pectinata* (*Bivalvia*: *Lucinidae*) with reference to hemoglobin in bivalves with symbiotic sulphur-oxidizing bacteria. *Mar Biol* **125**: 511–524.

Gill JW & Vogel HJ (1962) Lysine synthesis and phylogeny: biochemical evidence for a bacterial-type endosymbiont in the protozoon *Herpetomonas* (*Strigomonas*) *oncopelti*. *Biochim Biophys Acta* **56**: 200–201.

Gros O & Gaill F (2007) Extracellular bacterial association in gills of "wood mussels". *Cah Biol Mar* **48**: 103–109.

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Gros O, Darrasse A, Durand P, Frenkiel L & Mouëza M (1996) Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve *Codakia orbicularis*. *Appl Environ Microb* **62**: 2324–2330.

Gros O, De Wulf-Durand P, Frenkiel L & Mouëza M (1998) Putative environmental transmission of sulfur-oxidizing bacterial symbionts in tropical lucinid bivalves inhabiting various environments. *FEMS Microbiol Lett* **160**: 257–262.

Gros O, Duplessis MR & Felbeck H (1999) Embryonic development and endosymbiont transmission mode in the symbiotic clam *Lucinoma aequizonata* (*Bivalvia*: *Lucinidae*). *Invertebr Reprod Dev* **36**: 93–103.

Gros O, Liberge M, Heddi A, Khatchadourian C & Felbeck H (2003) Detection of the free-living forms of sulfide-oxidizing gill endosymbionts in the lucinid habitat (*Thalassia testudinum* environment). *Appl Environ Microb* **69**: 6264–6267.

Haygood MG & Davidson SK (1997) Small-subunit rRNA genes and *in situ* hybridization with oligonucleotides specific for the bacterial symbionts in the larvae of the bryozoan *Bugula neritina* and proposal of "*Candidatus endobugula sertula*". *Appl Environ Microb* **63**: 4612–4616.

Juniper SK & Sibuet M (1987) Cold seep benthic communities in Japan subduction zones: spatial organization, trophic strategies and evidence for temporal evolution. *Mar Ecol Prog Ser* **40**: 115–126.

Lechaire J-P, Freibourg G, Gaill F & Gros O (2008) *In situ* characterization of sulfur in gill-endosymbionts of the shallow water lucinid *Codakia orbicularis* (Linné, 1758) by cryo-EFTEM microanalysis. *Mar Biol* **154**: 693–700.

Le Pennec M, Diouris M & Herry M (1988) Endocytosis and lysis of bacteria in gill epithelium of *Bathymodiolus thermophilus*, *Thyasira flexuosa*, and *Lucinella divaricata*. (*Bivalvia*: *Mollusca*). *J Shellfish Res* **7**: 483–489.

Liberge M, Gros O & Frenkiel L (2001) Lysosomes and sulfide-oxidizing bodies in the bacteriocytes of *Lucina pectinata*, a cytochemical and microanalysis approach. *Mar Biol* **139**: 401–409.

Manz W, Arp G, Schumann-Kindel G, Szewzyk U & Reitner J (2000) Widefield deconvolution epifluorescence microscopy combined with fluorescence *in situ* hybridization reveals the spatial arrangement of bacteria in sponge tissue. *J Microbiol Meth* **40**: 125–134.

Pernthaler A, Pernthaler J & Amann R (2002) Fluorescence *in situ* hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Environ Microb* **68**: 3094–3101.

Polz MF & Cavanaugh CM (1995) Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site. *P Natl Acad Sci USA* **92**: 7232–7236.

Polz MF, Felbeck H, Novak R, Nebelsick M & Ott JA (1992) Chemoautotrophic sulfur-oxidizing symbiotic bacteria on marine nematodes: morphological and biochemical characterization. *Microbiol Ecol* **24**: 312–329.

Schweimanns M & Felbeck H (1985) Significance of the occurrence of chemoautotrophic bacterial endosymbionts in lucinid clams from Bermuda. *Mar Ecol Prog Ser* **24**: 113–120.

Stewart FJ, Newton IL & Cavanaugh CM (2005) Chemosynthetic endosymbioses: adaptations to oxic–anoxic interfaces. *Trends Microbiol* **13**: 439–448.

Taylor JD & Glover EA (2005) *Lucinidae* (*Bivalvia*) – the most diverse group of chemosymbiotic molluscs. *Zool J Linn Soc* **148**: 421–438.

Williams ST, Taylor JD & Glover EA (2003) Molecular phylogeny of the *Lucinoidea* (*Bivalvia*): non-monophyly and separate acquisition of bacterial chemosymbiosis. *J Mollus Stud* **70**: 187–202.

Zbinden M, Le Bris N, Gaill F & Compere P (2004) Distribution of bacteria and associated minerals in the gill chamber of the vent shrimp *Rimicaris exoculata* and related biogeochemical processes. *Mar Ecol Prog Ser* **284**: 237–251.

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