

Plasticity of symbiont acquisition throughout the life cycle of the shallow-water tropical lucinid *Codakia orbiculata* (Mollusca: Bivalvia)

Olivier Gros,^{1*} Nathalie H. Elisabeth,¹
Sylvie D. D. Gustave,¹ Audrey Caro² and
Nicole Dubilier³

¹UMR-CNRS 7138, Systématique-Adaptation-Evolution, Equipe « Biologie de la mangrove ». Université des Antilles et de la Guyane. UFR des Sciences Exactes et Naturelles, Département de Biologie. B.P. 592. 97159 Pointe-à-Pitre Cedex, Guadeloupe. France.

²UMR-CNRS 5119, Laboratoire Ecosystèmes Lagunaires, Université Montpellier II, 34095 Montpellier cedex 5, France.

³Max Planck Institute for Marine Microbiology, Celsiusstraße 1, 28359 Bremen. Germany.

Summary

In marine invertebrates that acquire their symbionts from the environment, these are generally only taken up during early developmental stages. In the symbiosis between lucinid clams and their intracellular sulfur-oxidizing bacteria, it has been shown that the juveniles acquire their symbionts from an environmental stock of free-living symbiont forms, but it is not known if adult clams are still competent to take up symbiotic bacteria from the environment. In this study, we investigated symbiont acquisition in adult specimens of the lucinid clam *Codakia orbiculata*, using transmission electron microscopy, fluorescence *in situ* hybridization, immunohistochemistry and PCR. We show here that adults that had no detectable symbionts after starvation in aquaria for 6 months, rapidly reacquired symbionts within days after being returned to their natural environments in the field. Control specimens that were starved and then exposed to seawater aquaria with sulfide did not reacquire symbionts. This indicates that the reacquisition of symbionts in the starved clams returned to the field was not caused by high division rates of a small pool of remaining symbionts that we were not able to detect with the methods used here. Immuno-

histochemistry with an antibody against actin, a protein involved in the phagocytosis of intracellular bacteria, showed that actin was expressed at the apical ends of the gill cells that took up symbionts, providing further evidence that the symbionts were acquired from the environment. Interestingly, actin expression was also observed in symbiont-containing cells of untreated lucinids freshly collected from the environment, indicating that symbiont acquisition from the environment occurs continuously in these clams throughout their lifetime.

Introduction

Chemosynthetic symbioses between sulfur-oxidizing bacteria and marine invertebrates have been described worldwide in a wide range of habitats from shallow water environments such as mangroves and sea grass beds to deep-sea hydrothermal vents and cold seeps (Dubilier *et al.*, 2008). The transmission of the symbiotic bacteria from one generation to the next plays a key role in the development and evolution of these associations, but only a few model systems have been studied in depth (Bright and Bulgheresi, 2010; Vrijenhoek, 2010). Two types of transmission modes have been described in chemosynthetic symbioses. Horizontal transmission of symbionts in which the bacteria are acquired from the environment was first shown in the tropical lucinid clam *Codakia orbicularis* (Gros *et al.*, 1996; 1997) and appears to be common in other lucinid host species as well (Gros *et al.*, 1998; 1999). Acquisition of symbionts from the environment has also been shown in the hydrothermal vent tube worm *Riftia pachyptila* (Nussbaumer *et al.*, 2006) and has been suggested to occur in deep-sea bathymodiolin mussels from vents and seeps (Won *et al.*, 2003). Vertical transmission of symbionts in which the bacteria are passed from one generation to the next through the gametes has been described in clams belonging to the deep-sea vent and seep vesicomyids [with rare cases of horizontal transmission (Stewart *et al.*, 2008)] and the shallow-water solemyids (Cary and Giovannoni, 1993; Cary, 1994; Krueger *et al.*, 1996).

In marine invertebrates that are obligately associated with horizontally transmitted endosymbionts, these are

Received 15 April, 2011; revised 12 March, 2012; accepted 18 March, 2012. *For correspondence. E-mail olivier.gros@univ-ag.fr; Tel. (+590) 590 48 30 06; Fax (+590) 590 48 32 81.

always taken up during early stages of their life cycle, as the hosts depend on these for their growth and health. There are two different ways in which the symbionts can mature with their hosts after being acquired: (i) they can be acquired during a short developmental stage early in the host's life and then multiply within the symbiont-containing cells or organs as shown in the vestimentiferan *R. pachyptila* (Nussbaumer *et al.*, 2006), or (ii) they can be continuously acquired throughout the life cycle of the host. A combination of these two is also possible, that is symbionts are continuously taken up but are also able to multiply within the host. Remarkably little is known about the acquisition of endosymbionts in adult animals, as most studies have focused on symbiont uptake during early developmental stages and it is generally assumed that infection is limited to the larval or juvenile life phase of most animals (Bright and Bulgheresi, 2010). In symbioses from chemosynthetic environments, only two studies have provided some indications for symbiont uptake in adult hosts. In aquarium experiments with bathymodiolin mussels, Kádár and colleagues (2005) showed that these might be able to take up symbionts from bacteria released by co-occurring mussels in the aquarium. In the bone-eating worm *Osedax mucofloris* from shallow-water whale falls, Verna and colleagues (2010) hypothesized that repeated infection events occur while the worms grow into the whale bones. However, to date there is no evidence that aposymbiotic (symbiont-free) adult hosts have the competence to take up their bacterial symbionts from their natural environment and the processes involved in adult symbiont acquisition have not been examined in detail. In this study we examined adult symbiont acquisition in the tropical shallow-water clam *Codakia orbiculata* from sea-grass sediments in the Caribbean. As most other lucinids, this clam harbours intracellular sulfur-oxidizing bacteria, and its symbionts are identical at the 16S rRNA level to the symbionts of *C. orbicularis* (Durand and Gros, 1996; Durand *et al.*, 1996). The free-living form of the *C. orbicularis* endosymbiont was previously detected in the sea-grass beds by PCR and fluorescence *in situ* hybridization (FISH) with symbiont-specific primers and probes (Gros *et al.*, 2003) as well as in the surrounding waters (O. Gros, pers. obs.). This indicates that these hosts have continuous access to their symbionts. To examine if *C. orbiculata* maintains its ability to acquire symbionts throughout its life cycle, we collected adult specimens from the field and deprived these of sulfide until symbionts were no longer detectable in their gill tissues. We returned these clams to the sea-grass sediments from which they were collected and used transmission electron microscopy (TEM), CARD-FISH, PCR, BrdU (5-bromo-2-deoxyuridin) labelling and immunohistochemistry to analyse their gills 2–15 days after replacing them in their natural environment.

Results and discussion

The gill structure of *C. orbiculata* freshly collected from *Thalassia testudinum* sea-grass sediments (T_0 individuals) was similar to that of *C. orbicularis* (Frenkiel and Mouëza, 1995) based on analyses of sections (Fig. 1) and CARD-FISH with a probe specific to the *C. orbiculata* symbiont. The lateral zone of each gill filament consisted of two main cell types (Figs 1 and 2A): the bacteriocyte cells filled with the intracellular bacterial symbionts (Figs 1, 2A and D, and 4A) and granule-containing cells without endosymbionts (Figs 1, 2A and 4A). The large bacteriocytes (up to 30 μm in length) were characterized by a rounded apical end in contact with the circulating sea-water and a cytoplasm filled with vacuoles containing single symbionts (Fig. 2D) as observed in other symbiotic lucinids (Frenkiel and Mouëza, 1995; Frenkiel *et al.*, 1996; Gros *et al.*, 1996; 2000; Brissac *et al.*, 2011).

The loss of symbionts was induced by keeping clams for 6 months in aquaria without sulfide, the energy source used by their symbionts to fix inorganic carbon. After 3 months, the gills of starved individuals contained markedly fewer symbionts (Fig. 2B and E). CARD-FISH signals after 3 months were much lower than in freshly collected clams confirming the clear decrease in intracellular bacteria per bacteriocyte in starved individuals (Fig. 6A and B). We assume that symbiont decrease was not achieved through the release of intracellular symbionts to the environment, as Brissac and colleagues (2009) were unable to detect symbionts in the aquaria of *C. orbiculata* starved for 30 days. It is therefore most likely that the symbionts were digested intracellularly. Numerous large lysosomes were observed in gills of starved *C. orbiculata* (Fig. 2E and F), an observation also described from starved *Bathymodiolus azoricus* (Kádár *et al.*, 2008).

After 6 months of starvation, no bacteria could be detected in the gills by TEM and CARD-FISH (Figs 2F and 4C) as well as by PCR analyses (Table 1) using the *C. orbicularis* symbiont specific primer (Gros *et al.*, 1996). TEM analyses showed that symbiont-containing cells were no longer present in the lateral zone (Figs 2C and 4C). Only two types of cells were observed: granule-containing cells (Fig. 2C) and small 'empty' cells of up to 12 μm without bacterial symbionts (Fig. 2F). The latter possessed numerous secondary lysosome-like structures and mitochondria in their cytoplasm (Fig. 2F). The morphology of these cells was highly similar to gill bacteriocytes in individuals starved for 3 months that contained a few endosymbionts, indicating that the 'empty' cells in 6 month starved individuals were empty bacteriocytes. This conclusion is further supported by their similarity to undifferentiated cells in the gills of aposymbiotic juveniles of *C. orbicularis* (Gros *et al.*, 1996; 1998). The cellular

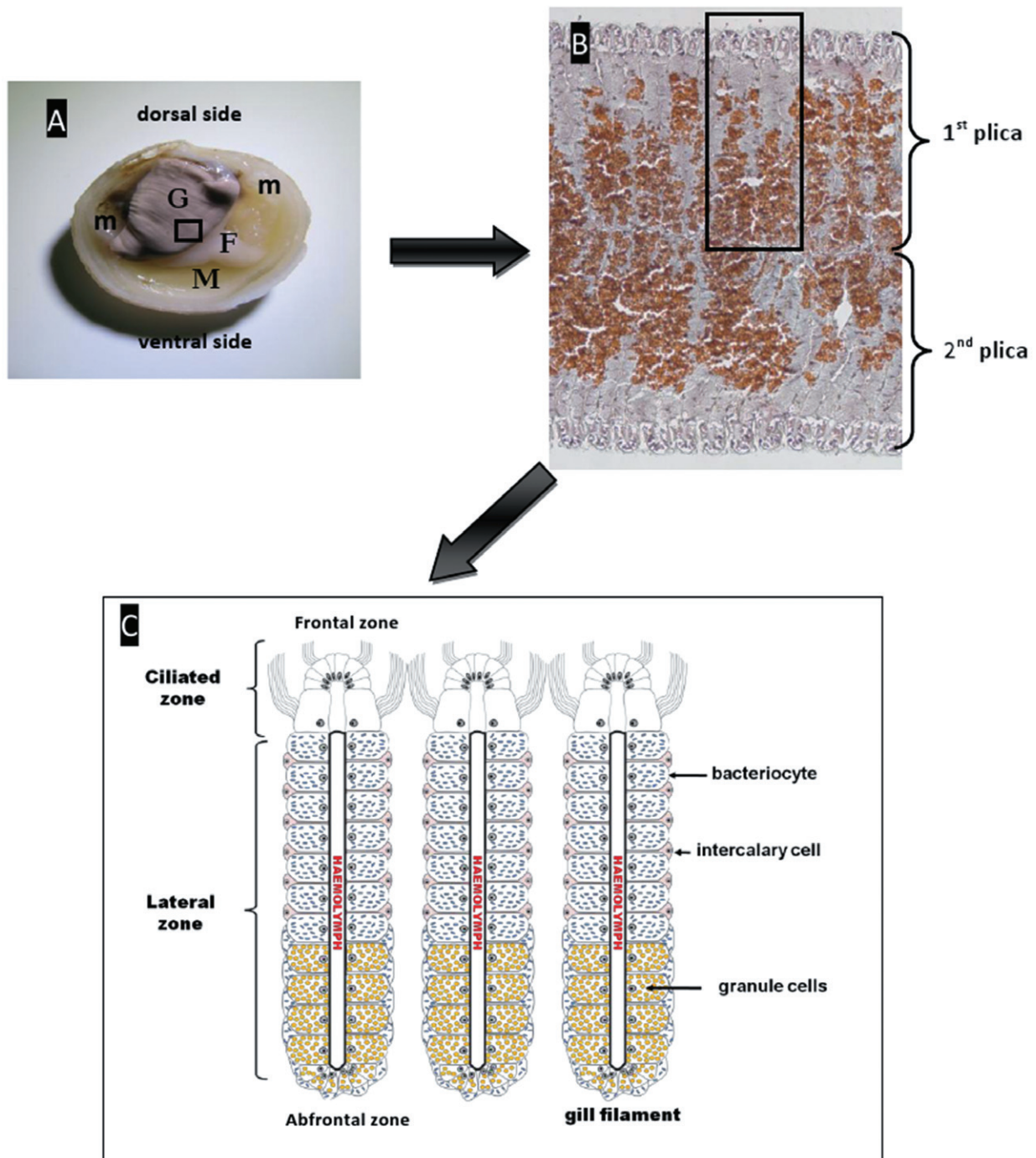


Fig. 1. Diagram of the symbiont-bearing gill tissues in a freshly collected *Codakia orbiculata* adult individual. A. Clam with left shell removed. The gill (G) tissues are greatly enlarged compared with bivalves without symbionts. F, foot; M, mantle; m, adductor muscle. B. Transverse section through the gill showing the two plicae, each consisting of multiple gill filaments. C. Diagram of three gill filaments from the 1st plica. Each filament consists of a ciliated zone that never contains bacterial symbionts and a lateral zone that constitutes the main part of the filament and accounts for the thickness of the gill. This lateral zone consists of epithelial cells organized around a blood lacuna containing the haemolymph. Three cell types occur in the epithelium: (i) The dominant cell type is the symbiont-bearing bacteriocyte (BC) characterized by a large cytoplasmic volume filled with conspicuous inclusions, the bacterial symbionts. (ii) Intercalary cells (stars) are interspersed between the bacteriocytes and are characterized by a trumpet shape with an apical nucleus. These two cells are mainly found in the outer part of the gills (called the frontal zone), while the inner part of the gills (called the abfrontal zone) mainly consists of granule cells (GC) that contain large membrane-bound inclusions in their cytoplasm. These granular cells never harbour bacteria.

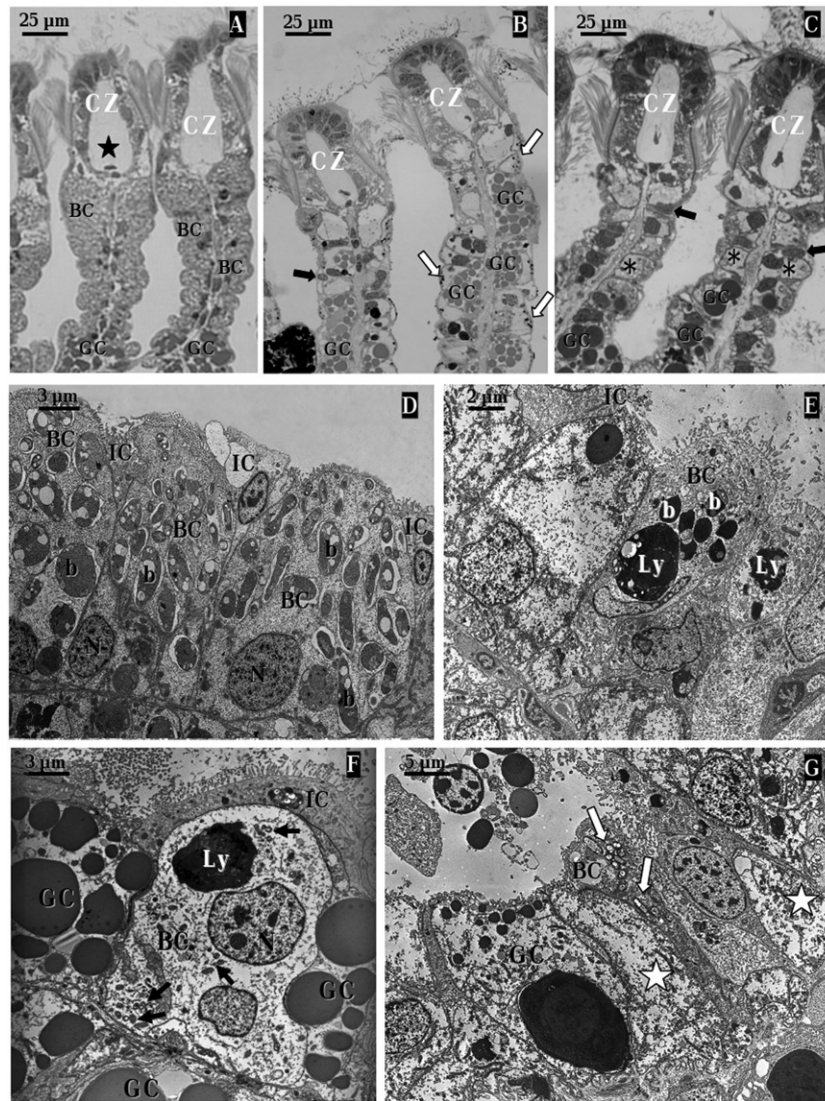


Fig. 2. Ultrastructural analyses of loss and reacquisition of intracellular symbionts in *C. orbiculata* gills. (A–C: light micrographs; D–G: TEM micrographs).

A. Each gill filament from a freshly collected adult individual is characterized by (i) a ciliated zone (CZ) devoid of bacterial symbionts that is organized along a collagen axis (star) and by (ii) the lateral zone dominated by large bacteriocytes (BC) filled with symbiotic bacteria, and granule-containing cells (GC) further away from the ciliated zone.

B. Gill filaments from an adult individual after 3 months of starvation. While the morphology of the ciliated zone (CZ) is similar to that of freshly collected specimens, the lateral zone is strongly modified. Bacteriocytes are much smaller and contain only a few bacteria (visible as black dots) mainly at their apical poles (white arrows). Granule-containing cells (GC) are now the most prevalent cell type in the lateral zone. Intercalary cells, characterized by their apical nucleus, are indicated by black arrows.

C. Gill filaments from an adult individual after 6 months of starvation. Bacteriocytes are no longer present and the lateral zone is dominated by large granule-containing cells (GC) and a few undifferentiated cells with rounded apical poles that are most likely 'empty' bacteriocytes (asterisks). Intercalary cells, characterized by their apical nucleus, are indicated by black arrows.

D. Gills of a freshly collected *C. orbiculata* individual are mainly composed of bacteriocytes (BC) that have a basal nucleus (N) in contact with the blood lacuna of the filament axis. These bacteriocytes possess a rounded apical pole with a broad contact to the sea-water filtered through the gill tissues. The cytoplasm is filled with vacuolated bacteria (b) that are usually individually enclosed. Intercalary cells (IC), characterized by a trumpet shape and an apical nucleus, are regularly interspersed among bacteriocytes.

E. After 3 months of starvation, the bacteriocytes (BC) contain only a few small intracellular bacteria (b) at the cell periphery, and larger sized symbionts are no longer present. Bacteriocytes now contain large lysosomes (Ly) presumably involved in the digestion of symbionts and are flanked by undifferentiated cells without symbionts that are most likely 'empty' bacteriocytes.

F. In a *C. orbiculata* specimen starved for 6 months, only a few residual bacteriocytes (BC) remain in the lateral zone and are characterized by an empty cytoplasm containing a few organelles such as mitochondria (small arrows), a nucleus (N), and large lysosomes (Ly). These cells correspond to the empty cells marked with an asterisk in Fig. 2C. GC, granule-containing cell; IC, intercalary cell.

G. In gill filaments from a *C. orbiculata* specimen starved for 6 months and returned to the field for four days, a few intracellular bacteria (arrows) are located at the apical poles of bacteriocytes (BC). Most of the cells in the lateral zone are still devoid of bacteria (empty stars). Granule cells (GC) contain less granules than those of starved individuals.

Table 1. Experimental re-infection of starved aposymbiotic *C. orbiculata* adults under controlled conditions in the lab and in *Thalassia testudinum* sea grass bed.

	Starved individuals (no food, no sulfur added)		Starved individuals amended with food and w/o sulfides		Starved individuals amended in sulfides and w/o food		Starved individuals amended in Na ₂ S and food		Starved individuals put back in their natural environment	
	PCR	CARD-FISH	PCR	CARD-FISH	PCR	CARD-FISH	PCR	CARD-FISH	PCR	CARD-FISH
6 months (E ₀)	-	-	-	-	-	-	-	-	-	-
E ₀ + 2d	-	-	-	-	-	-	-	-	-	-
E ₀ + 4d	-	-	-	-	-	-	-	-	-	-
E ₀ + 8d	-	-	-	-	-	-	-	-	-	-
E ₀ + 21d	-	-	-	-	-	-	-	-	-	-

w, weak signal.

organization of gill filaments in 6 month starved individuals differed clearly from freshly collected clams: in the starved clams granule-containing cells dominated the lateral zone (Fig. 2C). The colour of the gills progressively changed from creamy beige in freshly collected individuals to dark brown in 6 month-starved individuals. This colour change was most likely caused by the loss of elemental sulfur granules in the symbionts (Johnson and Fernandez, 2001; Lechaire *et al.*, 2008) as well as the loss of symbionts themselves.

During starvation, animals lost weight and the relative weight of gills, compared with whole tissues, decreased progressively (Fig. 3). Although lucinids can supplement their nutrition through filter-feeding, no food was added to the aquaria, so that the intracellular digestion of symbionts was the only source of nutrition during starvation.

Our morphological and molecular analyses support the conclusion that we were able to induce complete or nearly complete loss of symbionts in adult *C. orbiculata* by starving their symbionts of their energy source. While decreases in symbiont abundance have been shown in other chemosynthetic bivalves such as thyasirids (Dufour and Felbeck, 2006), vesicomysids (Goffredi *et al.*, 2004) and bathymodiolins (Kádár *et al.*, 2005) and more recently in the lucinid *C. orbicularis* (Caro *et al.*, 2007; 2009), experimental inducement of complete or nearly complete symbiont loss in adult hosts has only been described in the deep-sea hydrothermal vent mussel *B. azoricus* and in this study.

To examine if 6 month starved *C. orbiculata* adults were still able to take up symbionts from the environment, we returned the clams to their original collection site in sea-grass sediments. No bacteria were observed in individuals returned to the field for 2 days using FISH and TEM (Table 1). After 4 days, the gills of reintroduced clams clearly contained endosymbionts (Fig. 4D–F) while the gills of control individuals kept for up to 21 days in 0.2 µm filtered sea-water, or in 0.2 µm filtered sea-water amended with algae, Na₂S, or both algae and Na₂S did not (Table 1). There are two explanations for the presence of symbionts in individuals returned to their natural habitat: (i) the improvement of environmental conditions, such as access to reduced sulfur compounds provided symbionts that remained undetected in starved adults (due to detection limits of the methods used here) with the right conditions needed for rapid multiplication within their hosts. However, our control experiments in which starved adults were supplied with sulfide and no symbionts were found, even after 21 days, makes this explanation very unlikely. (ii) The more likely explanation is therefore, that the lucinid adults were able to reacquire symbionts from the environment by taking up competent free-living symbionts from the *T. testudinum* sediments. Our results presented below provide additional support for this conclusion.

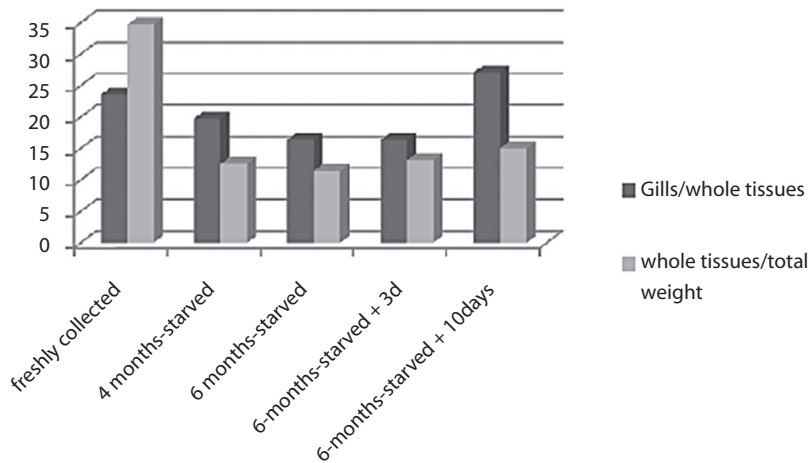


Fig. 3. Evolution of relative weight of (i) gills compared with the whole tissues (visceral mass + gills + muscles + mantle + foot) and (ii) whole tissues compared with total weight (whole tissues + shells) of *C. orbiculata* adults during starvation and after reintroduction to the *Thalassia testudinum* sea-grass bed. Three individuals were selected at random for each value presented.

In individuals returned to the field for 4 days, small numbers of envacuolated symbionts were observed in the apical regions of some bacteriocytes where the cells are exposed to the seawater circulating through the clam (Figs 2G and 4D). This suggests that the symbiotic bacteria were taken up by phagocytosis at the apical regions of the undifferentiated host cells and that these host cells progressively differentiated into bacteriocytes. In the same time, intercalary cells have always an apical nucleus and a trumpet shape with a narrow basal pole and they never acquire or maintain symbionts. The longer individuals were left in the seagrass beds, the higher the abundance of symbionts, with a clear distribution pattern of symbionts in the gill tissues observed over time (Fig. 4D–F). After 8 days in the field, bacteriocytes close to the ciliated zone where the seawater circulates were well filled with symbionts (Figs 4E and 6C). After 15 days, bacteriocytes further away from the ciliated zone also contained symbionts and their distribution resembled that observed in gills of freshly collected clams (Fig. 4F). The size of bacteriocytes as well as that of the symbionts increased over time until bacteriocytes did not differ morphologically from those of freshly collected specimens (Fig. 6A and C). At this stage, the macroscopic appearance of the gills returned to the normal condition observed in symbiotic host with a thick beige appearance and the relative weight of gills compared with the whole tissues was even higher than that of freshly collected clams (Fig. 3). This infection process is highly similar to that described in juveniles of *C. orbicularis* (Gros *et al.*, 1996; 1998).

To examine if massive intracellular multiplication of endosymbionts that were not detected in individuals starved for 6 months (instead of uptake of symbiont from the environment) could have led to bacteriocyte recolonization, we incubated the specimens returned to the field in BrdU to detect bacterial DNA synthesis. *Escherichia coli* cultures used as positive controls were clearly labelled

with this technique (Fig. 5A and B), as were host cells, particularly in the bridge zones of gill tissues (Fig. 5D), showing that BrdU labelling was possible with the histological methods used in this study. In contrast, no BrdU uptake was observed in the intracellular symbionts of clams returned to the field for 4, 8 and 15 days (Fig. 5C) despite their clear increase in the gill tissues based on CARD FISH (Fig. 4E and F). Dividing bacteria were not observed with CARD-FISH or TEM. This is consistent with previous studies in which only very few bacterial symbionts in divisional stages were observed in lucinids (Frenkiel and Mouéza, 1995; Gros *et al.*, 2000; Brissac *et al.*, 2011).

This could be interpreted as support for the conclusion that the massive increase in symbiont cell numbers in hosts returned to the field could not have been caused by their growth within host cells. However, it is also possible that the *C. orbiculata* symbionts are not able to take up BrdU, as known from other bacteria including chemosynthetic symbionts (Pflugfelder *et al.*, 2009). We therefore used an antiserum against F-actin to examine if this protein is expressed in clams returned to the field, as F-actin filaments are involved in the phagocytosis of intracellular bacteria (Rottner *et al.*, 2005). Our immunohistochemistry analyses showed that F-actin was expressed during the colonization process at the apical poles of most symbiont-containing cells throughout the lateral zone (Fig. 5F) while no actin could be detected at the apical poles of the bacteriocytes from starved individuals (not shown). The lack of actin expression at the apical poles of gill filaments in starved individuals suggests that 'empty bacteriocytes' only mobilize actin when exposed to the free-living symbiotic bacteria from the environment. We therefore conclude that symbiosis-competent bacteria from the environment enter by phagocytosis at the apical poles of bacteriocytes in a process similar to that described in juveniles of *C. orbicularis* (Gros *et al.*, 1996). Interestingly, F-actin was also expressed in some bacte-

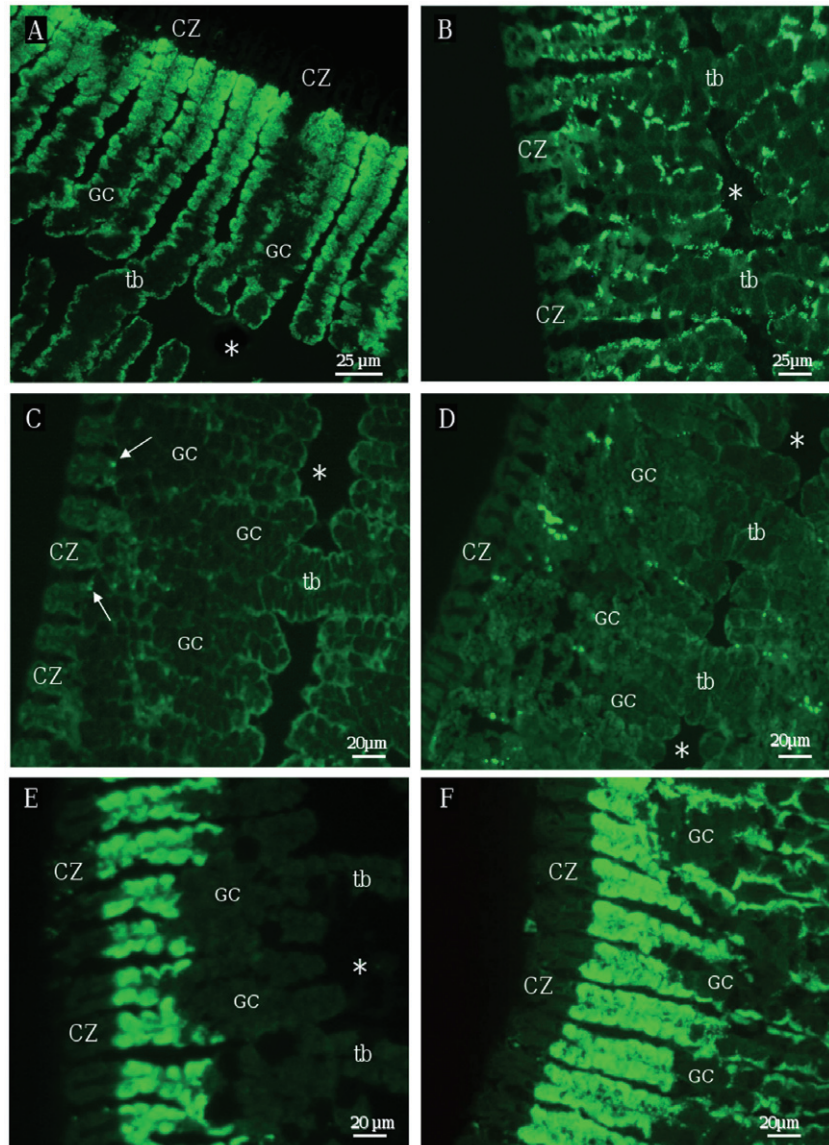


Fig. 4. CARD-FISH analyses with the symbiont-specific probe Symco2 of the loss and reacquisition of intracellular symbionts in *C. orbiculata* gills.

A. In a freshly collected *C. orbiculata* adult, bacteriocytes (visible in green due to the positive CARD-FISH signal from the endosymbionts) dominate the frontal zone of gill filaments and are filled with bacterial endosymbionts. Due to the lack of symbionts, no signal is present in the ciliated zone (CZ) and in the granule-containing cells (GC), which are mostly located in the abfrontal zone of the gill filament. Asterisk, blood lacuna; tb, tissue bridge characteristic of Eulamellibranchia bivalves.

B. In an individual starved for 3 months the number of bacterial endosymbionts (green hybridization signal) is greatly decreased and each bacteriocyte contains much less symbionts. Dark cells without green hybridization signals correspond to the granular cells.

C. In an individual starved for 6 months, no hybridization signal was visible throughout the entire gill tissues indicating that these were devoid of endosymbionts. Some background autofluorescence, identified by the lack of corresponding DAPI signals from bacteria, was observed (small arrow). The lateral zone mostly contained granule cells (GC) that appear dark due to the lack of symbiont hybridization signal.

D. In an individual starved for 6 months and returned to the field for 4 days, bacterial symbionts are now clearly present (green dots) although only in low abundance and most cells in the lateral zone are devoid of intracellular bacteria.

E. In an individual starved for 6 months and returned to the field for 8 days, the number of bacteriocytes (green due to symbiont hybridization signals) with intracellular symbionts has greatly increased. These are predominantly located below the ciliated zone (CZ) and the abfrontal part of the lateral zone is free of bacterial symbionts and only occupied by granule cells (GC).

F. 15 days after reintroduction to the field, a 6 month starved individual harbours numerous bacterial symbionts as visible in the strong hybridization signals (in green) throughout the lateral zone with the exception of the granular cells (GC) that are symbiont-free. The gills resemble those of freshly collected individuals (shown in Figs 3A and 5A).

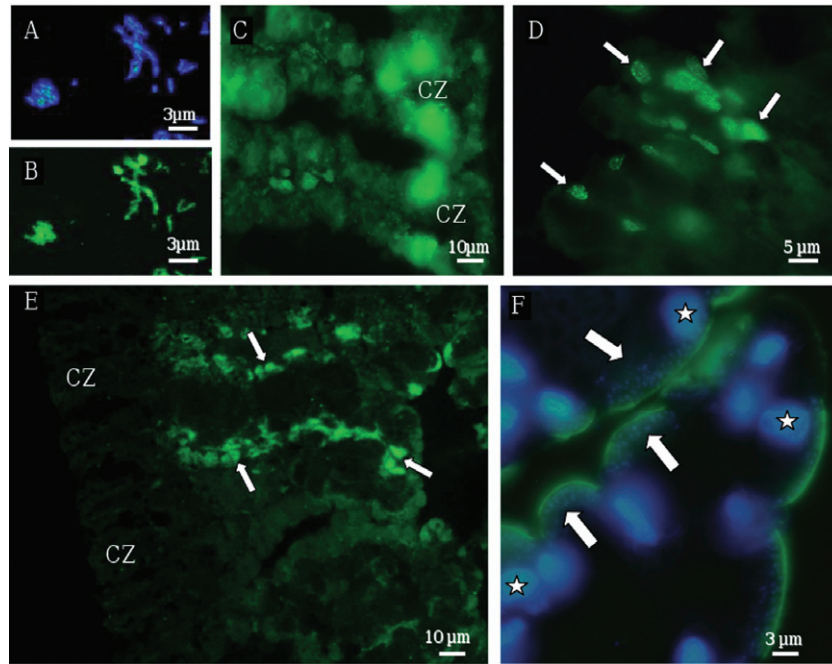


Fig. 5. Light micrographs showing cell proliferation and actin expression.

A and B. Log phase culture of *Escherichia coli*. Nearly all bacteria are labelled by the anti-BrdU antibody, indicating BrdU incorporation and thus, cell growth (B). (A) Same image as in (B) showing DAPI staining in blue.

C. In a specimen starved for 6 months and incubated in BrdU, no labelling with an anti-BrdU antiserum was observed.

D. Cell proliferation in gill filaments of an individual starved for 5 months and returned to the field for 4 days (2 h incubation in BrdU).

Numerous nuclei (arrows) from cells in the tissue bridge are positively labelled indicating replication of the host cells in this region of the gill filament.

E. Immunohistochemical analyses of actin expression in an untreated adult individual freshly collected from the field. Actin is expressed at the apical poles of only a few bacteriocytes (white arrows) suggesting that symbiosis-competent bacteria enter by phagocytotic processes under natural conditions. Other cell types in the gills are not positively hybridized indicating that actin expression is restricted to bacteriocytes.

F. Immunohistochemical analyses of actin expression in an individual starved for 6 months and returned to the field for 8 days. Actin is expressed at the apical poles of bacteriocytes suggesting that symbiosis-competent bacteria from the environment enter by phagocytotic processes involving actin mobilization. Bacterial symbionts (arrows) and host nuclei (stars) are stained with DAPI (shown in blue). Asterisk, blood lacuna; CZ, ciliated zone devoid of bacterial symbionts; tb, tissue bridge characteristic of Eulamellibranchia bivalves.

riocytes of untreated, freshly collected *C. orbiculata* clams (but not in other gill cells) (Fig. 5E). This suggests that symbiont uptake occurs continuously in adult hosts under natural conditions. If the actin expression observed in bacteriocytes had been caused by cytoskeleton activity, then we would have expected to see such expression more regularly distributed throughout the cells and also in starved individuals. Instead, we only observed actin expression in the apical poles of bacteriocytes from clams returned to the field and therefore we conclude that this expression is involved in phagocytosis of symbionts from the environment.

Gill cells of experimentally starved *C. orbiculata* adults were able to take up symbiosis-competent bacteria from the environment after as much as 10 months of starvation (N.H. Elisabeth, pers. obs.). Furthermore, symbiont acquisition by starved adults was successful for more than 15 independent assays between 2005 and 2010 (O. Gros, unpubl. data) at different times of the year showing that

symbionts can be taken up throughout the year independently of seasons.

Thus in *C. orbiculata*, and presumably other lucinids, the environmental acquisition of endosymbionts does not appear to be limited to a short period during post-larval development as in other chemosynthetic hosts such as hydrothermal vent tube worms (Nussbaumer *et al.*, 2006) but can occur continuously throughout the lucinid's adult lifetime. This would provide these hosts with considerable flexibility in their natural environment. They could use their symbionts as a food source by digesting them when sulfide becomes too limiting to provide sufficient energy for carbon fixation and reacquire fresh symbionts when sulfide concentrations become more favourable. It is also possible that these two strategies coexist in lucinids, so that symbiont digestion and uptake from the environment are continuous parallel processes in adult hosts. Population genetic studies of endosymbionts from hosts collected from a wide range of environmental conditions are

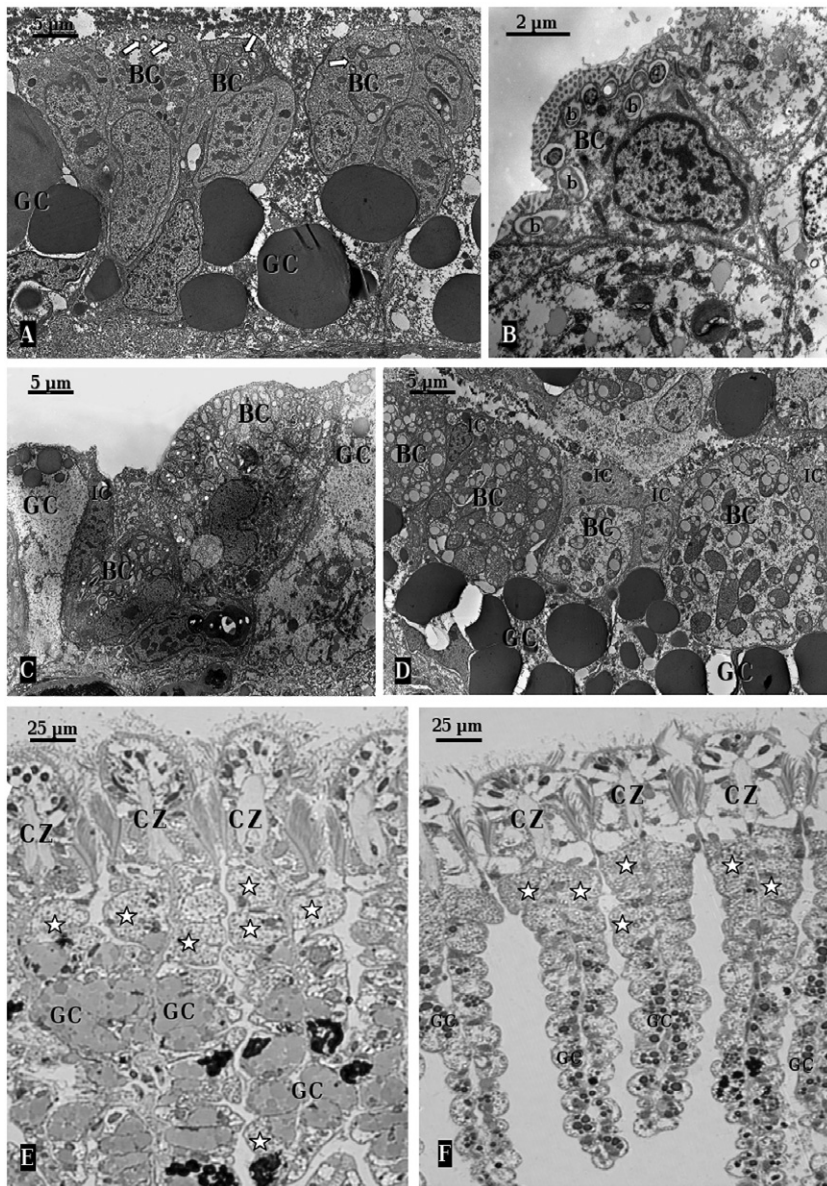


Fig. 6. Ultrastructural analyses of endosymbiont acquisition in adults starved for 6 months and then returned to the field. A–D TEM micrographs, E–F light micrographs.

A. Starved *C. orbiculata* specimen returned to the field for 4 days. The lateral zone contains numerous bacteriocytes (BC), each populated by a few bacteria (arrows). Granule-containing cells (GC), are still present in considerable numbers in the lateral zone.

B. Higher magnification focusing on a bacteriocyte (BC) in a starved specimen returned to the field for 4 days. The distribution of the newly acquired bacterial symbionts (b) is typical with these mostly located at the apical poles of the bacteriocyte suggesting a phagocytosis-like process from the environment. The symbionts are small (~1 µm) and larger symbionts commonly present in gills from freshly collected *C. orbiculata* individuals (e.g. Fig. 2D) were not observed.

C. Through the lateral zone of a starved individual returned to the field for 8 days, the bacteriocytes (BC) are more similar to those of freshly collected specimens with a rounded apical pole in broad contact with the circulating sea-water. The cytoplasm is filled with envacuolated bacteria (b) with mostly only a single symbiont in each vacuole.

D. The bacteriocytes (BC) of a starved individual returned to the field for 15 days now dominate the lateral zone and are highly similar to those of freshly collected specimens, with the cytoplasm filled with both small and large bacterial symbionts. Intercalary cells (IC), characterized by a trumpet shape and an apical nucleus, are regularly interspersed among the newly differentiated bacteriocytes.

E. In the gill filaments from a starved individual returned to the field for 8 days, numerous bacteriocytes (stars) with bacteria are present close to the ciliated zone (CZ) of the gill filaments.

F. The lateral zone of gill filaments from a starved individual returned to the field for 15 days now appears highly similar to that of freshly collected specimens (Fig. 2A) with large bacteriocytes (stars) full of intracellular bacteria dominating the frontal zone of gill filaments.

needed to better understand the contribution of continuous symbiont acquisition to the life cycles of natural host populations.

Experimental procedures

Sampling site

Adult *C. orbiculata* (Montagu, 1802) individuals of 15–20 mm shell length were collected by hand from *T. testudinum* sea-grass sediments in the 'Ilet Cochon' in Guadeloupe, French West Indies, Caribbean. A total of 15 starvation experiments were conducted throughout the year with clams collected

during all seasons. After starvation (see below), individuals were returned to their original collection site for symbiont recolonization experiments.

Aposymbiosis inducement

A total of seventy freshly collected *C. orbiculata* were kept in 50 l plastic containers with 0.22 µm filtered seawater at 25°C for up to 6 months. The seawater was kept oxygenated with an aquarium pump and renewed twice a week to avoid accumulation of ammonium released by the clams. Every month, three clams were randomly selected and their gills fixed and embedded as described below under CARD-FISH.

CARD (catalysed reporter deposition) FISH

Gills were fixed for 1–3 h at 4°C in 2% paraformaldehyde in 0.2 µm filtered sea water. The specimens were then rinsed three times for 10 min each at room temperature (RT) in 0.2 µm filtered seawater and dehydrated in an ascending ethanol series and stored at –20°C until embedding in paraffin. Four µm thick sections were placed on precoated slides from Sigma before hybridization. Paraffin was removed before hybridization with toluene, and sections were rehydrated in a decreasing ethanol series. FISH and CARD-FISH were performed as described previously (Pernthaler *et al.*, 2001; 2002). The *C. orbicularis*-symbiont specific probe Symco2 (5'-TACAGAGGGTCGCCAACCCGTG-3') (Gros *et al.*, 1996) was used to examine symbiont loss and uptake, and labelled with fluorescein for FISH and with horseradish peroxidase for CARD-FISH. Bacterial cell membranes were permeabilized 10 min using HCl (0.2 M at RT) followed by 10 min in Tris-HCl (20 mM at RT) then proteinase K (0.5 µg ml⁻¹, 5 min at 37°C), and finally endogenous peroxidases were inhibited with HCl (0.01 M at RT) for 12 min. Hybridizations were performed using 50% formamide, and signals were amplified using a buffer containing carboxyfluorescein (FITC). Positive controls were done on gill sections obtained from freshly collected *C. orbiculata* individuals using the universal bacterial probe EUB 338 (Sekar *et al.*, 2004) while negative controls were done using the probe NON338 (Wallner *et al.*, 1993) to check for false positive signals after hybridization. Slides were mounted with cytomation fluorescent mounting medium (DAKO, France) and visualized under an epifluorescence microscope Eclipse 80i (Nikon, France). Three clam individuals per sampling time point were analysed (monthly samples from the starvation incubations and 0, 2, 4, 8 and 15 days from the reinfection experiments described below). Two FISH or CARD-FISH analyses per clam individual were conducted on gill sections separated by at least 250–300 µm, with a mean of 30 gill filaments analysed per section. The FISH micrographs presented here are representative of all of the clams examined.

Transmission electron microscopy (TEM)

Gill pieces from each individual examined with FISH, i.e. three individuals per sampling time point were prefixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M pH 7.2 cacodylate buffer adjusted to 900 mOsm with NaCl and CaCl₂ to improve membrane preservation. After a brief rinse, tissues were stored in the same buffer at 4°C. Gills were dissected, fixed for 45 min at RT in 1% osmium tetroxide in the same buffer, then rinsed in distilled water and post-fixed with 2% aqueous uranyl acetate for 1 h before embedding and observation as described (Gros *et al.*, 1998). Two grids containing 4–5 ultrathin sections were observed per individual; the corresponding semi-thin sections were also examined for overview analyses. The TEM micrographs presented in this study are representative of all of the clams examined.

Experimental reinfection of starved *C. orbiculata* adults

Adult clams had no detectable symbionts after starvation for 6 months (see *Results*, Table 1). The 6 month starved indi-

viduals were used for the following 2 week reinfection experiments: (i) 30 individuals were returned to their original collection site and randomly collected by hand for each sampling time. Before returning the clams, their valves were painted blue with acrylic paint (Humbrol) to recognize them among the naturally occurring bivalves at the site. For each reinfection sampling time (0, 2, 4, 8 and 15 days), clams were collected at random for BrdU incubations (5 individuals per sampling time) and immunohistochemical analyses of actin (1 per sampling time). For controls, four batches containing five clams each were kept in the laboratory for 21 days in (ii) 0.22 µm filtered seawater, in (iii) 0.22 µm filtered seawater amended every 2 days with sulfide (final concentration of 50 µM Na₂S), (iv) in 0.22 µm filtered seawater amended daily with algae as food (a mix of *Nannochloropsis* sp. and *Isochrysis tahiti* at 1000 cells ml⁻¹) and (v) in 0.22 µm filtered seawater amended with both sulfide and algae.

BrdU incubations

To identify proliferating active cells, sections of gill tissues were prepared as described above for CARD-FISH and incubated with 5-bromo-2-deoxyuridin (BrdU), a synthetic nucleoside that is an analogue of thymidin. BrdU is incorporated into the newly synthesized DNA of replicating cells substituting for thymidin during DNA replication. A culture of *E. coli*, a Gram-negative bacterium, was used as a positive control. It was grown and continuously shaken in Tripcase Soy Broth (TSB) medium at 37°C for 3 h until log phase. Then, 200 µg ml⁻¹ of BrdU (Sigma) was added to the culture for 2 additional hours under the same conditions. Bacterial cells were fixed in 2% paraformaldehyde (final concentration) in PBS for 3 h at 4°C, centrifuged 2 min at 8000 g, the supernatant discarded and the bacterial pellet resuspended in 1 × PBS (phosphate-buffered saline) and stored at 4°C before use. Clams from the reinfection experiments were incubated with BrdU (Sigma) at 200 µg ml⁻¹ for 2 h (4 individuals per sampling time), and a fifth was kept in 0.2 µm filtered seawater as a negative control. Gills were fixed for 3 h in 2% paraformaldehyde at 4°C and treated as described below.

BrdU and actin detection

Gills for actin analyses were fixed in 4% paraformaldehyde for 24 h at 4°C. The gill tissues fixed for BrdU and actin analyses were dehydrated, embedded, sectioned, de-waxed and rehydrated as described above for CARD-FISH. Gill sections and smear slides from the *E. coli* cultures were incubated for 2 h at RT in solution A [0.1 M PBS solution with 2% bovine serum albumin (BSA) and 0.3% Triton X-100]. Then, slides were incubated in the primary antibody (anti-BrdU monoclonal antibody from Dako at 650 mg l⁻¹ or anti-F-actin antibody from Stressgen), diluted 1:500 in solution A for 2 h at RT. After rinsing twice in 1 × PBS for 10 min, slides were incubated in a secondary antibody (HRP-conjugated goat anti-mouse IgG from AnaSpec for BrdU or HRP-conjugated goat anti-rabbit IgG from Stressgen), and diluted 1:200 in solution A for 1 h at RT. Slides were rinsed again in 1 × PBS and incubated in amplification buffer with FITC. Sections were covered with a solution containing Fluorepre and 4'6-

diamino-phenylindole dihydrochloride (DAPI) and observed with an Eclipse 80i Nikon microscope.

PCR experiments

DNA was extracted from gill tissue pieces of lucinid specimens using a standard chloroform-isoamyl alcohol extraction protocol, and genes encoding bacterial 16S rRNA were amplified using the *C. orbicularis*-specific primer set (Symco1-1492r) during 30 PCR cycles as described previously (Gros et al., 1996).

References

- Bright, M., and Bulgheresi, S. (2010) A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* **8**: 218–230.
- Brissac, T., Gros, O., and Merçot, H. (2009) Lack of endosymbiont release by two Lucinidae (Bivalvia) of the genus *Codakia*: consequences for symbiotic relationships. *FEMS Microbiol Ecol* **67**: 261–267.
- Brissac, T., Mercot, H., and Gros, O. (2011) Lucinidae/sulfur-oxidizing bacteria: ancestral heritage or opportunist association? Further insights from Bohol Sea (the Philippines). *FEMS Microbiol Ecol* **75**: 63–76.
- Caro, A., Gros, O., Got, P., De Wit, R., and Troussellier, M. (2007) Characterization of the population of the sulfur-oxidizing symbiont of *Codakia orbicularis* (Bivalvia, Lucinidae) by single-cell analyses. *Appl Environ Microbiol* **73**: 2101–2109.
- Caro, A., Got, P., Bouvy, M., Troussellier, M., and Gros, O. (2009) Effects of long-term starvation on a host bivalve (*Codakia orbicularis*, lucinidae) and its symbiont population. *Appl Environ Microbiol* **75**: 3304–3313.
- Cary, S.C. (1994) Vertical transmission of a chemoautotrophic symbiont in the protobranch bivalve, *Solemya reidi*. *Mol Mar Biol Biotechnol* **3**: 121–130.
- Cary, S.C., and Giovannoni, S.J. (1993) Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps. *Proc Natl Acad Sci USA* **90**: 5695–5699.
- Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6**: 725–740.
- Dufour, S.C., and Felbeck, H. (2006) Symbiont abundance in thyasirids (Bivalvia) is related to particulate food and sulphide availability. *Mar Ecol Prog Ser* **320**: 185–194.
- Durand, P., and Gros, O. (1996) Bacterial host specificity of Lucinacea endosymbionts: interspecific variation in 16S rRNA sequences. *FEMS Microbiol Lett* **140**: 193–198.
- Durand, P., Gros, O., Frenkiel, L., and Prieur, D. (1996) Phylogenetic characterization of sulfur-oxidizing bacterial endosymbionts in three tropical Lucinidae by 16S rDNA sequence analysis. *Mol Mar Biol Biotechnol* **5**: 37–42.
- Frenkiel, L., and Mouëza, M. (1995) Gill ultrastructure and symbiotic bacteria in *Codakia orbicularis* (Bivalvia, Lucinidae). *Zoomorphology* **115**: 51–61.
- Frenkiel, L., Gros, O., and Mouëza, M. (1996) Gill ultrastructure in *Lucina pectinata* (Bivalvia: Lucinidae) with reference to hemoglobin in bivalves with symbiotic sulphur-oxidizing bacteria. *Mar Biol* **125**: 511–524.
- Goffredi, S.K., Barry, J.P., and Buck, K.R. (2004) Vesicomid symbioses from Monterey Bay (Central California) cold seeps. *Symbiosis* **36**: 1–27.
- Gros, O., Darrasse, A., Durand, P., Frenkiel, L., and Mouëza, M. (1996) Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve *Codakia orbicularis*. *Appl Environ Microbiol* **62**: 2324–2330.
- Gros, O., Frenkiel, L., and Mouëza, M. (1997) Embryonic, larval, and post-larval development in the symbiotic clam *Codakia orbicularis* (Bivalvia: Lucinidae). *Invertebr Biol* **116**: 86–101.
- Gros, O., Frenkiel, L., and Mouëza, M. (1998) Gill filament differentiation and experimental colonization by symbiotic bacteria in aposymbiotic juveniles of *Codakia orbicularis* (Bivalvia: Lucinidae). *Invertebr Reprod Dev* **34**: 219–231.
- Gros, O., Duplessis, M.R., and 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., Frenkiel, L., and Felbeck, H. (2000) Sulfur-oxidizing endosymbiosis in *Divaricella quadrisulcata* (Bivalvia: Lucinidae): morphological, ultrastructural, and phylogenetic analysis. *Symbiosis* **29**: 293–317.
- Gros, O., Liberge, M., Heddi, A., Khatchadourian, C., and Felbeck, H. (2003) Detection of the free-living forms of sulfide-oxidizing gill endosymbionts in the lucinid habitat (*Thalassia testudinum* Environment). *Appl Environ Microbiol* **69**: 6264–6267.
- Johnson, M.A., and Fernandez, C. (2001) Bacterial symbiosis in *Loripes lucinalis* (Mollusca: Bivalvia) with comments on reproductive strategy. *J Mar Biol Assoc UK* **81**: 251–257.
- Kádár, E., Bettencourt, R., Costa, V., Santos, R.S., Lobo-da-Cunha, A., and Dando, P. (2005) Experimentally induced endosymbiont loss and re-acquirement in the hydrothermal vent bivalve *Bathymodiolus azoricus*. *J Exp Mar Biol Ecol* **318**: 99–110.
- Kádár, E., Davis, S.A., and Lobo-da-Cunha, A. (2008) Cytoenzymatic investigation of intracellular digestion in the symbiont-bearing hydrothermal bivalve *Bathymodiolus azoricus*. *Mar Biol* **153**: 995–1004.
- Krueger, D.M., Gustafson, R.G., and Cavanaugh, C.M. (1996) Vertical transmission of chemoautotrophic symbionts in the bivalve *Solemya velum* (Bivalvia: Protobranchia). *Biol Bull* **190**: 195–202.
- Lechère, J.-P., Frébourg, G., Gaill, F., and Gros, O. (2008) *In situ* characterization of sulphur in gill-endosymbionts of the shallow water lucinid *Codakia orbicularis* (Linné, 1758) by high-pressure cryofixation and EFTEM microanalysis. *Mar Biol* **154**: 693–700.
- Nussbaumer, A.D., Fisher, C.R., and Bright, M. (2006) Horizontal endosymbiont transmission in hydrothermal vent tubeworms. *Nature* **441**: 345–348.
- Pernthaler, A., Pernthaler, J., and Amann, R. (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Environ Microbiol* **68**: 3094–3101.
- Pernthaler, J., Glöckner, F.O., Schönhuber, W., and Amann,

- R. (2001) Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes. *Method Microbiol* **30**: 208–226.
- Pflugfelder, B., Cary, S.C., and Bright, M. (2009) Dynamics of cell proliferation and apoptosis reflect different life strategies in hydrothermal vent and cold seep vestimentiferan tubeworms. *Cell Tissue Res* **337**: 149–165.
- Rottner, K., Stradal, T.E.B., and Wehland, J. (2005) Bacteria-host-cell interactions at the plasma membrane: stories on actin cytoskeleton subversion. *Dev Cell* **9**: 3–17.
- Sekar, R., Fuchs, B.M., Amann, R., and Pernthaler, J. (2004) Flow sorting of marine bacterioplankton after Fluorescence *in situ* Hybridization. *Appl Environ Microbiol* **70**: 6210–6219.
- Stewart, F.J., Young, C.R., and Cavanaugh, C.M. (2008) Lateral symbiont acquisition in a maternally transmitted chemosynthetic clam endosymbiosis. *Mol Biol Evol* **25**: 673–687.
- Verna, C., Ramette, A., Wiklund, H., Dahlgren, T.G., Glover, A.G., Gaill, F., and Dubilier, N. (2010) High symbiont diversity in the bone-eating worm *Osedax mucofloris* from shallow whale-falls in the North Atlantic. *Environ Microbiol* **12**: 2355–2370.
- Vrijenhoek, R.C. (2010) Genetic diversity and connectivity of deep-sea hydrothermal vent metapopulations. *Mol Ecol* **19**: 4391–4411.
- Wallner, G., Amann, R., and Beisker, W. (1993) Optimizing fluorescent *in situ* hybridization of suspended cells with rRNA-targeted oligonucleotide probes for the flow cytometric identification of microorganisms. *Cytometry* **14**: 136–143.
- Won, Y.J., Hallam, S.J., O'Mullan, G.D., Pan, I.L., Buck, K.R., and Vrijenhoek, R.C. (2003) Environmental acquisition of thiotrophic endosymbionts by deep-sea mussels of the genus *Bathymodiolus*. *Appl Environ Microbiol* **69**: 6785–6792.