Marine Biology (2003) 142: 57–66
DOI 10.1007/s00227-002-0921-7

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Interspecific infection of aposymbiotic juveniles of *Codakia orbicularis* by various tropical lucinid gill-endosymbionts

Received: 8 April 2002 / Accepted: 22 July 2002 / Published online: 16 October 2002

Abstract Previous molecular phylogenetic analyses have shown that five tropical lucinid species living in or near *Thalassia testudinum* seagrass beds are colonized by the same bacterial symbiont species. In addition, a new lucinid species belonging to the genus *Anodontia*, which inhabits reducing sediment found near seagrass beds and in mangrove swamps, has been included in the present study. Endosymbiosis in *Anodontia alba* was examined according to symbiont phylogenetic and gill ultrastructural analysis. Phylogenetic analysis showed that partial 16S rDNA sequences of *A. alba*– and *Codakia orbicularis*–symbionts were 100% identical at all nucleotide positions determined, suggesting that *A. alba* also harbors the same symbiont species as *C. orbicularis* (and, consequently, as *C. orbiculata*, *C. pectinella*, *Linga pensylvanica* and *Divaricella quadrisulcata*). Based on light and electron microscopy, the cellular organization of the gill filament appeared similar to those already described in other lucinids. The most distinctive feature is the lack of "granule cells" in the lateral zone of *A. alba* gill filaments. In order to confirm the single-species hypothesis, purified fractions of gill bacterial symbionts obtained from the gills of each of the six tropical lucinids cited above were used to infect aposymbiotic juveniles of *C. orbicularis*. In each case, aposymbiotic juvenile batches were successfully infected by the gill-endosymbiont fractions, whereas, during the experiments, juveniles from the negative control were still uninfected. These experimental data confirm the phylogenetic data and also demonstrate that chemoautotrophic bacterial endosymbionts from their host cells can colonize aposymbiotic juveniles. The conclusion also follows that intracellular gill-endosymbionts still have the capacity to recognize and colonize new host generations. Lucinids provide a unique model for the study of sulfide-oxidizing symbiosis, even if symbionts remain unculturable.

Communicated by S.A. Poulet, Roscoff

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Introduction

Endosymbioses between sulfide-oxidizing bacteria and marine invertebrates, first described in deep-sea hydrothermal vent fauna (Cavanaugh et al. 1981; Felbeck et al. 1981), are distributed across a broad range of host taxa in marine environments (Fenchel and Finlay 1989; Fisher 1990; Reid 1990; Felbeck and Distel 1992; Cavanaugh 1994; Polz et al. 1994). The greatest diversity of these intracellular thioautotrophic symbioses is found among the Bivalvia; in this class members of five families, i.e. Lucinidae, Mactridae, Solemyidae, Thyasiridae and Vesicomyidae (Southward 1986; Fisher 1990; Reid 1990; Distel et al. 1994), harbor gill-endosymbionts.

In the family Lucinidae, all species examined to date, despite inhabiting different environments, harbor sulfide-oxidizing symbiotic bacteria inside bacteriocytes that are regularly distributed throughout the lateral zone of their gill filaments (for review see Gros 1997). As these sulfide-oxidizing symbionts have not been cultured, comparisons of rRNA gene sequences have been particularly useful in their characterization; such sequences can be obtained without isolating the bacterial symbiont. All of the thioautotrophic symbionts examined to date are unambiguously confined to the gamma subdivision of the Proteobacteria. Symbionts of the bivalve superfamily Lucinacea form a distinct monophyletic lineage specifically associated with the host family (Distel et al. 1994). However, the same symbiont 16S rDNA sequence was

recently found in five tropical lucinids inhabiting the same low-sulfide environment (*Thalassia testudinum* seagrass beds): *Codakia orbicularis*, *C. orbiculata*, *C. pectinella*, *Linga pensylvanica* (Durand and Gros 1996; Durand et al. 1996) and *Divaricella quadrisulcata* (Gros et al. 2000). These observations strongly suggest that these five host species harbor the same bacterial species in their gill filaments. Moreover, the symbiont transmission mode has been demonstrated to be environmental in *C. orbicularis*, according to molecular data and through experimental infection studies using aposymbiotic juveniles (Gros et al. 1996a, 1998a,b). The same transmission mode has been strongly suggested in the other tropical lucinids, because symbiont-specific DNA cannot be amplified by PCR (polymerase chain reaction) from mature gonads of adult specimens (Gros et al. 1998a, 2000). Thus, all of the previously described lucinids, including *Lucina pectinata* and *Lucinoma aequizonata* (Gros et al. 1998a, 1999), have been hypothesized to acquire their symbionts through free-living forms of gill-endosymbionts from the environment.

In this investigation, we identified and characterized, using 16S rDNA (DNA encoding rRNA) sequencing, the bacterial endosymbiont of *Anondontia alba* collected in Guadeloupe (French West Indies). In addition, we studied the cellular organization of gill filaments of this newly examined lucinid and compared various ultrastructural features with those of other tropical lucinid gill filaments previously described. Finally, in order to confirm the phylogenetic data and check the recognizing specificity of the lucinid host for its symbiont, we placed aposymbiotic juveniles of *C. orbicularis* in contact with purified gill-endosymbiont fractions from five other lucinid species known to harbor the same bacterial symbiont species.

Materials and methods

Organisms

Specimens of *Anodontia alba* (Link, 1807), identified according to Warmke and Abbott (1962) and Abbott (1974), were collected in Guadeloupe from the reduced part of shallow-water seagrass beds. They were maintained in 5-μm-filtered seawater until fixation and DNA extraction, which were performed within 48 h.

Aposymbiotic juveniles of *Codakia orbicularis* were obtained as previously described (Gros et al. 1997). After metamorphosis, juveniles were kept in 30-l tanks supplied with a UV sterilizer (Rena UV Compact 9 W) in order to limit bacterial and protist growth. Seawater exchange was limited to 10–20% once a week.

DNA extraction, PCR amplification and sequencing

Nucleic acids from symbiont-containing gill and symbiont-free foot tissues (negative control) were independently extracted from two specimens as described elsewhere (Durand and Gros 1996; Durand et al. 1996). 16S rDNA sequences were determined directly from PCR amplification products. PCR reaction mixtures included 1× reaction buffer (Promega, France), dNTPs at 0.25 mM, 2 mM $MgCl_2$, 0.2 μg each of universal bacterial 16S primers 27f and 1492r (Lane 1991), 0.1 μg of template DNA and 2.5 U of *Taq* polymerase (Promega) in a total volume of 50 μl. Initial denaturation at 95°C for 4 min was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, followed by a 7-min final extension at 72°C. Negative controls using water and foot DNA were always run as a precaution against laboratory-derived contamination. PCR products were examined by horizontal electrophoresis in 0.8% agarose gel.

PCR products of three independent amplifications were pooled for each sequencing reaction in order to reduce nucleotide misincorporation errors introduced by *Taq* polymerase, then separated from primers and unincorporated dNTPs using a QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer's protocol. PCR products were directly sequenced by using only the original PCR primers (27f, 1492r) and the *Taq* DyeDeoxy terminator cycle sequencing method (ABI Prism sequencer, Perkin-Elmer, USA), according to the manufacturer's instructions. Sequencing reaction products were electrophoretically separated using a Perkin-Elmer ABI 373A DNA sequencer.

Phylogenetic analysis

Sequences were manually aligned with published sequences from previously described thioautotrophic symbionts by using the ae2 sequence editor (Larsen et al. 1993). Regions of ambiguous or uncertain alignment were eliminated from consideration. A total of 686 nucleotide positions were utilized in this analysis. Phylogenetic analyses were performed as described previously (Durand and Gros 1996; Durand et al. 1996). Phylogenetic analyses were performed using the following programs, contained in the PHYLIP 3.5 package (Felsenstein 1989).

Evolutionary distances were estimated by using DNADIST with Jukes and Cantor corrections. Phylogenetic trees were constructed using NEIGHBOR. Maximum parsimony analysis was performed using DNAPARS. Bootstrap values based on analysis of 100 trees were calculated using the programs SEQBOOT and CONSENSE. Bootstrap values $>50\%$ are given, but are considered to support the grouping of organisms in an associated node only at values $>75\%$ (Zharkikh and Li 1992).

Purified bacterial fractions

Purified bacterial fractions were prepared from gills of six tropical lucinids: *A. alba*, *C. orbicularis*, *C. orbiculata*, *C. pectinella*, *Linga pensylvanica* and *Divaricella quadrisulcata*.

Small pieces of gills (approximately 0.5 g) were dissected, washed with sterilized seawater and crushed using a pestle homogenizer in an Eppendorf tube. Subsequently, 1 ml of sterile seawater was added, the crushed tissue was filtered through 26 μm nylon mesh to remove large tissular pieces, and the suspension was centrifuged at 100 *g* for 1 min. The supernatant was then centrifuged at room temperature at 700 *g* for 2 min. The final pellet was resuspended in sterile seawater for use in experimental infections.

To check the possibility of infection by the free-living symbiotic form present on the surface of the gills, all the bacteria located outside the gills were killed before the purification of gill-endosymbionts. Under aseptic conditions, small pieces of delaminated gills were washed four times in sterilized seawater, then placed in sterilized seawater containing 100 ppm NaClO for 15 min. The pieces were then rewashed three times in sterilized seawater in order to remove NaClO, which could kill the endosymbionts when released after crushing. Then, 1 ml of the last washing bath was used as inoculum in Marine Broth culture medium (DIFCO Laboratories, Mich., USA) and incubated at 37°C for 4 days. Pieces of gills were then crushed in sterile conditions, and the protocol described above was applied to obtain the purified bacterial fraction.

Experimental infection of aposymbiotic juveniles by the purified bacterial fraction from lucinid species

A total of 15 to 20 aposymbiotic juveniles (0.5–1.3 mm) were placed in 1 l glass dishes filled with sterile seawater and containing

a 2 mm thick layer of sterile sand (< 200 μm grain diameter) as substrate. To each experimental batch, 1 ml of the purified bacterial gill-endosymbiont fraction was added. Two weeks later, a second inoculation of the purified bacterial fraction was added to each batch. Juveniles were kept in contact with the purified bacterial gill-endosymbionts during 1 month without exchange of water.

Meanwhile, 15–20 aposymbiotic juveniles were kept in a 1 l glass dish filled with sterile seawater and containing sterile sand during experimentation, as negative controls. Simultaneously, aposymbiotic juveniles were placed in crude sand (collected from seagrass beds of *Thalassia testudinum*) for the same period as positive controls (Gros et al. 1996a, 1998b), in order to check if juveniles can be infected.

All the juveniles were sampled 1 month after the first addition of the purified symbiont fraction and prepared for TEM analysis.

Transmission electron microscopy preparation

Juveniles were prefixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2, adjusted to 900 mosmol l^{-1} with NaCl and $CaCl_2$) to improve membrane preservation. After a brief rinse, they were fixed for 45 min at room temperature in 1% osmium tetroxide in the same buffer, then rinsed in distilled water and postfixed with 2% aqueous uranyl acetate for one more hour (Hayat 1970).

Specimens were then rinsed in distilled water and decalcified overnight in 1% aqueous ascorbic acid at room temperature. Juveniles were subsequently dehydrated through a graded ethanol series and embedded in Epon-Araldite according to Mollenhauer (in Glauert 1975). Sections were cut using an Ultracut E Reichert ultramicrotome; semi-thin sections (500 nm thick) were stained with 0.5% toluidine blue in 1% borax and thin sections (60 nm thick) were contrasted 30 min in 2% aqueous uranyl acetate and 10 min in 0.1% lead citrate (Reynolds 1963) before examination in a TEM Hitachi H-8000 at 100 kV.

Scanning electron microscopy preparation

Both intact and delaminated gill tissue were fixed in 2.5% glutaraldehyde in seawater for 24 h at room temperature. After a rinse in 0.1 M cacodylate buffer (pH 7.2, adjusted to 1000 mosmol l^{-1} by NaCl), gill pieces were dehydrated in a graded acetone series and critical point dried using CO_2 as transitional fluid. Then, they were sputter coated with gold before observation in a SEM Hitachi S-2500 at 20 kV.

Results

Gill filament structure

The beige, homorhabdic, single demibranchs of *Anodontia alba* possess direct and reflected lamellae linked by lacunar, tissular bridges delimiting a large interlamellar space. Each gill filament is composed of three distinct zones: (1) a short, ciliated zone (very similar to those previously described in other lucinids); (2) a relatively long, intermediary zone composed of large, electron-lucent cells containing numerous mitochondria; and (3) the lateral zone, which is responsible for the thickness of the gill filament (Fig. 1A). This lateral zone is organized as a simple epithelium composed of three cell types: (1) mucocytes, which are mostly located in the abfrontal part; (2) intercalary cells, characterized by their trumpet shape and a nucleus in an apical position; and (3) bacteriocytes, representing the most abundant cell type of the lateral zone, which always appear to be filled with bacterial symbionts (Fig. 1C, D). No "granule cells" as described in *Codakia orbicularis* (Frenkiel and Mouëza 1995) were observed in the four specimens examined either by light or electron microscopy.

SEM observations show that intercalary cells encroach upon the apical area of the adjacent bacteriocytes, restricting their contact with pallial seawater (Fig. 1B).

Bacteria are usually individually enclosed, or are sometimes seen to be dividing within vacuoles, which are probably produced by the host. These endosymbionts are usually rod-shaped, with the characteristic double membrane of gram-negative bacteria. They are large (3–4.5 μm) and variously shaped, probably due to the section orientation. The bacterial cytoplasm contains numerous, slightly polygonal, glycogen-like particles, which are strongly positive to the periodic acid–Schiff reaction (data not shown), and numerous sulfur granules located in the periplasmic space, which appear refractive in phase contrast. Depending on the individual examined, the periplasmic granules usually reported as "empty vesicles" can also appear as electron-dense, membrane-bound vesicles (Fig. 1D).

Symbiont phylogeny

PCR amplifications performed with universal eubacterial 16S rDNA primers on each gill tissue sample produced DNA fragments of the expected size (about 1500 bp, as determined by agarose gel electrophoresis). No amplification products were obtained with foot DNA as template. Direct sequence analysis indicated that the gill PCR products contained a single detectable sequence. Each of the symbiont 16S rDNA samples was then sequenced independently. A total of 686 nucleotides were sequenced from the *A. alba* symbiont, corresponding to positions 8–338 and 1126–1510 of the *Escherichia coli* nomenclature (Brosius et al. 1981). In each case, 16S rDNA sequences from the symbionts of the two specimens were identical at all positions determined, indicating that the symbiont population in this newly examined tropical lucinid is composed entirely, or at least predominantly, of a single symbiont species.

Sequence analysis of the 16S rDNA of the *A. alba* symbiont showed it to be identical at all 686 nucleotide positions determined to the previously examined *C. orbicularis*–symbiont and, consequently, to *C. orbiculata*–, *C. pectinella*–, *Linga pensylvanica*–, and *Divaricella quadrisulcata*–symbionts (Fig. 2). Thus, it is highly probable that these symbionts represent a single bacterial species (Fox et al. 1992).

Experimental infection of aposymbiotic juveniles by the purified bacterial fraction from lucinid species

The gill filaments of aposymbiotic juveniles are composed of ciliated and intermediary zones similar to those

previously described (Gros et al. 1997, 1998b). The lateral zone, which appears free of intracellular bacteria (Fig. 3A), is composed of four cell types: mucocytes, "granule cells" with osmiophilic inclusions, intercalary cells that encroach upon the apical area of adjacent cells, and undifferentiated cells characterized by a basal nucleus and a cytoplasmic volume containing few organelles, such as mitochondria. The undifferentiated cells represent the putative bacteriocytes.

The fact that the positive control was infected by symbiosis-competent bacteria from the sediment demonstrates that the aposymbiotic juveniles used in this experiment were able to initiate symbiosis and, consequently, were susceptible to being infected. All the aposymbiotic juveniles placed in contact with the purified gill-endosymbiont fractions were successfully infected after 1 month of exposure. Thus, aposymbiotic juveniles of *C. orbicularis* were infected by gill-endosymbionts prepared from six lucinid species: *A. alba*, *C. orbicularis*, *C. orbiculata*, *C. pectinella*, *Linga pensylvanica* and *Divaricella quadrisulcata* (Fig. 3B–D). Most of the juveniles revealed more endosymbiotic bacteria in the lateral zone of their gill filaments than the positive control placed in contact with crude sand collected from seagrass beds. During the same time, juveniles from the negative control were still aposymbiotic, indicating that no contamination occurred in the laboratory during the experiments.

The culture media inoculated with 1 ml of the last washing bath following the treatment of the gill surface by NaClO were negative after 4 days of incubation at 37°C. The juveniles placed in contact with such fractions

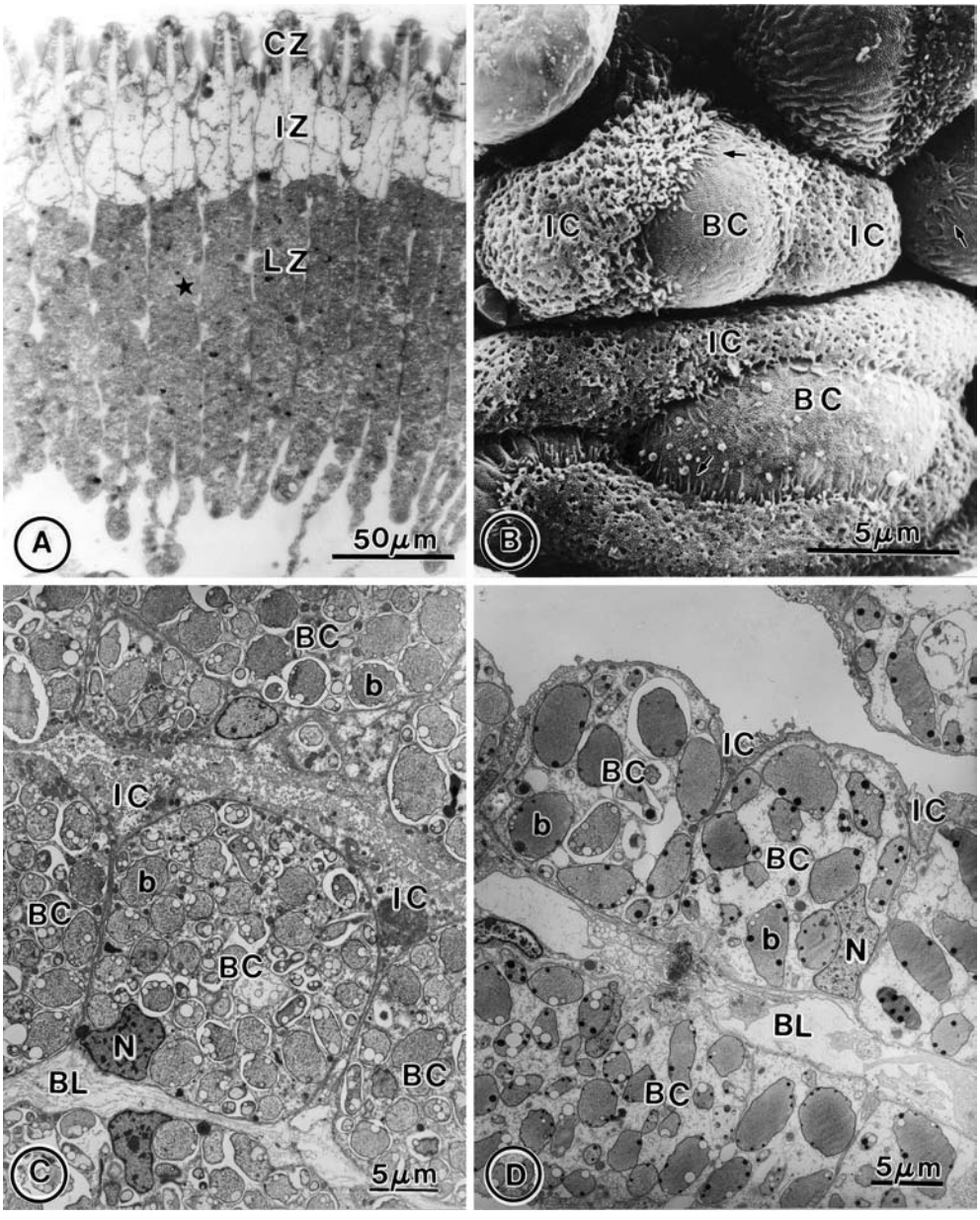

Fig. 1A–D *Anodontia alba*. Structure of the gill. **A** Semithin transverse section of the outer lamina. Each gill filament is composed of a ciliated zone (*CZ*), a relatively long intermediary zone (*IZ*) and a lateral zone (*LZ*), which contains bacteriocytes (*star*). Light microscopy: **B** intercalary cells (*IC*) expand over the apical pole of bacteriocytes (*BC*) by marginal expansions (*arrows*), limiting their contact with pallial seawater. SEM: **C, D** bacteriocytes possess a basal nucleus (*N*) near the blood lacuna (*BL*) of the gill filament axis. Intercalary cells are characterized by a trumpet-shaped extension over the apical pole of the bacteriocytes. Bacterial symbionts (*b*) occupy the main part of the cytoplasmic volume; they are mostly individually enclosed and possess numerous periplasmic sulfur granules, which are usually electron lucent (in C), but can appear as osmiophilic granules (in D) depending on the individual analyzed

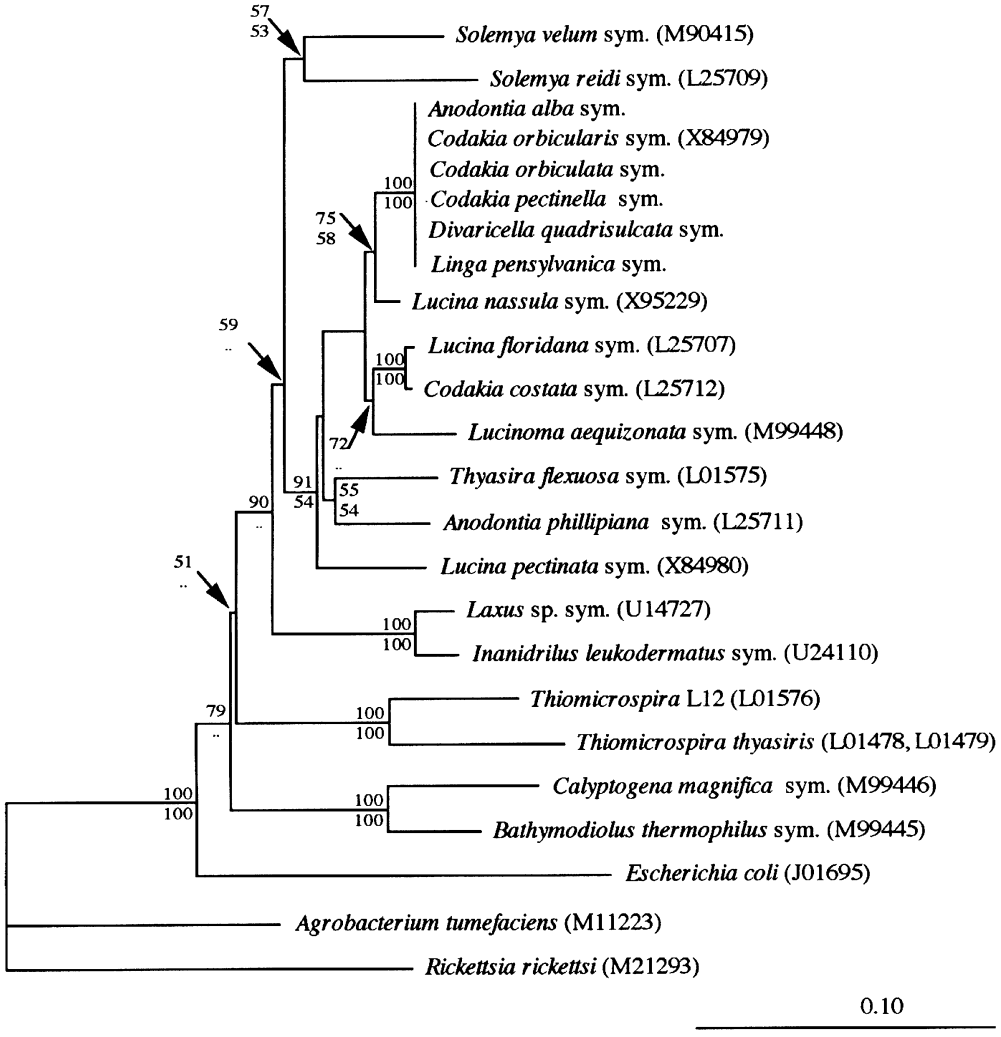

Fig. 2 Phylogenic tree (based on partial 16S rDNA sequences) from lucinid endosymbiotic bacteria, and selected symbiotic and free-living representatives of the class Proteobacteria. Bootstrap values for selected nodes that are supported in > 50 of 100 trees by distance analysis (*upper numbers*) and parsimony analysis (*lower numbers*) are shown. *Scale bar*: 0.1 nucleotide substitutions per sequence position

were positively infected, indicating that the infections observed in the juveniles are due to the gill-endosymbionts rather than environmental bacteria present on the gill surface.

Discussion

Gill ultrastructure and phylogeny

The gill filament organization in *Anodontia alba* is very similar to that previously described in various lucinids (Giere 1985; Distel and Felbeck 1987; Frenkiel and Mouëza 1995; Frenkiel et al. 1996; Gros et al. 1996b, 2000). The ciliated and intermediary zones are also very similar to those previously described in various lucinid species with the same ciliated cell types. Intermediary cells appear to form the narrow aperture of the bacteriocyte channel, controlling the water flow along the apical pole of bacteriocytes from the mantle cavity into the interlamellar space, as described by Distel and Felbeck (1987) for *Lucinoma aequizonata*.

The most important features in the gill filament organization of *A. alba* are observed in the lateral zone and involve various identified cell types. Mucocytes are relatively scarce when compared with the gill structure of other lucinids, such as species from the genus *Codakia*. They are only located at the abfrontal end of the gill filaments instead of being intermingled with bacteriocytes and granule cells as in *Codakra orbicularis*, in which they are distributed throughout the lateral zone (Frenkiel and Mouëza 1995), or even at both ends of the gill filaments as in *Linga pensylvanica* (Gros et al. 1996b) and *Lucina pectinata* (Frenkiel et al. 1996). Bacteriocytes are distributed all along the lateral zone, intermingled with intercalary cells, as described in other lucinids (Frenkiel et al. 1996; Gros et al. 1996b); however, no "granule cells" could be observed in the lateral zone of *A. alba*, unlike *C. orbicularis*, in which bacteriocytes occupy only the most superficial one-third of the lateral zone and granule cells the remaining two-thirds. The lack of this typical cell type suggests physiological differences between host species, with probable consequences for the gill-endosymbionts inside the bacteriocyte.

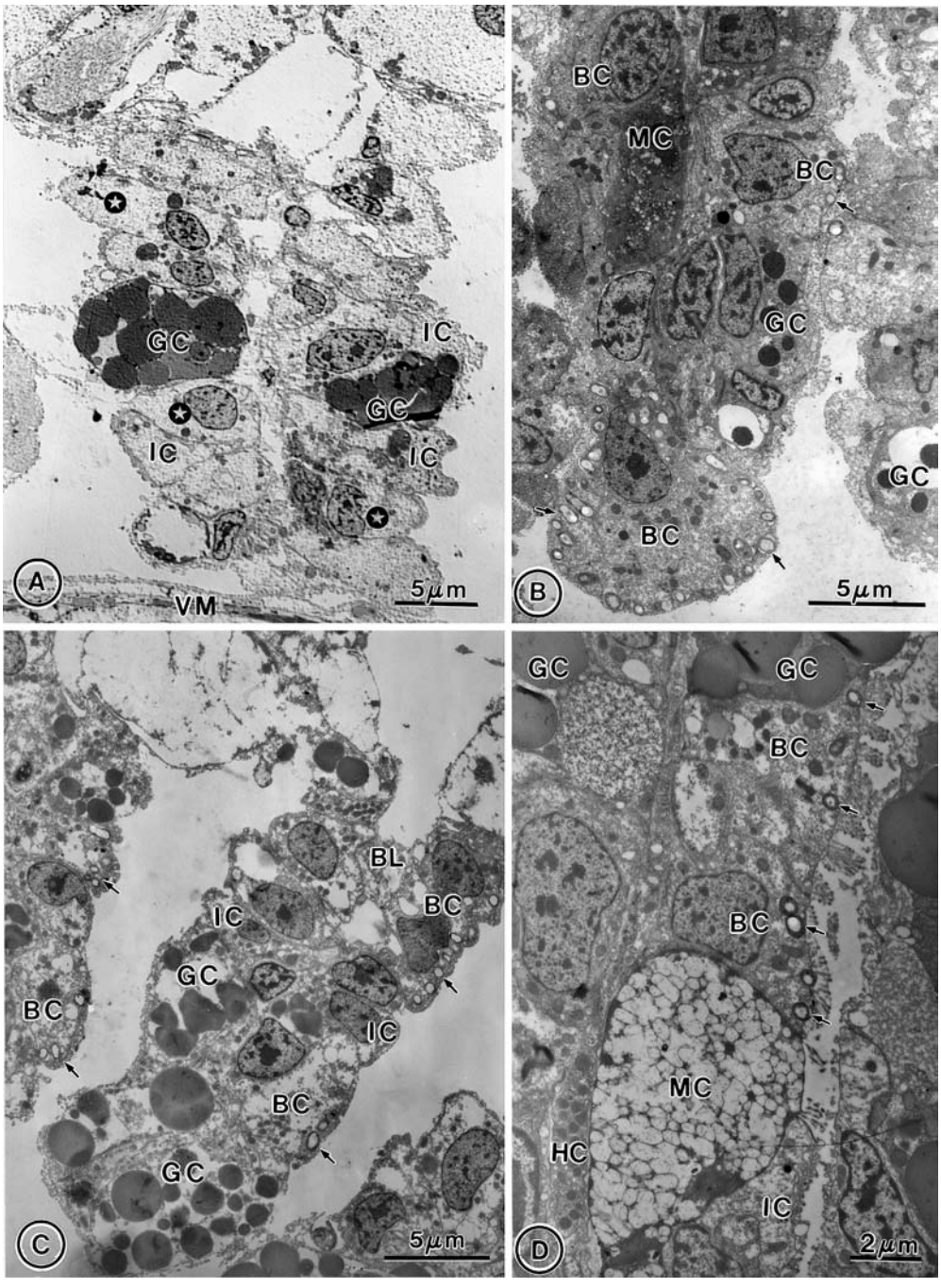

Fig. 3A–D *Codakia orbicularis*, *Anodontia alba*, *Divaricella quadrisulcata*. Experimental infection of aposymbiotic juveniles. **A** Lateral zone of a gill filament in an aposymbiotic juvenile (800 μm shell length). Granule cells (*GC*), intercalary cells (*IC*) and undifferentiated cells, which represent the putative bacteriocytes without bacteria (*star*), are the main components of the lateral zone (*VM* visceral mass). **B** Lateral zone of a gill filament in an aposymbiotic juvenile (1 mm shell length) placed in contact with the purified gill-endosymbiont fraction prepared from an adult individual of *C. orbicularis*. The undifferentiated cells are now differentiated in bacteriocytes (*BC*), which possess numerous endosymbiotic bacteria principally located at the periphery of the cell (*arrows*) (*MC* mucocyte). **C** Lateral zone of a gill filament in an aposymbiotic juvenile (1 mm shell length) placed in contact with the purified gill-endosymbiont fraction prepared from an adult individual of *A. alba*. Thioautotrophic endosymbiotic bacteria (*arrows*) are located at the periphery of cells, which are bacteriocytes (*BL* blood lacuna). **D** Lateral zone of a gill filament in an aposymbiotic juvenile (900 μm shell length) placed in contact with the purified gill-endosymbiont fraction prepared from an adult individual of *D. quadrisulcata*. The cytoplasmic volume of the bacteriocyte is partially occupied by envacuolated bacteria (*arrows*) (*HC* hemocyte)

A. alba usually lives in highly reduced mangrove swamps. However, the specimens of *A. alba* studied in this paper were collected in highly reduced sediment near seagrass beds of *Thalassia testudinum*, known to harbor *C. orbicularis* and most of the other lucinid species (Jackson 1973). Some histological sections were also made from one fixed specimen collected 15 years ago in mangrove sediment in Guadeloupe, in order to check whether some structural differences could be related to environmental factors. No structural differences could be identified between these specimens, suggesting a stable gill filament organization regardless of ecological conditions.

Notwithstanding the structural differences observed between the gills of *A. alba* and *C. orbicularis*, these two species seem to harbor the same bacterial symbiont species. 16S rDNA phylogenetic analysis indicated that the *A. alba*–symbiont was related to the gamma subdivision of the Proteobacteria. More specifically, this sequence fell within the distinct cluster containing the 16S rRNA sequences of all symbionts from bivalves of the superfamily Lucinacea. This phylogenetic congruence among hosts and symbionts has already been described by Distel et al. (1994). Previous investigations have also shown that the specificity of the association between host bivalve and chemoautotrophic symbionts was

unique to each host species (Distel et al. 1994): one bacterial symbiont species for one invertebrate host. Nevertheless, our results show that at least six species of lucinid bivalves representing four genera (*Anodontia*, *Codakia*, *Linga* and *Divaricella*) harbor the same bacterial species inside their bacteriocytes. So, these results do not support the previous concept of a monospecific association between marine invertebrate hosts and their chemoautotrophic symbionts. Other associations between marine animal hosts and non-chemoautotrophic symbiotic microorganisms examined to date also display this moderate level of specificity (see for review Gros et al. 1998b). Previous studies based on DNA–DNA hybridization have suggested that the two vestimentiferans *Riftia pachyptila* and *Tevnia jerichonana* harbor the same bacterial species (Edward and Nelson 1991). More recently, these results were confirmed; the chemoautotrophic symbionts from different genera (*Oasisia*, *Riftia*, *Ridgea* and *Tevnia*) collected from various hydrothermal vent sites presented identical 16S rRNA gene sequences (Feldman et al. 1997; Laue and Nelson 1997).

The fact that *A. alba*, which lives both in highly reduced area of *Thalassia testudinum* environments as well as in the black, reduced sediments of mangrove swamps, harbors the same gill-endosymbiont species as *C. orbicularis*, which only lives in the *T. testudinum* beds, could explain the positive PCR amplifications obtained from mangrove sediments by using a primer set specific for *C. orbicularis*-symbionts (authors' unpublished data). These earlier results were difficult to analyze prior to the symbiont phylogenetic analysis of this lucinid species.

Previous experimental infections of aposymbiotic juveniles of *C. orbicularis* (Gros et al. 1996a, 1998b) using crude sediment collected from seagrass beds as inoculum have suggested the presence of a free-living symbiont stock in the environment, probably in the sediment. The symbiont transmission mode was investigated in *A. alba* only by PCR from mature gonads, because of the inability to induce spawning in this species and, consequently, to obtain larvae (authors' unpublished results). We used the same protocol as previously described for other lucinid species known to harbor the same bacterium (Gros et al. 1996a, 1998a, 2000). No specific symbiont 16S rDNA target was amplified from the mature individuals tested (three males and two females). The lack of PCR amplifications from gonads in comparison to the successful amplifications obtained from symbiont-containing gill tissues suggests that the symbiont transmission mode is environmental for *A. alba*. This new observation seems to confirm the previous hypothesis (Gros et al. 1998a) stating that the transmission mode of gill-endosymbionts in bivalves is environmental in Lucinidae and vertical in Solemyidae and Vesicomyidae (Cary and Giovannoni 1993; Cary 1994; Krueger et al. 1996), according to the bivalve family.

Due to the fact that this free-living symbiont form also remains unculturable (authors' personal observations), it is not known whether or not the free-living, symbiosis-competent bacteria participate in some of the numerous microbial loops known to exist in the environment. Research is in progress in our laboratory to detect the free-living symbiont form in the environment by fluorescent in situ hybridization (FISH) and to establish its importance in the sediment microbial community.

Experimental infection of aposymbiotic juveniles using the purified bacterial fraction from lucinid species

Endosymbiotic associations, especially those concerning symbionts which are environmentally transmitted, require highly specialized host-symbiosis mechanisms of communication and recognition. They are essential for the establishment of the symbiosis by free-living, symbiosis-competent bacteria. Usually, these mechanisms are thought to be so specific that only one bacterial species can be accepted by aposymbiotic hosts. In tropical lucinids, the phylogenetic analysis of symbionts has shown that at least six lucinid species harbor the same bacterial species. In this case, is the host selective at the infraspecific level? Can a lucinid host accept any symbiosis-competent strains from the same bacterial species, or could it select some of them while refusing others?

In order to address these questions, we placed aposymbiotic juveniles of *C. orbicularis* in contact with purified endosymbionts from the gills of adult specimens of six lucinid species. Aposymbiotic juvenile batches successfully acquired gill-endosymbionts from all six of the lucinid hosts separately tested. In order to decrease the possibility of contamination from free-living bacterial symbionts located on the surface of gills, we used NaClO solution to kill most of the bacteria present on the gills used in the experiments. All the extracellular, heterotrophic, cultivable bacteria were killed before purification (and, consequently, the putative free-living symbiont form, which is a gram-negative bacterium as are most marine bacteria), as shown by the microbial culture experiments. The fact that juveniles placed in contact with such gill-endosymbiont fractions were positively infected suggests that the infections are due to the gill-endosymbionts rather than environmental bacteria present on the gill surface. Thus, it is really an interspecific infection of the *C. orbicularis* juveniles that was observed here.

The results presented here suggest that lucinid hosts do not distinguish between intraspecific and interspecific symbiont strains. All strains can colonize the gill filaments of aposymbiotic juveniles of one recipient species: *C. orbicularis*. The most important control made by the host is probably to check the species level of the bacteria present in the environment before initiating the colonizing process (Fig. 4). Any bacterial strain belonging to the right symbiotic bacterial species can be accepted by the host. In the sepiolid squid–*Vibrio*

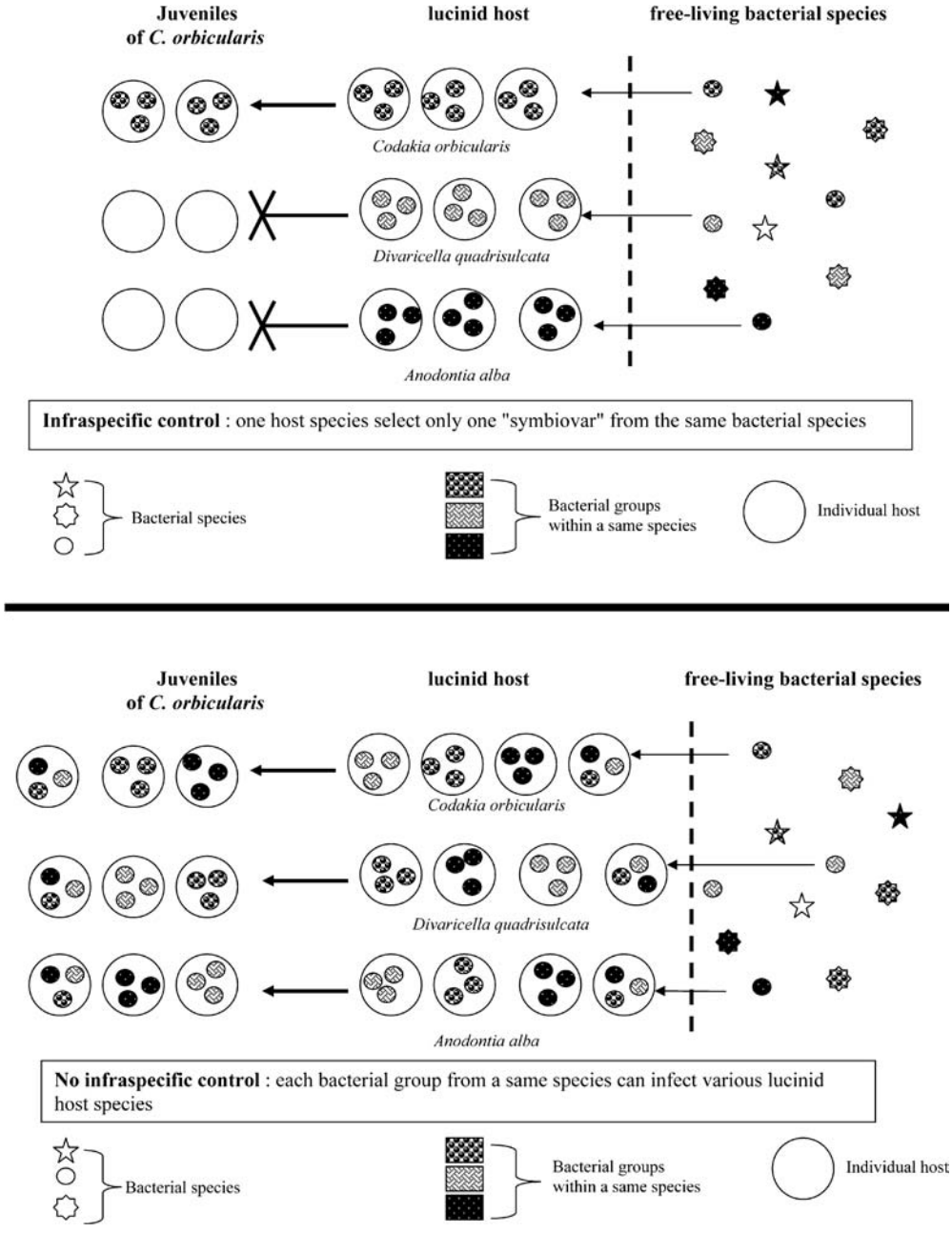

Fig. 4 Two hypotheses on the level of control by the host. Only the second hypothesis (*lower panel*) is confirmed by the interspecific infection of aposymbiotic juveniles of *Codakia orbicularis* by various tropical lucinid gill-endosymbionts

symbiosis, intraspecific symbionts are preferred to interspecific symbiont strains, and there is also a hierarchy of symbiont competency that could reflect the phylogenetic relationships of the partners (Nishiguchi et al. 1998). More recently, infections by intraspecific *Vibrio fischeri* isolated from *Euprymna* host species exhibited preferential colonization of *Euprymna* aposymbiotic juveniles over interspecific *V. fischeri* symbionts isolated from sepiolid taxa, while all strains used can colonize the light organ equally. Moreover, there are subspecies-level interactions, like the pronounced competitive advantage of intraspecific *V. fischeri* strains over other interspecific strains (Nishiguchi 2002).

The data presented here, which were obtained for single inoculation assays, could be completed by simultaneous infections using interspecific strains (isolated from different host species), in order to determine whether competitive interactions among symbiont strains may occur in lucinid hosts, as suggested in the squid model (Nishiguchi 2002). The use of molecular, hypervariable markers to differentiate unculturable symbiont strains associated with experimental infections of aposymbiotic juveniles will help to investigate this point.

The results presented in this paper also demonstrate that an intracellular gill symbiont can establish a symbiotic relationship with a new host generation when released into the environment. Similar observations have previously been made regarding *Rhizobium* species and legume plants, in which intracellular symbionts could be

cultured from root nodules and used to inoculate uninfected plants (Pelmont 1993). Moreover, from the enriched fraction, the bacterial gill-endosymbionts have to modify their metabolic pathways from an intracellular lifestyle, within a controlled environment, to pathways adapted to a free-living lifestyle. These modifications include the expression of genes involved in specific processes important for free-living symbiosis-competent bacteria such as those of cellular recognition or chemotaxis in order to find receptive host cells for establishing symbiosis. Thus, the lucinid symbionts probably possess a full genome allowing a free life in the environment, unlike symbionts involved in ancient symbioses which cannot survive outside their host cell. These data, in association with the symbiont transmission mode, indicate that chemoautotrophic symbiosis in the family Lucinidae is relatively recent when compared with other bivalve families in which symbionts are vertically transmitted (Peek et al. 1998). Consequently, it would be interesting to study the phylogenetic relationships between species within the family Lucinidae.

The metabolic pathway modifications cited above are generally accompanied by some structural changes in the bacterial symbionts. The most remarkable is bacterial cell size. In tropical lucinids, the gill-endosymbionts are generally large (up to 5 μm) and characterized by sulfur granules located in the periplasmic space (Frenkiel and Mouëza 1995; Gros et al. 1996b, 2000). However, in previous experimental infection studies of aposymbiotic juveniles of *C. orbicularis* using unsterilized sediment, the bacteria that penetrated, by endocytosis, the apical pole of undifferentiated cells located in the lateral zone rarely exceeded 1 μm in length (Gros et al. 1998b). In present experiments, the inoculum prepared from symbiont-bearing tissues contained gill-endosymbionts typical in their ultrastructural characteristics (see above for more details). However, the bacterial symbionts observed in the gill cells of the juveniles were generally small (< 1 μm in length), often with a coccoid shape similar to that observed in experiments done with bacteria from sediment (Gros et al. 1996a, 1998b). Thus, symbiont ultrastructure appears to reflect modifications made during extracellular life. This phenomenon has already been described for bacteria belonging to the Rhizobiaceae family, in which species are known to have a particular shape inside their host cells and a classical rod shape when they are cultured in vitro (Pelmont 1993).

Another interesting observation is that this symbiont species was able to colonize species known to have different gill ultrastructures. For example, only individuals from the *Codakia* genus possess numerous "granule cells" in the lateral zone of their gill filaments; no "granule cells" were detected in *A. alba* or *L. pensylvanica*. Some particular organelles like peroxysomes can be detected in the bacteriocytes of *L. pensylvanica*. Intercalary cells generally encroach upon the apical area of adjacent bacteriocytes, restricting differently their contact with pallial seawater. On the whole, these observations could suggest different intracellular environments for the endosymbionts between the host species. Despite the host origin, the symbionts were able to infect *C. orbicularis* juveniles, thus showing a real capacity to adapt.

According to the experimental infection results presented here, *C. orbicularis* definitively represents a unique model for investigations of symbiotic relationships between sulfide-oxidizing bacteria and bivalves as: (1) aposymbiotic juveniles can be obtained from the laboratory and (2) it is possible to infect them either by the free-living symbiont form from crude sediment or by purified symbiotic bacteria prepared from adult gills. The last option is preferable, because it enables the use of an inoculum composed entirely, or at least predominantly, of a single bacterial species, in contrast to the mixed bacterial population found in crude sand collected from *Thalassia testudinum* sediments. Also, unsterilized sediments pose the risk of experimental infections, as, in addition to the free-living symbiont form, opportunistic bacteria, protists, annelids, crustacean larvae and other organisms can be unintentionally introduced that might impact the survival of aposymbiotic juveniles (authors' personal observations). Thus, even if chemoautotrophic endosymbionts remain unculturable, it is possible, with the *C. orbicularis* model, to carry out experimental infections with a well-defined inoculum. However, to date, we do not know the percentage of intracellular bacterial symbionts that are able to adapt and establish symbiosis with new host generations.

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