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Environmental Transmission of a Sulfur-Oxidizing Bacterial Gill Endosymbiont in the Tropical Lucinid Bivalve *Codakia orbicularis*

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***Codakia orbicularis* is a large tropical member of the bivalve mollusk family *Lucinidae* which inhabits shallow-water sea-grass beds (*Thalassia testudinum* environment) and harbors sulfur-oxidizing endosymbiotic bacteria within bacteriocytes of its gill filaments. When a *C. orbicularis*-specific 16S rDNA (DNA encoding rRNA) primer is used with a bacterium-specific 16S rDNA reverse primer in amplifications by PCR, the primer set was unsuccessful in amplifying symbiont DNA targets from ovaries, eggs, veligers, and metamorphosed juveniles (600 μm to 1 mm in shell length) cultivated in sterile sand, whereas successful amplifications were obtained from gill tissue of adult specimens and from metamorphosed juveniles (600 μm to 1 mm in shell length) cultivated in unsterilized sea-grass bed sand. To ascertain the presence of the symbiont target in juveniles, restriction fragment length polymorphism analysis, Southern blotting, and transmission electron microscopy were used. Specific hybridizations and observation of endosymbiotic bacteria in the gills of numerous juveniles cultivated in unsterilized sea-grass bed sand showed that the sulfur-oxidizing endosymbionts of *C. orbicularis* are environmentally transmitted to the new generation after larval metamorphosis.**

Since their discovery by Felbeck et al. (13) in deep-sea hydrothermal vent fauna, symbioses between bivalves and sulfur-oxidizing chemoautotrophic bacteria located in their gill cells have been identified in various environments (2, 12). In the shallow water environment of tropical sea-grass beds, the dominant species of bivalves belong to the family *Lucinidae* (21), in which every species of the eight genera examined to date contain sulfur-oxidizing endosymbiotic bacteria (29) inside modified gill cells named bacteriocytes. According to Le Pennec et al. (24) and Cary et al. (7), symbiont transmission in invertebrates may occur by one of these three ways: (i) vertical transmission, i.e., from parents to offspring, which may include incorporation of symbionts in or on the gametes; (ii) horizontal transmission, which involves the spread of symbionts between contemporary hosts; and (iii) environmental transmission, which involves the reinfection of the new host generation from an environmental stock of free-living symbiont form.

The ability to cultivate *Codakia orbicularis* from fertilization to 2-mm-long juveniles offers a unique opportunity to examine the process of symbiont transmission in the *Lucinidae*. Preliminary results have shown that metamorphosed larvae cultivated in the presence of sterile sand as the substratum did not acquire normal gill filaments with intracellular bacterial symbionts compared with wild juveniles of the same size (17). The addition of 1 μM sulfide, the estimated concentration in lucinid habitats (8, 14), or the addition at regular intervals of a sulfide crystal buried in sterile sand did not permit *C. orbicularis* to acquire their symbionts either (25). These first observations, based on transmission electron microscopy (TEM) views of reared juveniles, needed new investigations to understand the endosymbiont transmission mode.

In a previous study, the *C. orbicularis* endosymbiont was characterized by using 16S rRNA gene sequence analysis (11), the main objective of which was to understand the phylogenetic relationships between this lucinid gill endosymbiont and the other gill endosymbionts sequenced previously. The knowledge of the nearly complete 16S rDNA (DNA encoding rRNA) sequence of the *C. orbicularis* symbiont permitted the development of molecular probes enabling the detection of symbiont-specific target sequences.

In this study, we checked the presence of chemoautotrophic endosymbionts during all development stages of *C. orbicularis* by using molecular technologies in association with TEM studies in two series of batches cultivated in sterile or unsterilized sand.

MATERIALS AND METHODS

Bacterial strains. The microorganisms investigated in this study were *Vibrio alginolyticus* (cultivated on TCBS agar [Difco Laboratories, Detroit, Mich.] slopes at 30°C) and bacterial gill endosymbionts of tropical Lucinidae listed in Table 1.

TABLE 1. Members of the tropical bivalve mollusk family *Lucinidae* harboring sulfur-oxidizing endosymbiont in their gills used in this study

Species	Location	Habitat	Symbiont 16S rDNA previously sequenced (reference)
Codakia costata	Guadeloupe	Sea-grass bed	9
Codakia orbicularis	Guadeloupe	Sea-grass bed	10,11
Linga pensylvanica	Martinique	Sea-grass bed	11
Lucina pectinata	Guadeloupe	Mangrove swamp	11

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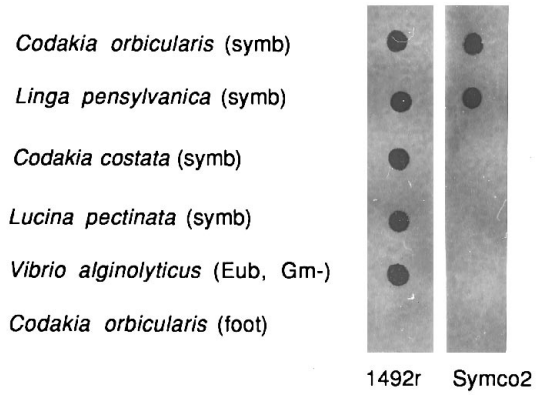

FIG. 1. Oligonucleotide probe specificity determined by dot blot hybridization. Membranes were separately probed with the *C. orbicularis* symbiont (symb)-specific probe (Symco2) and the bacterium-specific probe (1492r).

Specimen culture. Adult specimens of *C. orbicularis* were collected by hand from shallow water sea-grass beds in Guadeloupe in the French West Indies. They were kept in filtered (pore size, 5 μm) and UV-treated seawater until spawning, which was induced within 24 h.

Gametes were obtained by injection into the visceral mass of 0.3 ml of a 4 mM serotonin (5-hydroxytryptamine creatinine sulfate complex; Sigma Chemical Co., St. Louis, Mo.) solution in filtered (pore size, 0.22 μm) seawater (16, 19). During embryological development, completed within 48 h (1), and larval development, no antibiotics were used and the water was changed every day until the pediveliger stage. After this developmental stage, the water was changed every other day. Induction of metamorphosis of the larvae was obtained by the addition of a sterile sand fraction smaller than 200 μm in grain size. After metamorphosis (about 5 weeks after fertilization), juveniles were distributed into two batches; one was cultivated in sterile sand, and the other was cultivated in unsterilized sand obtained from the same area of a shallow-water sea-grass bed. Four independent batches of postmetamorphosed larvae were cultivated in sterile and unsterilized sand.

DNA extraction. Nucleic acids from symbiont-containing gill tissues, a symbiont-free foot, and *V. alginolyticus* were prepared as described elsewhere (11). DNA extraction from ovaries, eggs, D-larvae (48-h-old veligers), and juveniles (postmetamorphosed larvae) were performed by several procedures (6, 11, 30) and by using the Isoquick nucleic acid extraction kit (ORCA Research Inc., Bothell, Wash.) as described in the manufacturer's instructions. Before PCR amplification, DNA concentration and purity in each sample were determined spectrophotometrically.

Oligonucleotide probe and primers. The nearly complete 16S rDNA sequence (1,502 nucleotides) of the *C. orbicularis* endosymbiont was determined previously (11) and deposited in the EMBL data library under accession number X84979. Computer-assisted analysis of bacterial 16S rDNA databases identified two regions that were specific to the *C. orbicularis* endosymbiont; the first one, Symco1 (5′-TGATACTGCCTCACTAGAG-3′; *Escherichia coli* positions 638 to 656 [3]), was used as a PCR forward primer with the bacterium-specific reverse primer 1492r (23); the second, Symco2 (5′-TACAGAGGGTCGCCAACCCGTG-3′; *E. coli* positions 1247 to 1268), was used as a probe for hybridization. Oligonucleotide probes for hybridization were labeled by using the digoxigenin oligonucleotide 3′-end labeling kit (Boehringer GmbH, Mannheim, Germany) as described in the manufacturer's instructions.

PCR conditions. PCR amplification was performed with a 25-μl reaction volume containing 200 μM each deoxynucleoside triphosphate, 2.5 μl of a 10× reaction buffer (Bioprobe Systems, Montreuil, France), 25 pmol of each primer, 0.5 U of Hi *Taq* DNA polymerase (Bioprobe Systems), and 25 ng of DNA. Samples were amplified with a DNA thermal cycler (model; 480 Perkin-Elmer Inc., Norwalk, Conn.) under the following conditions: initial denaturation at 94°C for 8 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min, followed by a final elongation time of 7 min at 72°C. Five microliters of each PCR sample was used for a second amplification under the same conditions. PCR products were examined by horizontal electrophoresis with an 0.8% agarose gel.

The specificity of the primer set Symco1–1492r was determined in a series of amplification reactions (25 cycles) with a variety of nonhomologous templates extracted from one bacterial strain and from symbiont-containing gill tissue of other tropical members of the bivalve mollusk family *Lucinidae* (Table 1). The sensitivity of the primer set to detect target sequences in a mixture of prokaryotic and eukaryotic DNAs was determined through a series of amplification reactions (two times 25 cycles) in which *C. orbicularis* symbiont template concentrations were titrated from 1 ng to 1 pg at 10-fold dilutions.

Restriction fragment analysis. The amplification products were digested with the endonuclease *Hin*fI (Sigma) as described in the supplier's instructions. *Hin*fI was chosen on the basis of a restriction map predicted from the sequence of the 16S rRNA gene amplified from *C. orbicularis* gill tissue. The restriction fragment pattern resulting from *Hin*fI activity was specific to this sequence, as determined by a search of the 16S rRNA sequences of all the other chemoautotrophic symbionts. The positions (*E. coli* numbering) of the restriction sites are 836, 1034, 1338, 1355, and 1475. They generate fragments of the following sizes: 304, 198, 198, 120, 35, and 17 bp. Reactions were performed with a 20-μl volume with 15 μl of amplified DNA and compared by 1.5% agarose gel electrophoresis with the restricted gill amplification product.

DNA blotting. Five hundred nanograms of genomic DNA per dot was immobilized on Biohylon Z neutral nylon membrane (Bioprobe Systems). For Southern blotting, 10 μl of whole PCR products was electrophoresed on an 0.8% agarose gel, whereas 15 μl of restricted DNA products was run on a 1.5% agarose gel. Southern blotting was performed on Biohylon Z neutral membranes (Bioprobe Systems) after alkaline denaturation (1.5 M NaCl, 0.5 M NaOH) and neutralization (1.5 M NaCl, 1 mM EDTA, 0.5 M Tris-HCl) of the agarose gel.

Hybridizations. Membranes were prehybridized for 2 h at 68°C in 20 ml of a hybridization buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% sodium dodecyl sulfate (SDS), and 1% blocking reagent (Boehringer). Hybridization was carried out overnight at 42°C in a sealed bag containing 5 ml of hybridization buffer with 26 ng of digoxigenin-labeled probe per ml. Membranes were carefully washed two times at 37°C for 5 min each with 2× SSC buffer containing 0.1% SDS. Immunological detection of the labeled DNA probes was obtained by use of the DIG nucleic acid detection kit (Boehringer) as described in the manufacturer's instructions.

TEM preparation. Juveniles of *C. orbicularis* were prefixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer adjusted to pH 7.2 and to 1,000 mOsM with 0.4 M NaCl, 2 mM $CaCl_2$ being added for better membrane preservation. After a brief rinse, they were fixed at room temperature for 45 min in 1% osmium tetroxide in the same buffer and postfixed in 2% aqueous uranyl acetate for an additional hour. Pieces were decalcified overnight in 1% ascorbic acid at room temperature prior to dehydration through an ascending series of ethanol and propylene oxide. After embedding in an Epon-Araldite resin mixture by the method of Mollenhauer described by Glauert (20), sections were cut on a Leica Ultracut E ultramicrotome. Thin sections, 60-nm thick, were contrasted for 10 min in lead citrate before being observed in a Hitachi H-8000 TEM at 100 kV.

RESULTS

Probe specificity assessment. The bacterium-specific probe (1492r) hybridized to all bacterial DNA samples, demonstrating probe accessibility to bacterial 16S rDNA. Conversely, *C. orbicularis*-specific probe Symco2 hybridized specifically to DNA samples extracted from *C. orbicularis* and *Linga pensylvanica* gills (Fig. 1).

PCR test. PCR amplification (25 cycles) performed with the primer set Symco1–1492r produced an expected single band of 872 bp from DNAs extracted from *C. orbicularis* and *Linga pensylvanica* gills, whereas no amplification products were detected with DNAs extracted from *Codakia costata* or *Lucina pectinata* gills, from symbiont-free tissues, or from *V. alginolyticus* (Fig. 2).

This primer set can successfully amplify the *C. orbicularis* symbiont target DNA from 10 pg of a mixed population of prokaryotic and eukaryotic genomic DNA extracted from symbiont-containing gill tissues (Fig. 3A). After Southern blotting and hybridization with the specific probe Symco2, the detection limit was not improved (Fig. 3B).

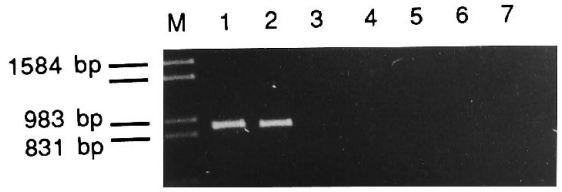

FIG. 2. Specificity of PCR experiments. The symbiont-specific primer Symco1 was used with the bacterium-specific primer 1492r in amplifications of genomic DNA extracted from a variety of nonhomologous templates. Lanes: M, DNA marker; 1, *C. orbicularis* gill; 2, *Linga pensylvanica* gill; 3, *C. costata* gill; 4, *Lucina pectinata* gill; 5, *C. orbicularis* foot; 6, *V. alginolyticus*; 7, negative control.

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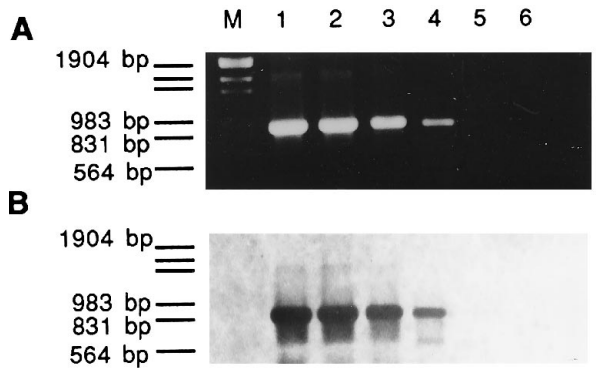

FIG. 3. Agarose gel electrophoresis (A) and Southern hybridization (B) with the symbiont-specific probe Symco2 of PCR products. *C. orbicularis* symbiont template DNA was titrated from 1 ng to 1 pg at 10-fold dilutions. Lanes: M, DNA marker; 1, 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg; 6, negative control.

PCR analysis of bacterial colonization. The symbiont-specific primer set was used in a series of PCR experiments to detect the *C. orbicularis* symbiont 16S rDNA target in various DNA samples. To examine the possibility that contaminating substances might inhibit the amplification reaction, 25 ng of mixed DNA extracted from symbiont-containing gill tissue was added to the PCR mix. These control reactions produced successful amplifications only for DNA extracted with the Isoquick nucleic acid extraction kit, indicating that this DNA extraction protocol produces a DNA sufficiently pure for PCR amplification (Fig. 4). Repeated attempts to amplify the *C. orbicularis* symbiont DNA target from bulk DNA extracted and purified from ovaries, eggs, D-larvae, and postmetamorphosed larvae cultivated in sterile sand were unsuccessful. The only successful amplifications were obtained from juveniles cultivated in unsterilized sand and from gills of adult specimens (Fig. 4).

Length polymorphism analysis of PCR-amplified DNA. To control the specificity of amplified fragments, each positive amplification was restricted with the endonuclease *Hin*fI. We can detect on the agarose gel three major bands (304 bp, 198 bp [due to two fragments of identical size], and 120 bp). Restriction fragment length polymorphism analysis indicated that the products amplified from juveniles cultivated in unsterilized sand were similar to those obtained from the gill tissue (Fig. 5A). Moreover, Southern blotting and hybridization with the symbiont-specific probe Symco2 showed a hybridization signal for the 304-bp fragment (Fig. 5B), confirming the identity of the PCR products amplified from the juveniles cultivated in unsterilized sand with those obtained from the gill tissue of a mature bivalve.

TEM analysis. Data from PCR were confirmed by the observation of thin sections of whole juveniles (600 μm to 1 mm shell length) cultivated in sterile and unsterilized sand. In juveniles cultivated in sterile sand, no bacteria are found in the gill filaments which appear truncated (Fig. 6A and C); the lateral zone is shortened without bacteriocytes (Fig. 6C), whereas the ciliated and intermediary zones appear identical to the gill filaments of wild subadults. No views of a putative cryptic form of a gill endosymbiont are observed either in gill filaments or in other larval tissues. On the other hand, TEM observations of juveniles cultivated in unsterilized sand show that their gill filaments are identical to those of wild subadults of similar size. Thus, they possess well-developed ciliated and intermediary zones, and the lateral zone consists of all the cell types found in gill filaments of wild specimens, i.e., granule cells, intercalary cells, mucous cells, and bacteriocytes (Fig. 6B and D). Bacteriocytes have a rounded apical pole with microvilli directly in contact with seawater; the endosymbiont bacteria, individually enclosed in vacuoles at the periphery of the cell, are salient in the microvilli border (Fig. 6B and 7A). Inconspicuous intercalary cells are interspersed between bacteriocytes (Fig. 7A). Sulfur-oxidizing endosymbionts are rod shaped, up to 2.5 μm in length, and have the characteristic double membrane of gram-negative bacteria; their nuclear area is identified as a well-defined network (Fig. 7B). Periplasmic sulfur globules are frequent, and the bacterial cytoplasm contains numerous non-membrane-bound irregular inclusions which may be glycogenic storage granules (Fig. 7B).

DISCUSSION

In the present paper, the environmental transmission mode of the gill-endosymbiotic bacteria was demonstrated in a member of the bivalve mollusk family *Lucinidae*, *C. orbicularis*, by using molecular techniques in association with electron microscopy. Previous studies of endosymbiotic chemoautotrophic associations in marine organisms demonstrated two types of transmission: a vertical transmission reported for some members of the families *Vesicomyidae* and *Solemyidae* (5–7) and an environmental transmission described for the vestimentiferan species *Riftia pachyptila* and *Ridgea piscesae* (7). Until now, cases of environmental transmission have never been described for *Bivalvia.*

The specific primer set for *C. orbicularis* symbiont (Symco1–1492r) amplifies the target sequence from bulk DNA extracted from *C. orbicularis* and *Linga pensylvanica* gills, both of them known to harbor the same symbiont (11). DNA extracted from *V. alginolyticus* and from the gills of *C. costata* and *Lucina pectinata* was not amplified by our PCR test, a result also expected because of the previous phylogenetic characterization of *C. costata* and *Lucina pectinata* endosymbionts based on 16S rDNA sequence analysis (9, 11).

The detection limit of our PCR experiments was about 10 pg of target DNA in a mixture of eukaryotic and prokaryotic DNAs extracted from symbiont-containing host tissue. Since the genomic DNA of a bacterium is about 10 fg, we can estimate that we successfully detected and amplified about 1,000 bacteria. Moreover, we worked with a mixed genomic DNA for which the ratio of prokaryotic DNA to total (eukaryotic and prokaryotic) DNA was unknown but which undoubtedly decreases the detection level, which could be well under 1,000 bacteria. The fact that the detection limit after Southern blotting and hybridization with the specific probe Symco2 was not improved would strengthen this conclusion.

The fact that strong amplification was obtained from postmetamorphosed larvae cultivated in unsterilized sand, while no amplification was obtained from ovaries, eggs, D-larvae, and postmetamorphosed larvae cultivated in sterile sand, strongly

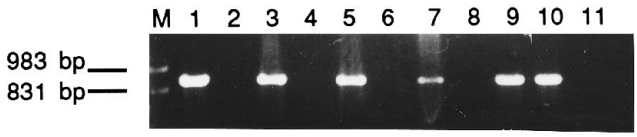

FIG. 4. PCR detection of *C. orbicularis* symbiont with target DNA prepared with the Isoquick nucleic acid extraction kit (ORCA Research). Lanes: M, DNA marker; 1, gill; 2, ovary; 3, ovary plus 25 ng of gill DNA; 4, eggs; 5, eggs plus 25 ng of gill DNA; 6, D-larvae; 7, D-larvae plus 25 ng of gill DNA; 8, juveniles cultivated in sterile sand; 9, juveniles cultivated in sterile sand plus 25 ng of gill DNA; 10, juveniles cultivated in unsterilized sand; 11, negative control.

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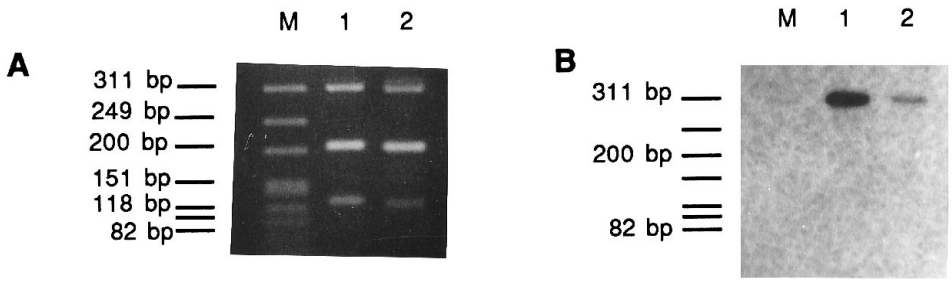

FIG. 5. Agarose gel electrophoresis (A) and Southern hybridization (B) with the symbiont-specific probe Symco2 of *Hin*fI-digested fragments from the PCR primer pair Symco1–1492r. Lanes: M, DNA marker; 1, *C. orbicularis* gill; 2, juveniles cultivated in unsterilized sand.

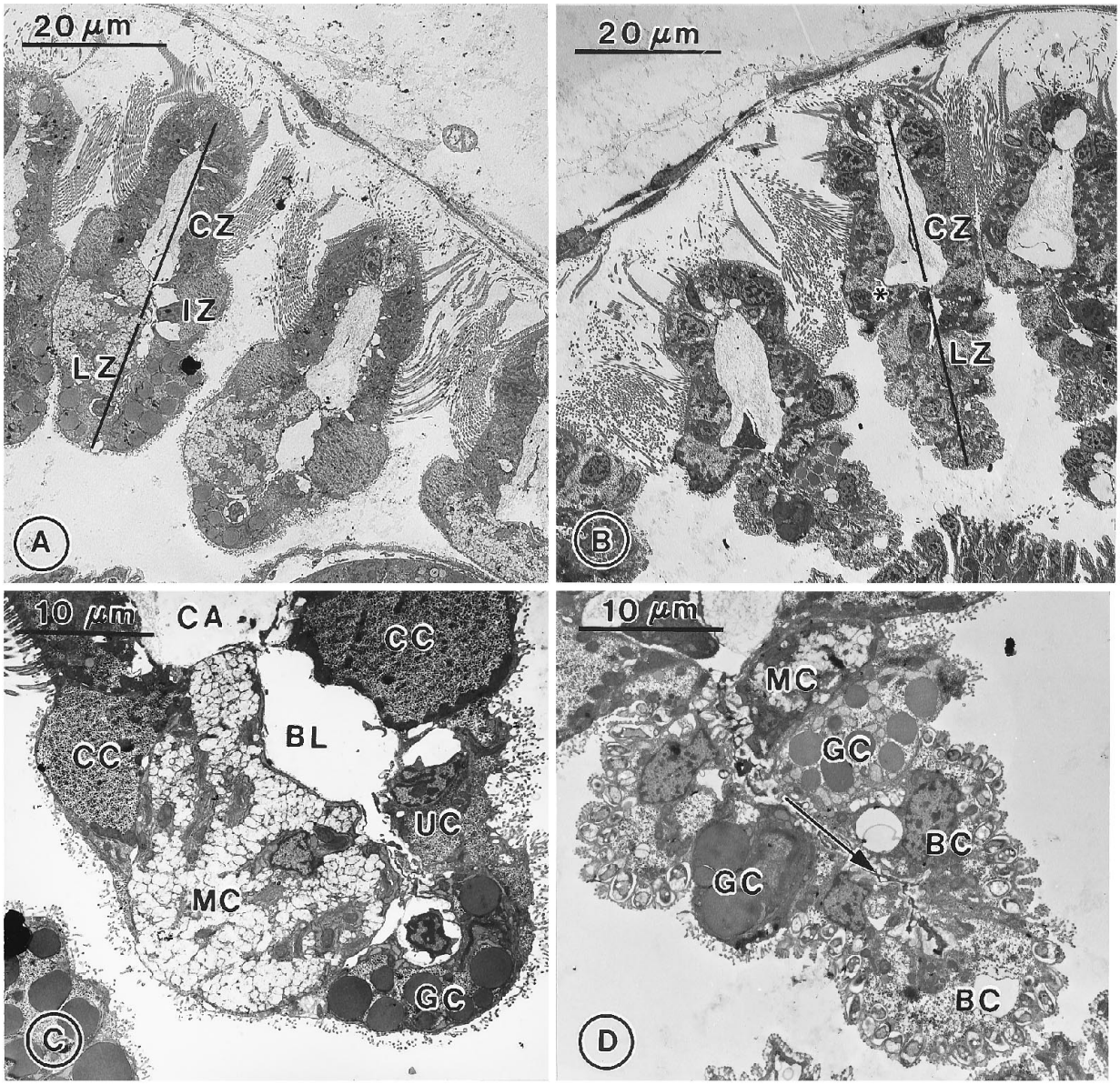

FIG. 6. TEM of gill filaments in 600-µm (shell length) larvae. (A) Low magnification of whole gill filaments of a larva cultivated in sterile sand; (B) low magnification of whole gill filaments of a larva cultivated in unsterilized sand (the intermediary zone is marked by a star); (C) higher magnification of the lateral zone of a gill filament from larva cultivated in sterile sand; (D) higher magnification of the lateral zone of a gill filament from larva cultivated in unsterilized sand (the lateral-zone axis is shown by an arrow). Abbreviations: BC, bacteriocytes; BL, blood lacuna; CA, collagen axis; CC, clear cell of the intermediary zone; CZ, ciliated zone; GC, granule cells; IZ, intermediary zone; LZ, lateral zone; MC, mucous cells; UC, undifferentiated cells.

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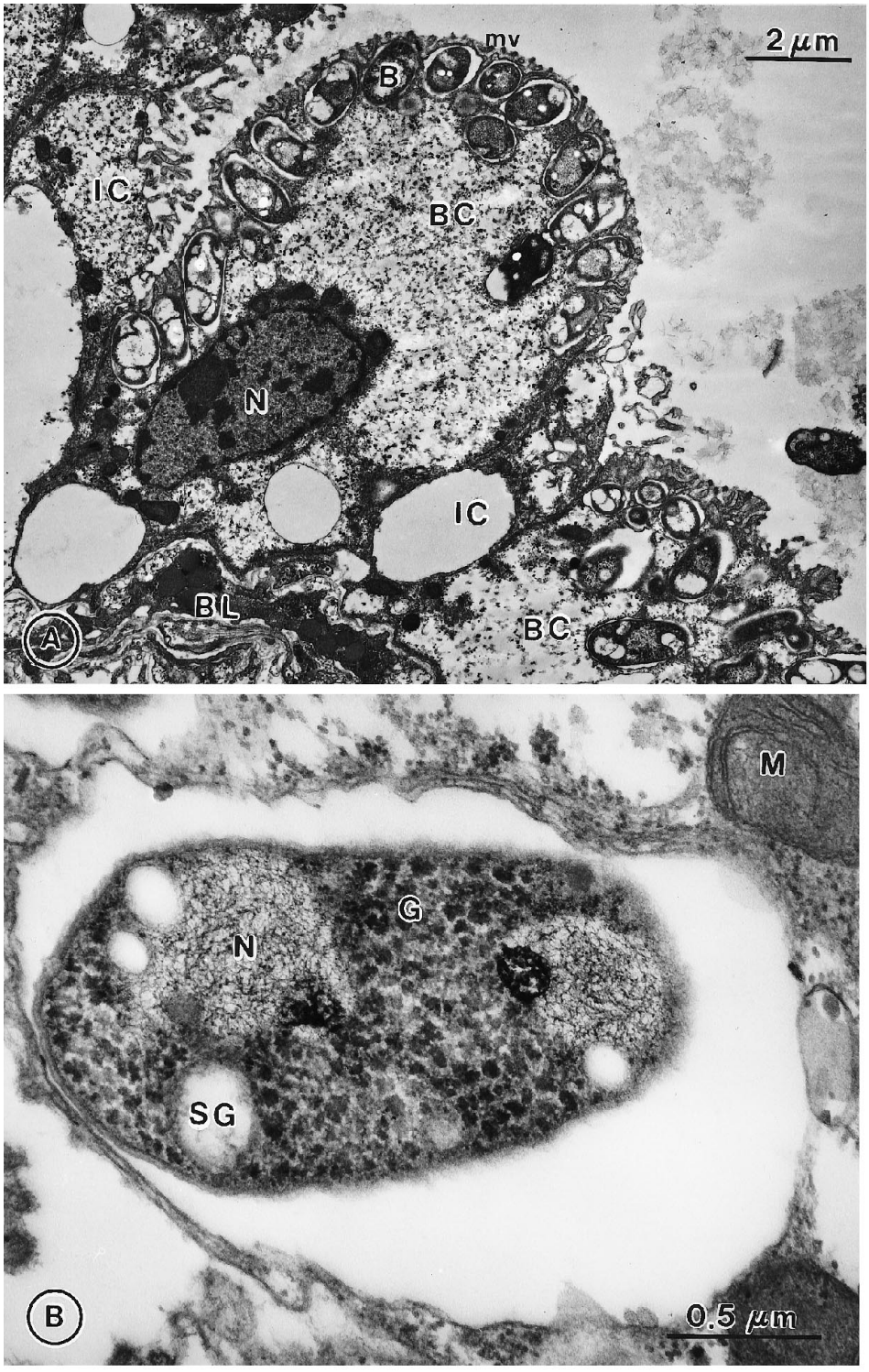

FIG. 7. (A) TEM of endosymbiotic bacteria located at the periphery of a bacteriocyte in a 600-μm (shell length) larva cultivated in unsterilized sand; (B) TEM of a bacterium endosymbiont in a single vacuole. Abbreviations: B, bacteria; BL, blood lacuna; G, glycogenic storage; IC, intercalary cells; M, mitochondria; MV, microvilli; N, nuclear area; SG, periplasmic sulfur globules.

suggests that the transmission of the *C. orbicularis* symbionts is not vertical but possibly horizontal or more likely from an environmental stock of a free-living symbiont form.

Cary and Giovannoni (6) showed that follicle cells surrounding primary oocytes of two members of the family *Vesicomyidae*, *Calyptogena pacifica* and *Calyptogena magnifica*, contained endosymbiotic bacteria. The infection of follicular cells ought to be carefully checked since, in some species, it may play a

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role in mechanisms for the inoculation of oocytes. This strategy was also reported for *Tunicates* (4) and for certain sponges in which, during spawning, bacterium-containing follicle cells are shed with oocytes (18). For *C. orbicularis*, no bacteria were observed in either oocytes, follicular cells (15), or spermatozoa (26). The lack of PCR amplification with the ovary suggests that the follicle cells of the mature female gonad do not contain the 16S rDNA symbiont target. Moreover, the fact that postmetamorphosed juveniles (600 μm to 1 mm shell length) cultivated in sterile sand do not house endosymbiotic bacteria in their gill filaments rules out the hypothesis that an endospore-like stage exists which is present in eggs or during embryological and larval development and which is resistant to nucleic acid extraction and to the PCR conditions described.

We successfully established symbiosis in four independent batches of *C. orbicularis* by adding unsterilized sand to the culture after metamorphosis of larvae, about 1 month after fertilization. The whole embryological and larval development seems to occur without the presence of chemoautotrophic symbionts. No significant growth difference was observed between juveniles cultivated in sterile and unsterilized sand, at least until the shell length reached 1 mm. These results suggest that the association between the bivalve and the endocellular sulfur-oxidizing bacteria is not obligate for larval and early-postlarval development. However, no adult specimens of *C. orbicularis* have been found without symbiotic bacteria in their gills, which implies that the association between this bivalve species and the chemoautotrophic bacteria takes place at some postmetamorphic developmental stage.

In TEM views, gill filaments of juveniles cultivated in unsterilized sand appear very similar to those of wild specimens of *C. orbicularis* with a shell length of 2 mm collected in sea-grass beds (17) with regard to their general organization as well as the ultrastructure of their sulfur-oxidizing endosymbiont. The localization of symbionts in bacteriocytes appears quite different in both wild and cultivated juveniles when compared with their localization in adults (17). In juveniles, the endocellular bacteria are mostly at the periphery of the bacteriocytes, a site which is suggestive of an endocytosis mechanism for the colonization of the bacteriocytes by free-living symbiont forms present in the environment of the postmetamorphosed larvae.

The experimental infestation of *C. orbicularis* larvae cultivated with unsterilized sand collected in sea-grass beds as the substratum validates the hypothesis that endosymbionts appear to be environmentally transmitted to the new generation through free-living symbiont forms, probably located in the sand. However, attempts to amplify *C. orbicularis* symbiont 16S rDNA from DNA extracted from sea-grass sand failed. Extraction of DNA from sea-grass sediment always resulted in coextraction of humic compounds which interfere with DNA detection and amplification. This contamination seems to inhibit the DNA polymerase in PCR, as it has been shown previously (22, 27, 32). Research is in progress to amplify the *C. orbicularis* target DNA from sea-grass bed sand and to cultivate the *C. orbicularis* symbiont free-living form.

The environmental transmission mode might imply some complex recognition mechanisms between the bivalve host and the free-living symbiont form, perhaps similar to those which occur between *Rhizobium* species and legume plants (28, 31). New investigations will be necessary to identify these recognition mechanisms and to understand how host-symbiont relationships are unique and invariant in time and in geographical range.

Research is in progress to determine the bacterial transmission mode in other lucinid species sharing the *C. orbicularis* habitat. The understanding of the transmission mode in these other members of the *Lucinidae* may provide relevant information on the relationships between the gill-endosymbiotic bacteria and the bivalve host.

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