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Embryonic development and endosymbiont transmission mode in the symbiotic clam *Lucinoma aequizonata* (Bivalvia: Lucinidae)

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Summary

Lucinoma aequizonata is a large lucinid clam which lives in reducing mud around 500 m deep. Adults harbor intracellular chemoautotrophic sulfur-oxidizing bacteria in specialized gill cells called bacteriocytes. The embryonic and early larval development of *L. aequizonata* is described by using light and scanning electron microscopy. Gametes were obtained by injection of 0.2 ml of 4 mM serotonin solution in seawater into the posterior adductor muscle. The oocytes, 200 μm in diameter, are surrounded by a glycoprotein capsule which gives to the egg a total diameter of 500 μm. The development which occurs at 10 °C is slow. The first polar body is detected 2.5 h after contact between sperm and oocytes (T_0+2.5 h), and the first cleavage begins 10 h later (T_0+12.5 h). The following successive cleavages produce a nonciliated morula, then a ciliated gastrula which begins to rotate within the egg-capsule at T_0+4.5 days. At this stage, the first shell pellicle appears on the dorsal side of the embryo. At T_0+8 days, the trochophore larvae develop discrete ciliary bands which constitute the prototroch. Typical straight-hinge veligers, D-shaped larvae, hatch from the egg-capsule 12 days after fertilization. The newly hatched larvae are 240 μm in length and 200 μm in height, and the straight hinge 150 μm long. To elucidate the symbiont transmission mode, two symbiont-specific primers were designed and used in amplifications by PCR. This primer set was unsuccessful in amplifying symbiont DNA targets from mature gonads, spawned oocytes, eggs, and veligers whereas successful amplifications were obtained from symbiont-containing gill tissues. These data rule out the vertical transmission mode and strongly suggest that the symbionts are environmentally transmitted to the new host generation in *L. aequizonata* as for all tropical lucinids examined to date.

Key words: Embryology, Lucinidae, transmission mode, SEM, PCR

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Introduction

Lucinoma aequizonata is a lucinid clam that lives in reduced mud along the western coast of the Americas from California to Chile (Abbott, 1974). The clam is found burrowed in reduced sediment that contains sporadic pockets of sulfide (Cary et al., 1989). Structural (Distel and Felbeck, 1987) and biochemical (Distel and Felbeck, 1988) studies have shown that the adult clams have intracellular sulfur-oxidizing bacteria in the gill tissue. The endosymbionts utilize energy derived from the oxidation of environmental sulfur compounds to drive carbon fixation. The reduced organic matter is later made available to the heterotrophic host (Felbeck et al., 1981; Cavanaugh, 1983; Felbeck, 1983).

Chemoautotrophs are commonly found as symbionts in bivalves that inhabit reducing environments. They have been found housed in bacteriocytes in all species of Lucinidae, Solemyidae, and Vesicomydae examined. Some species in the families Mytilidae and Thyasiridae and one species of Mactridae have also been reported to contain chemoautotrophs [for review see Reid (1990) and Gros (1997)].

Larval development has been described in several species of Lamellibranchia with as much detail as possible by using light microscopy. Most recent studies use electron microscopy to describe the various steps of development [for review see Gros et al. (1997)].

There has been a heightened interest to obtain larvae and juveniles from animals containing sulfur-oxidizing bacteria. This partly stems from the desire to (1) confirm the symbiont transmission mode inferred from PCR data using mature gonads and (2) study the establishment of the symbiont in host tissue. Progress in this area has been limited due to the difficulty in raising the animals from eggs to the adult stage in the laboratory. Since 1974, only two protobranchs and two eulamellibranchs have been successfully raised; two solemyids *Solemya reidi* (Gustafson and Reid, 1986; 1988a) and *S. velum* (Gustafson and Lutz, 1992), one thyasirid, *Thyasira gouldi* (Blacknell and Ansell, 1974), and one lucinid *Codakia orbicularis* (Gros et al., 1997).

Symbiont transmission studies on the two cultured solemyid species have shown that the symbionts are vertically transmitted to the new host generation and are therefore present in all the life stages of the animal (Cary, 1994; Krueger et al., 1996). The symbiont transmission mode in *T. gouldi* has not been investigated. Recently, Gros et al. (1997) reported that the lucinid *C. orbicularis* could be reared in the laboratory without symbionts using sterilized sand. PCR and TEM observations have shown that the symbionts are acquired from the sediment of seagrass beds during the juvenile stage and are not vertically transmitted from parent to offspring (Gros et al., 1996). To date, this is the only chemoautotrophic system where aposymbiotic animals are available.

The complete development and the symbiont transmission mode in *C. orbicularis* (Gros et al., 1996, 1997) stimulate an interest to compare these data with those obtained from lucinids inhabiting different environments. In the present paper we describe, by using light and scanning electron microscopy, the embryonic development of the Pacific deep water lucinid *L. aequizonata* from spawning to the veliger stage. We used symbiont-specific primers and the polymerase chain reaction (PCR) on mature gonads and newly hatched larvae to infer the symbiont transmission mode in *L. aequizonata*.

Materials and Methods

Sampling

L. aequizonata was collected from the Santa Barbara Channel (34°9′37″ N; 120°01′17″W) in April and (34°09′27″ N, 120°04′27″W) in September 1997 at a depth of 500 m with an otter trawl. The adult clams were kept alive for several months in an aquarium with sediment from their natural habitat and running filtered seawater. The aquarium water temperature ranged from 6°C to 13°C during the course of the experiments.

Rearing

All phases of this study were conducted at 10°C and in 0.45 μm filtered seawater. Before spawning induction the clams were removed from the sediment in the aquarium, their shells were scrubbed clean, and they were held overnight in filtered seawater. To induce release of gametes, each individual was injected into the posterior adductor muscle with 0.2 ml of 4 mM serotonin (5-hydroxytryptamine creatine sulfate complex, Sigma) in 0.22 μm filtered seawater (Matsutani and Nomura, 1982; Gibbons and Castagna, 1984). After injection, the clams were put in individual glass dishes filled with seawater. Spawned oocytes were pipetted into fresh seawater, and they were fertilized with sperm at a density of 15–25 sperm per oocyte. After the appearance of the first polar body, eggs were washed on a nylon screen (100 μm mesh) to eliminate excess sperm, then placed in 2- or 20-l tanks at a density adjusted to 300 egg liter^{-1}. No antibiotics

or aeration were used during the embryonic and larval development. Major bacterial contamination was prevented by changing 50–75% of the water every 3 days.

Oocytes, embryos, and larvae were measured with an ocular micrometer and photographed with an Olympus BH-2 microscope. To observe the jelly coat, a few drops of 2% aqueous Janus Green were added to 5 ml of seawater containing oocytes or embryos [modified from Costello and Henley (1971)]. Embryos and D-shaped larvae were sampled every 10–12 h from fertilization to hatching for scanning electron microscopy (SEM) analysis. They were fixed 2 to 3 h at 4 °C in a solution of 2% glutaraldehyde-1% paraformaldehyde in cacodylate buffer (0.2 M cacodylate buffer pH 7.2, adjusted to 900 mOsM with 0.6 M NaCl). The samples were then rinsed 5 min in the same buffer before dehydration in an ascending acetone series. After critical point drying (Biorad E 3,000), the specimens were mounted on tape, gold-palladium coated in a Technics Hummer sputter coater, and examined in a Cambridge 360 SEM microscope at 20 kV accelerating voltage.

DNA extraction and PCR assay

DNA was extracted from mature reproductive tissues, eggs, and 12-day-old larvae. Ovaries that were light pink in color and plump were considered mature. Testis were considered ripe when they appeared cream in color and full. Samples (~25 mg of each tissue type) were prepared using the QIAmp Tissue Kit from Qiagen according to the manufacturer's instructions. The only exception was that the samples were incubated for 19 h at 55 °C after the addition of proteinase K. DNA extracted from foot and gill tissue served as a negative and positive control, respectively.

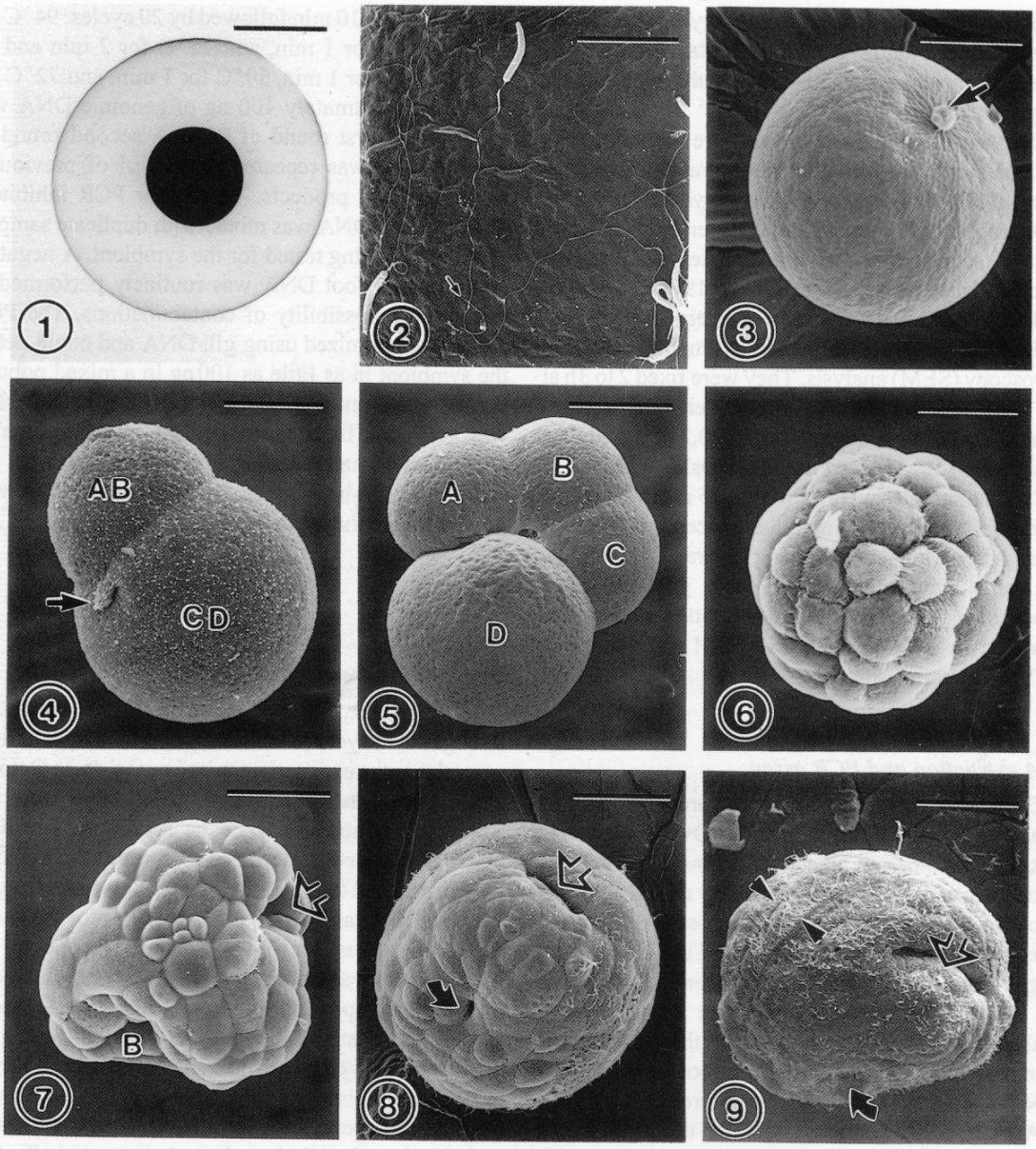
A PCR assay was used to infer the presence or absence of the symbiont in mature reproductive tissue, oocytes, and 12-day-old larvae. Symbiont specific PCR primers were constructed using sequence data taken from the 16S ribosomal DNA (Distel et al., 1994). The primers were designed to amplify 1069 base pairs of the gene: 28F (5′-AGGAGGGCTTGCTCTCTT-3′) and 1097R (5′-CACCACTATGCGCTGCAA-3′). The reaction mixture contained 50 μl of the reaction (1× Perkin Elmer Amplitaq Gold buffer), 2.5 U Amplitaq gold polymerase (Perkin Elmer), 3.0 mM of $MgCl_2$, 10 mM of dNTP mix (Promega), and 30 pmol of each primer. Two rounds of PCR (35 cycles each) were repeated for each DNA sample using a Perkin Elmer thermocycler. The following cycling parameters were used: 94 °C for 10 min followed by 20 cycles: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min and 15 cycles: 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min. Approximately 100 ng of genomic DNA was used for the first round of PCR. A second round of amplification was repeated using 1 μl of previously amplified PCR products. To test for PCR inhibitors 100 ng of gill DNA was mixed with duplicate samples of the DNA being tested for the symbiont. A negative control using foot DNA was routinely performed to rule out the possibility of contaminations. The PCR assay was optimized using gill DNA and could detect the symbiont in as little as 100 pg in a mixed population of symbiont and host gill DNA. Gonadal DNA was amplified from seven females and three males. Due to the small number of veligers obtained per fertilization, D-larvae from different spawnings were pooled before the DNA extraction.

Results

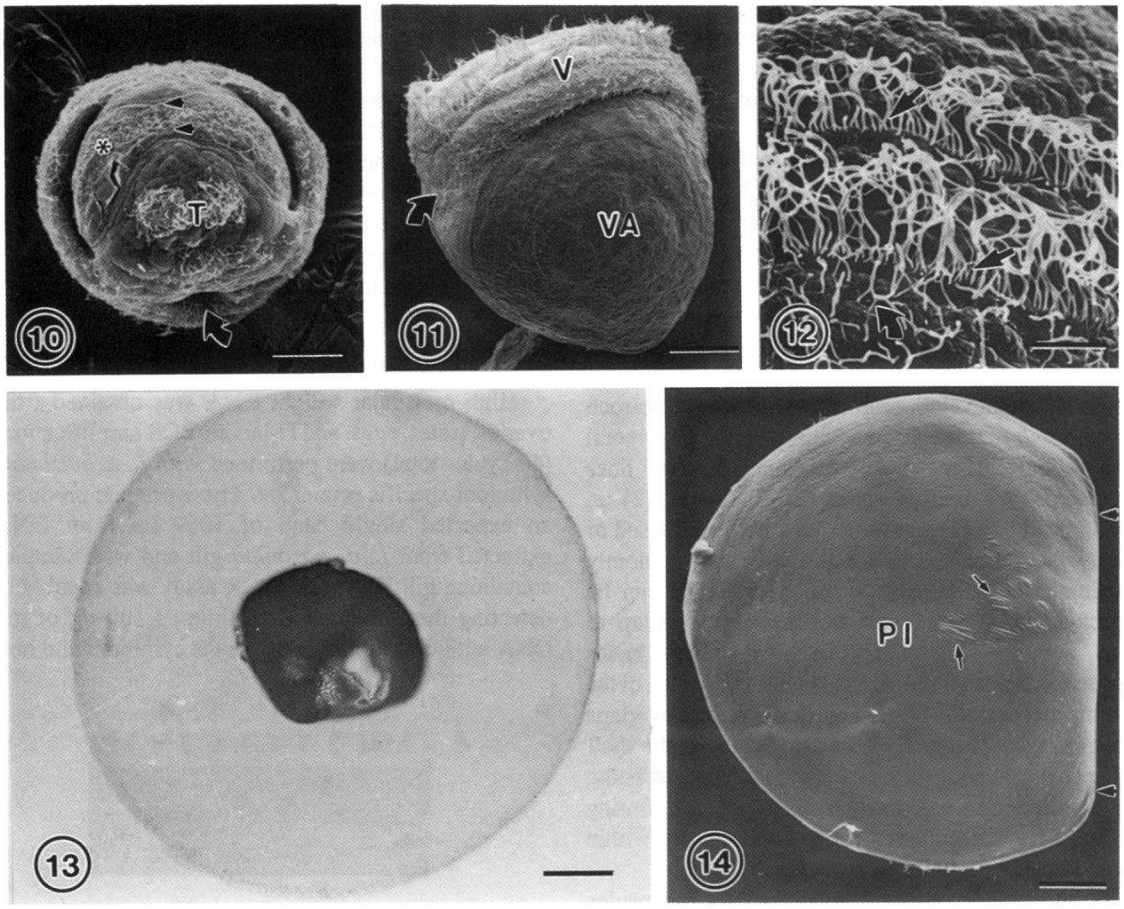
Embryonic development

L. aequizonata responded to serotonin stimulation throughout the year in laboratory conditions, even after several months in aquaria. On one occasion, in October 1996, four females and five males were observed spawning spontaneously in the aquarium. Fertilized eggs were placed in sterile seawater, but the embryos only developed to the 32-cell stage. Serotonin triggers the release of gametes 2 h after injection for the males and at the earliest 4 h for the females. The oocytes are positively buoyant, optically dense and are released as clusters that appear pale yellow to light pink in color. Before fertilization, the oocytes have a yolk-mass diameter of 200 μm and are surrounded by a single layer of jelly giving them a final diameter of 500 μm (Fig. 1). The sperm are most often emitted individually but may occasionally be released as sperm balls. The sperm are motile, upon contact with seawater, with slow pendular movements. They are characterized by a sickle shaped head about 10 μm long from the tip of the acrosome to the distal end of the middle piece. Sperm have an overall length of ~70 μm when measured from the tip of the head to the end of the tail (Fig. 2).

During fertilization, many sperm are found attached to the jelly-coat. The first polar body appears approximately 2 h after the sperm contact (T_0+2 h) in a position opposite to sperm puncture (Fig. 3). The first cell division (T_0+12 h) results in two unequal blastomeres, AB and CD (Fig. 4). The polar body is in



Figs. 1–9. Embryonic stages from fertilization to early trochophores. 1, light microscopy; 2–9, SEM. Fig. 1. Unfertilized oocyte with its typical large jelly coat. Scale bar, 200 μm. Fig. 2. Sperm lying on the surface of an egg. The head is curved and the long tail is devoid of undulating membrane. The end of the tail is indicated by a small arrow. Scale bar, 15 μm. Fig. 3. Egg with the first polar body (arrow) emitted 2.5 h after sperm contact. Scale bar, 60 μm. Fig. 4. Two-cell embryo 12.5 h after fertilization with unequal blastomeres (AB and CD). The polar body (arrow) is in the cleavage plane. Scale bar, 60 μm. Fig. 5. Four-cell embryo with three equal blastomeres (A, B, C) and a larger one (D). Scale bar, 60 μm. Fig. 6. Non ciliated morula 3 days after fertilization (T_0+3 d). Scale bar, 60 μm. Fig. 7. The gastrula, differentiated at T_0+4 d, is characterized by a blastopore (B) and the shell field invagination in the dorsal region (open arrow). Micromeres and macromeres are still well defined. Scale bar, 60 μm. Fig. 8. Late gastrula at T_0+4.5 d with a small round blastopore (curved arrow) anteriorly and crescent shaped shell-field invagination (open arrow) posteriorly. Scale bar, 60 μm. Fig. 9. Early trochophore at T_0+5.5 d. The prototroch consists of two bands of cilia (arrow heads) ; the shell-field invagination (open arrow) begins its evagination and appears as a transverse pad. The blastopore is indicated by a curved arrow. Scale bar, 60 μm.



Figs 10–14. Late trochophore to hatching. Figs. 10–12, 14 SEM. Fig. 13, light microscopy. Fig. 10. Postero-dorsal view of a late trochophore at T_0+8 d. The wrinkled shell organic pellicle (asterisk) is dumbbell shaped; the hinge line is delineated by arrowheads. The mouth is indicated by a curved arrow. T, telotroch. Scale bar, 40 μm. Fig. 11. Intermediary stage between typical trochophore and early veliger at T_0+9.5 d. Ventral view. Mouth, curved arrow; V, velum; VA, valve. Scale bar, 40 μm. Fig. 12. Details of the prototroch showing the double row of long cilia composing the preoral band (straight arrows) and short cilia randomly distributed from the adoral and the postoral bands (curved arrow). Scale bar, 10 μm. Fig. 13. Early veliger at T_0+11 d enclosed in the jelly coat just before hatching. Scale bar, 100 μm. Fig. 14. D-shaped veliger larva just after hatching (T_0+12 d) characterized by a large and smooth prodissoconch I (PI) which has short pointed triangles (small arrows) similar to the stellate marks from the punctate stellate pattern described by Carriker and Palmer (1989) in newly hatched veligers of *Crassostrea virginica*. The long straight hinge line is indicated by arrow heads. Scale bar, 40 μm.

the cleavage plane and no polar lobe was observed. The second cleavage, at right angle to the first, produces the four-cell stage (T_0+27 h) which is characterized by three equal blastomeres (A, B, C) and a larger (D) blastomere (Fig. 5). The AB cell divides first, which results in two equal blastomers, followed by unequal cleavage in the CD cell.

During embryonic development, cell division is slow. Successive cleavages result in a non-ciliated morula (Fig. 6), 3 days after sperm contact (T_0+3 d), then in a non-ciliated blastula. At the initial stage of gastrulation (T_0+4 d), two invaginations can be observed (Fig. 7); the large blastopore positioned at the vegetal pole and the shell field invagination [as defined by Eyster and Morse (1984)] located dorsally to the blastopore. After 4.5 days the embryos begin to rotate slowly within the jelly coat due to the presence of cilia. During gastrulation, the blastopore is displaced anteriorly over the ventral side due to the growth in the dorsal region of the embryo (Fig. 8). After 5.5 days, some cilia increase in length, become organized in a crown of cilia and are visible through the jelly-coat

under a light microscope. The blastopore becomes reduced in size while the shell-material gradually expands in the shell field and appears as transverse pad in the postero-dorsal region of the early trochophore (Fig. 9).

At T_0+8 d, the shell material progressively grows and folds into right and left valves which expand over the body compressing the trochophore laterally (Fig. 10). The first cilia of the telotroch appear in the posterodorsal region behind the shell material which has the appearance of a saddle with a wrinkled surface. The bilateral symmetry becomes obvious and the hinge line is apparent (Fig. 10).

A functional velum originating from the prototroch is composed of three discrete ciliary bands: (1) preoral cilia (about 20 μm long), distributed in two lines separated by a narrow space (20 μm wide); (2) an adoral band and (3) a postoral band both composed of short cilia (about 5 μm long) which are randomly distributed (Fig. 11). No inner preoral cilia can be detected. At T_0+9.5 d, late trochophores develop to early veligers (Fig. 12). The velar ciliary bands increase in density. The mouth is masked by cilia of the adoral and postoral bands and the telotroch overlaps the anal region. From T_0+9.5 d until hatching, the shell valves enlarge until they enclose the whole soft body; the larvae become negatively buoyant and sink. During all this time, the embryos rotate continuously within the enlarged perivitelline space (Fig. 13).

Straight-hinge veligers hatch from the egg capsules 12 days after fertilization. The newly hatched larvae of *L. aequizonata* are ~240 μm in length and ~200 μm in height. The straight hinge is approximately 150 μm long (Fig. 14). The first shell, prodissoconch I, is large, smooth, and has a short pointed triangular pattern. At 10°C, the early veligers do not swim in the water column but seem to lay quietly on the bottom. However, under a light microscope the velum can be occasionally seen protruding from the opened valves or during short movements. During the beginning of their larval development, veligers do not need to be fed. They are nourished by the numerous vitelline platelets derived from the yolk mass of the oocyte. Five weeks after hatching, the mean size of larvae is similar to newly hatched D-shaped larvae resulting from poor growth during the larval development. Such 5-week-old larvae still possess abundant vitelline platelets and there is no hint of pedal anlage. On several trials, larvae died progressively from that stage on and we could not obtain metamorphosis.

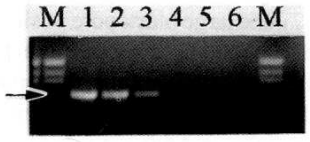


Fig. 15. PCR experiment. Sensitivity of the *L. aequizonata* symbiont specific primers after 2×30 cycles of amplification. Lanes: M, DNA markers; 1, 100 ng of gill DNA; 2, 10 ng of gill DNA; 3, 1 ng of gill DNA; 4, 100 pg of gill DNA; 5, 10 pg of gill DNA; 6, 1 pg of gill DNA.

Gill endosymbiont transmission mode

High molecular weight DNA was obtained from ovaries, testes, eggs, and D-larvae. PCR amplifications (60 cycles total) were performed with *L. aequizonata* symbiont-specific primer set. These primers produced an expected single band of 1069 bp from DNA extracted from *L. aequizonata* gill and with samples containing gill DNA. The PCR assay was capable of detecting the symbiont in as little as 100 pg of gill DNA with the genomes of both the symbiont and host

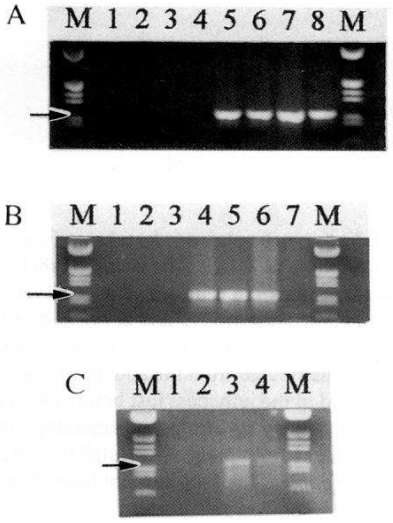


Fig. 16. PCR detection of the 1069 bp *L. aequizonata* symbiont targets in ovaries (A), testes (B), and newly hatched D-larvae (C). Arrows in all panels point to the 947 bp fragment. (A) Lanes: M, DNA markers; 1, female #1; 2, female #2; 3, female #3; 4, female #4; 5, female #1+gill DNA; 6, female #2 + gill DNA; 7, female #3 + gill DNA; 8, female #4 + gill DNA. (B) Lanes: M, DNA markers; 1, male #1; 2, male #2; 3, male #3; 4, male #1 + gill DNA; 5, male #2 + gill DNA; 6, male #3 + gill DNA. (C) Lanes: M, DNA markers; 1, 60 ng of larval DNA; 2, 100 ng of larval DNA; 3, 60 ng of larval DNA + gill DNA; 4, 100 ng of larval DNA + gill DNA.

(Fig. 15). Despite repeated attempts, no amplification products were detected in foot (negative control), ovaries, testes, oocytes and D-shaped larvae (Fig. 16).

Discussion

Embryonic development

Spawning induction, which is the crucial step for any experimental development, can be obtained successfully in the lucinid *L. aequizonata* through the injection of serotonin solution into either adductor muscles or into the visceral mass. Serotonin stimulation has been shown to induce spawning in several marine bivalves (Matsutani and Nomura, 1982; Gibbons and Castagna, 1984; Frenkiel and Mouëza, 1988; Gros et al., 1997). It is a powerful spawning inducer in the two lucinid species *L. aequizonata* (this study) and *Codakia orbicularis* (Frenkiel and Mouëza, 1988; Gros et al., 1997). However, not all the lucinid species release gametes when exposed to serotonin. For example, induced spawning could not be obtained with serotonin injection in the lucinids *Linga pensylvanica* and *Lucina pectinata* (pers. obser.) during the entire breeding period.

Serotonin induced release of gametes for both sexes in *L. aequizonata*. The sperm balls observed following the injection of serotonin solution are similar to those observed from *C. orbicularis* (Alatalo et al., 1984). They are most likely immature sperm attached to their nurse cells. Such emissions could potentially be reduced by lowering the dose of serotonin as was found in *C. orbicularis* (Frenkiel and Mouëza, 1988). The spermatozoon of *L. aequizonata,* with an overall length of 70 μm from head to tail, is as long as the longest bivalve sperm previously described in *Scrobicularia plana* (Sousa et al., 1989). No ultrastructural studies were performed on the sperm, but scanning electron micrographs indicate that the tail of the sperm of *L. aequizonata* is devoid of the undulating membrane that is found along the tail of the *C. orbicularis* sperm (Mouëza and Frenkiel, 1995). This finding is not surprising since other tropical lucinids such as *L. pensylvanica* and *L. pectinata* (Mouëza, pers. com.) are also devoid of an undulating membrane. The middle piece of the sperm from *L. aequizonata*, which is not well defined in SEM pictures, is probably composed of elongated mitochondria as described by Mouëza and Frenkiel (1995) in *C. orbicularis*.

Oocytes of *L. aequizonata* are surrounded by a large jelly-coat. A similar structure has been described in several bivalve families including Articidae (Lutz et al., 1982), Cardiidae (Creek, 1960), Laternulidae (Ansell and Harvey, 1997), Lucinidae (Alatalo et al., 1984; Gros et al., 1997), Pandoridae (Allen, 1961), Pectinidae (Hodgson and Burke, 1988), Semelidae (Hughes, 1971; Frenkiel and Mouëza, 1979), Solemyidae (Gustafson and Reid, 1986, 1988a; Gustafson and Lutz, 1992), Thyasiridae (Blacknell and Ansell, 1974), and Veneridae (Loosanoff and Davis, 1950; Ansell, 1961). In *L. aequizonata*, the jelly coat appears to be composed of only one layer whereas in *C. orbicularis* the jelly coat was composed of an inner layer overlying the vitelline membrane and an outer layer distinguishable using phase contrast (Gros et al., 1997). This jelly coat, already present around mature oocytes in the ovary, is probably composed of glycoproteins and proteoglycans synthesized by the oocyte itself during vitellogenesis, as described in *L. pectinata* by Frenkiel et al. (1997).

Spawned oocytes are positively buoyant, so they concentrate at the top of the tanks even after fertilization and during the first week of embryonic development. It would be interesting to check the presence of encapsulated embryos at various depths by using plankton hauls. In the wild, eggs and encapsulated embryos that are positively buoyant might be involved in passive transport by water currents. This would provide an effective dispersal phase in the life cycle and could provide a way for a wide distribution of larvae along the Californian coasts. This is the first report of positively buoyancy of oocytes in the bivalve superfamily Lucinacae. However, it is difficult to ascertain whether it is related to the particular environment of *L. aequizonata*. The positively buoyancy of the oocytes could be due to the abundance of lipidic yolk platelets and/or to the nature of the jelly coat. Biochemical analysis will be necessary to compare the composition of the positively buoyant eggs of *L. aequizonata* with that of negatively buoyant eggs of other shallow water lucinid species. Embryos of *L. aequizonata* become progressively negatively buoyant and sink returning to the adult habitat. This phenomenon could be due to (1) the use of vitelline lipids during the development, (2) the modification of hydration of the proteoglycans of the jelly coat, and/or (3) the development of the shell.

An important step in the organogenesis of bivalves is the shell formation which occurs during the second part of embryonic development. However, only a few studies describe this process. Shell field invagination has been described in early trochophores of *Spisula solidissima* (Eyster and Morse, 1984). Conversely,

Gros et al. (1997) described it in early gastrulas before the differentiation of the prototroch in the lucinid *C. orbicularis*. Mouëza et al. (1998) also described the same developmental sequence in the venerid *Anomalocardia brasiliana*. In *L. aequizonata*, shell field invagination begins at the gastrula stage well before the differentiation of the prototroch and has a morphogenetic sequence similar to that observed in lucinid and venerid species. Then, the shell material evaginates in early trochophores as a transverse pad which becomes dumbbell-shaped before enclosing the soft body of the early veliger.

In the late trochophores or early veligers, the velum appears to be composed of three distinct ciliary bands (preoral, adoral, and postoral bands), which are very similar to those previously described in the velum of the tropical lucinid *C. orbicularis* (Gros et al., 1997). However, in most bivalve species studied, the velar crown is generally composed of four distinct ciliary bands: an inner preoral band made of single short cilia, an outer preoral band made of a double row of long cirri, an adoral and a postoral bands both made of single cilia randomly distributed (Waller, 1981; Hodgson and Burke, 1988; Cragg, 1989; Mouëza et al., 1999). However, there are not enough descriptions available to really conclude if the velum without the inner preoral band is specific to the bivalve family Lucinidae.

From the fifth day of embryonic development, the embryos rotate slowly but continuously in an enlarged perivitelline space until hatching. In all species known to have an intracapsular development, the duration of encapsulation (from 18 h to more than 50 days after fertilization) and the developmental stage at hatching (from gastrula to fully metamorphosed juveniles) are variable without apparent familial or environmental trends [for review, see Gros et al. (1997)]. *L. aequizonata*, which hatches as a veliger D-shaped larva after 12 days of embryonic development at 10 °C, show a similar duration of the embryonic development as other cold-water bivalve species. Thus, *Thyasira gouldi* reaches the trochophore stage in 4 days and the shelled larval stage 12 days after fertilization at 10 °C (Blacknell and Ansell, 1974) compared with 5 days and 18 days for *Laternula elliptica* at 0 °C (Ansell and Harvey, 1997). Whereas the embryonic development is slower for *L. aequizonata* than for *C. orbicularis*, both developmental patterns look similar.

Five weeks after hatching, larvae are still D-shaped with a straight hinge and a shell length similar to that of newly hatched veligers meaning a poor growth of the prodissoconch II which is limited to a narrow fringe around the prodissoconch I. According to the large diameter of the oocytes with an abundant vitelline supply and to the slow growth during larval development, the development of *L. aequizonata* may be classified as lecithotrophic. According to Ockelmann (1965), lecithotrophic development is correlated with a short pelagic life and with the absence, or poor development, of the prodissoconch II. The development of *L. aequizonata,* while possessing features of lecithotrophic development, is characterized by a long planktonic life. Such a developmental pattern appears similar to that described in *C. orbicularis* (Gros et al., 1997) and observed also in other lucinid species as *L. pensylvanica* and *L. pectinata* (Mouëza, pers. comm.). However, the rearing conditions did not meet *L. aequizonata* larval needs well enough to obtain metamorphosis. Thus, we cannot ascertain whether the developmental pattern described for *C. orbicularis* may be generalized for the family Lucinidae.

Gill endosymbiont transmission mode

Previous studies of bivalves harboring sulfur-oxidizing bacteria in their gill cells demonstrated two types of bacterial symbiont transmission. A vertical transmission mode has been reported in the solemyids *Solemya reidi* (Cary, 1994) and *S. velum* (Krueger et al., 1996), and the vesicomyids *Calyptogena magnifica*, *C. phaseoliformis*, and *C. pacifica* (Cary and Giovannoni, 1993; Cary et al., 1993), and an environmental transmission has been recently demonstrated in the tropical lucinid *Codakia orbicularis* (Gros et al., 1996).

The primer set for *L. aequizonata* symbiont specifically amplifies target sequence from bulk DNA extracted from *L. aequizonata*. This result was expected because of the previous phylogenetic characterization *L. aequizonata*-endosymbiont based on 16S rDNA sequence analysis (Distel et al., 1988, 1994). The detection limit of our PCR experiments was about 100 pg target-DNA in a mixture of eukaryotic and prokaryotic DNAs extracted from symbiont-containing host tissue. We worked with a mixed genomic DNA for which the ratio prokaryotic DNA/total (eukaryotic and prokaryotic) DNA was unknown. Comparable PCR experiments have been performed with vertically transmitted symbionts and no problem of detection occurred in the bivalves gonads with template concentration similar or below to those used in this study (Cary et al., 1993; Cary, 1994).

The fact that the symbiont 16S ribosomal gene was not amplifed from ovaries, testis, eggs, and D-larvae

strongly suggests that the transmission mode of the *L. aequizonata*-symbiont is not vertical but environmental as for the five other lucinid species analyzed to date (Gros et al., 1996, 1998a). The infection of *L. aequizonata* with its symbiont is probably similar to the symbiont acquisition in *C. obicularis*. In the case of *C. obicularis*, the symbiont is acquired from a free living stock during the juvenile stage (Gros et al., 1996, 1998b). The absence of PCR product from the endosymbionts in eggs and in 12-day-old newly hatched veligers rules out the vertical transmission mode of chemoautotrophic bacterial symbionts in *L. aequizonata*. It is important in research on the symbiont transmission mode in marine invertebrates harboring uncultivable symbionts to check not only the gonads but also the eggs and larvae. Spawned oocytes help us rule out the hypothesis of a symbiont transfer from the female hosts during spawning. The ability to check fertilized eggs helps rule out the possibility that the sperm contribute the symbiont during the fertilization process. Finally the availability of D-larvae indicates that the symbionts are not brought by host gametes or do not infect embryos during the first cleavages of the embryonic development. In summary, the ability to check gonads, eggs, and larvae allows us to be more definite concerning the symbiont transmission mode for *L. aequizonata* than for other lucinid species in which fertilization and development are not available (Gros et al., 1998a).

Even though serotonin is a good spawning inducer for *L. aequizonata*, the number of oocytes emitted is limited to a few thousand per female against 2–6 million oocytes per female in *C. obicularis* (Frenkiel and Mouëza, 1988). Moreover, the developmental rate is lower in *L. aequizonata*. Thus, at the end of the embryonic development, which is the longest known in lucinids, we were never able to obtain more more than 100 or 200 D-larvae per batch. This number is too small to obtain complete development and to get enough juveniles to perform experimental infection to confirm the symbiont transmission mode in *L. aequizonata*.

In the present paper, the environmental transmission mode of the gill-endosymbiotic bacteria was strongly suggested in the lucinid *L. aequizonata* by using PCR amplifications of symbiotic DNA from mature gonads, oocytes, and newly hatched veligers. The results obtained from a lucinid species living in a very different environment to that of the tropical lucinids previously analyzed are consistent with the hypothesis put forward by Gros et al. (1998a) according which the symbiont transmission mode could be specific to the host bivalve family independent of environmental conditions or host metabolic pathways.

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