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Endosymbiotic bacteria in the bivalve *Loripes lacteus*: Localization, characterization and aspects of symbiont regulation

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ABSTRACT

Loripes lacteus inhabits reduced sediments in shallow waters and harbors chemotrophic sulfur-oxidizing bacteria in its gills. In this study, a combination of molecular (16S rRNA analysis, real time PCR and fluorescence in-situ hybridization) and microscopy approaches (confocal and transmission electron microscopy) was used to characterize this symbiotic association and evaluate factors potentially involved in endosymbiont acquisition and regulation. 16S rRNA sequencing revealed the presence of two phylotypes of endobacteria specifically associated with clam gills. The more abundant phylotype is a close relative to thiotrophic symbionts found in several symbiotic *Lucinidae*, while the second phylotype clustered with bacteria not known to be involved in symbiotic association. Endobacteria were not detected in other organs including gonads strongly suggesting a horizontal mode of transmission of symbionts to aposymbiotic juveniles. Clams were exposed to 3 weeks of starvation, which caused a significant decrease in the relative abundance of both phylotypes as compared to fresh animals suggesting a lytic degradation of endobacteria within clam gills. In addition, endosymbiont-depleted clams were not able to re-acquire their symbionts once moved back to "optimal" conditions. These results highlight the need for a better understanding of the molecular mechanisms that mediate acquisition, regulation and control of endosymbiotic bacteria by *L. lacteus*.

1. Introduction

Chemoautotrophic symbioses in marine environment have been described in a wide range of habitats including shallow-water sediments, continental slope sediments, whale and wood falls and deep-sea hydrothermal vents (Cavanaugh et al., 2006; Dubilier et al., 2008). The number of groups of marine invertebrates that have developed symbiosis with chemosynthetic symbionts is considerable and underestimated due to the difficulty to explore deep-sea areas (see review by Dubilier et al., 2008 for more details). Among these groups, sponges, corals, platyhelminthes, nematodes, mollusks, annelids, or arthropods are representatives of hosts implicated in chemosynthetic symbiosis (Bulgheresi et al., 2006; Cary et al., 1997; Duperron et al., 2005; Lema et al., 2012; Morrow et al., 2012; Ott et al., 2004; Williams and McDermott, 2004).

In the same way, symbionts are also diverse as shown by their phylogeny, their metabolism or their mode of transmission (Dubilier et al., 2008). Progress in molecular biology methods, based on the sequencing of 16S rRNA gene, provided hints about the diversity of symbiotic proteobacteria (Brissac et al., 2009; Distel et al., 1988). Among these symbionts, some are able to use methane (methanotrophic symbionts or MOX) or hydrogen sulfide (sulfur-oxidizing symbionts or SOX) as energy source to produce biomass; some have been found to be located inside the host tissue (endosymbionts) and others (ectosymbionts) are closely associated to mucus covering external epithelia (Bulgheresi et al., 2006; Dufour, 2005). Some mollusk species harbor either a single endosymbiont strain, like *Bathymodiolus thermophilus* (SOX bacteria) and *Bathymodiolus childressi* (MOX bacteria) (Childress et al., 1986; Nelson et al., 1995) or have two symbionts (e.g. SOX and MOX bacteria), among them *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (Duperron et al., 2006, 2009; Fiala-Medioni et al., 2002). The mode of transmission to juvenile hosts and the level of interdependence of the two partners is another important "trait" used to characterize symbiosis. The transfer of symbionts from one generation to the following can be the result of either vertical or horizontal transmission. In vertically-transmitted symbiosis, symbionts are transmitted from parents to descendants mostly through the female germ line and the association between the host and the symbionts is permanent (Bright and Bulgheresi, 2010). Numerous examples of vertical transmission in invertebrates (Bright and Bulgheresi, 2010; Nakabachi et al., 2005) and more specifically in bivalves (Cary, 1994; Endow and Ohta, 1990; Krueger

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et al., 1996) have been documented. In the case of horizontal transmission, often facultative, aposymbiotic hosts acquire their symbionts directly from the environment (Nyholm and McFall-Ngai, 2004; Salerno et al., 2005).

Loripes lacteus Linnaeus, 1758 (synonym *L. lucinalis* Lamarck 1818) is a small bivalve from the group of *Lucinidae* harboring endosymbiotic bacteria localized in specialized gill cells, the bacteriocytes (Herry et al., 1989; Johnson and Fernandez, 2001; Johnson and Le Pennec, 1994). Based on enzymatic activity studies (especially the APS-reductase activity, see details in Cavanaugh, 1985), Herry et al. (1989) classified *L. lacteus* symbionts as chemoautotrophic sulfide-oxidizing bacteria. Like other lucinids, *L. lacteus* is a suspension filter-feeder also able to use metabolites released by bacterial symbionts (Herry et al., 1989; Johnson and Fernandez, 2001), and is suspected to digest its own symbionts as a mean of nutrient transfer (Johnson and Fernandez, 2001). Overall, the species (via their symbionts) is thought to contribute a significant proportion of seagrass bed's primary production (roughly 16%, see the discussion in Johnson et al., 2002).

Unlike symbiotic deep-sea species, *L. lacteus* is an easily accessible model to study chemosymbiosis. This species can be found in coastal areas from the intertidal zone to 150 m depth. Its geographical repartition includes the Mediterranean area and the east coasts of the Atlantic Ocean, from the Canary Islands to the Isle of Man (Allen, 1958). Another advantage of using *L. lacteus* as a model is the possibility of performing physiological experiments without any constraints of pressure or temperature making this species highly valuable. Previous studies have confirmed the symbiotic nature of *L. lacteus* (Herry et al., 1989; Johnson and Fernandez, 2001; Johnson and Le Pennec, 1994; Le Pennec et al., 1988; Mausz, 2008) but to our knowledge no recent research was done to characterize symbiont dynamics under various physiological conditions.

In this study, we used contemporary techniques to (1) determine symbiont(s) phylogeny and to (2) precisely localize their distribution in *L. lacteus* tissue. We further investigated symbiont dynamics (loss and re-acquisition) in response to environmental changes to better characterize this symbiotic association and determine symbiont mode of transmission.

2. Materials and methods

2.1. Clams and sediment

L. lacteus were sampled from Roscoff harbor on the English Channel (Brittany, France) in fall 2010. Clams were collected at low tide by digging reduced sediments from sea grass beds. Bivalves were randomly subdivided into 3 different groups. The first 2 groups were immediately used for DNA amplification (10 clams) and in situ hybridization studies (10 clams). Clams from the last group were used in a set of starvation and re-acquisition experiments (see below). In addition, sediments (3×50 ml tubes) were collected from the clam sampling site, and stored at −80 °C until DNA extraction.

2.2. DNA extraction and amplification

Ten clams were immediately dissected to separate gills (known to be the main organ harboring symbionts in *L. lacteus*; Herry et al., 1989), the visceral mass including gonads (organ known for symbiont storage in bivalves with vertical transmission of symbionts) and mantle (expected to be symbiont-free and used as negative control for real-time PCR). To limit external contamination of tissues with bacteria, samples were washed in a 5% hypochlorite in sea water for 3 min, and finally rinsed twice with filtered and sterilized sea water (FSW, 0.22 um). Samples were then stored at −80 °C or immediately processed as described below. Total DNA from symbiotic clams was extracted using the CTAB method according to the protocol described in Boutet et al. (2011).

In addition, DNA was extracted from reduced sediments using the NucleoSpin® Soil kit (Macherey-Nagel, Inc, Pennsylvania, USA) following the manufacturer's instructions. Public *L. lacteus* nucleotide databases available at the National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA) were screened for 16S rRNA sequences. Eight bacterial sequences (generated using universal 16S rRNA primers, see Fig. 4 for accession numbers) representing two *L. lacteus* gill symbionts (Mausz, 2008) were used to design 2 pairs of specific primers (Clone1 and 2 — forward and reverse, Table 1). Templates (DNA from clams or sediments) were then amplified to generate products for cloning following the general protocols described in Pales Espinosa et al. (2010). Briefly, the PCR reaction was carried out in an Eppendorf Mastercycler (ep gradient S) using GoTaq® DNA Polymerase (Promega, Madison, Wisconsin, USA) for 10 min of initial denaturation, followed by 35 cycles of denaturation (95 °C, 30 s), annealing (50 °C, 30 s), and extension (72 °C, 1 min), with an additional 10 min primer extension after the final cycle. Eight clones of each amplification (i.e. Clone 1 and 2) were then sequenced using an ABI 3730 automatic capillary sequencer and the ABI BigDye Terminator v.3.1 sequencing kit. The result of the sequencing of the 2 clones showed that the initial primers were specific and the design of new primer sets was not necessary.

2.3. Phylogeny

Partial 16S rRNA sequences of bacteria detected in *L. lacteus* gills were compared to bacterial sequences previously obtained from mollusk hosts living in different marine habitats (shallow and deep sea waters, 30 sequences total) including *L. lacteus* from Croatia, *Lucinidae*, *Mytilidae*, *Solemyidae*, *Thyasiridae*, and *Vesicomyidae* (see the review by Dubilier et al., 2008).

All sequences (43 in total) were imported into BioEdit (v7.0.9., Hall, 1999), aligned and cleared of ambiguous regions (i.e. containing gaps and/or poorly aligned). Average sequence length was 1220 bp except for some *L. lacteus* samples (13 sequences) for which only 385 bp was obtained. Modeltest (v3.6., Posada and Crandall, 1998) was then applied and results showed that the GTR + I + G model with AICc = 12199.43 was the best fitting one for maximum likelihood (ML) analysis. The analysis of the sequences was then performed using the GTR model implemented in the PhyML program (v2.4.4., Guindon and Gascuel, 2003). Reliability of internal branches was assessed using the bootstrapping method (100 bootstrap replicates). Graphical representation and edition of the phylogenic tree were performed with Treeview (v1.6.6., Page, 1996).

2.4. Real-Time PCR

The relative quantity of the two bacteria phylotypes in clam organs (i.e. gills, gonad, and mantle) was determined using Real-Time PCR

Table 1
List of primers and fluorescent in situ hybridization (FISH) probes used for the detection and quantification of *Loripes lacteus* endobacteria.

Primer/probe names	Primer sequences (5′–>3′)	References
RT-PCR and quantitative real-time PCR primers		
Clone1-16S-F (forward)	CAGCAGTGGGGAATATTGGAC	This study
Clone1-16S-R (reverse)	AAAGTTAACGTCAAGACCCAAGGT	This study
Clone2-16S-F (forward)	AGTAACGCGTAGGAATCTGC	This study
Clone2-16S-R (reverse)	ACTCTCCGAAAAGAACGGATC	This study
18S-F (forward)	CGCCGGCGACGTATCTTTCAA	This study
18S-R (reverse)	CTGATTCCCCGTTACCCGTTAC	This study
FISH probes		
LOR1-192	GGAAAGCGGGGGATC	This study
LOR2-196	AAGAACGGATCCCCTGCT	This study
EUB338 (positive control)	GCTGCCTCCCGTAGGAGT	Amann et al. (1990)
NON338 (negative control)	ACTCCTACGGGAGGCAGC	Wallner et al. (1993)

(qPCR) which is considered to be more reliable and accurate than fluorescent in situ hybridization (FISH) for the quantification of endosymbiont abundance in marine invertebrates (Loram et al., 2007), including bivalves (Fink, 2011).

Assays were carried out in a BioRAD Opticon Real-Time PCR with 4.6 μl of 1:200 diluted DNA (250 ng). The amplification was performed in a 10 μl reaction volume containing 2× Fast EvaGreen® qPCR Master Mix (Biotium, California, USA) and 100 nM of each specific primer (Table 1). The amplification was carried out as follows: initial enzyme activation at 94 °C for 15 min, then 40 cycles of 94 °C for 15 s and 60 °C for 1 min. A melting curve was generated at the end of each assay for quality control. PCR efficiency (E) was determined for each primer pair by determining the slope of standard curves obtained from serial dilution analysis of DNA. A fragment of 18S gene from the host was used as an internal PCR control. To compare the relative quantity (RQ) of each symbiont type in the three tissues, gill, gonad and mantle, we used the comparative Ct method (Livak and Schmittgen, 2001) using the formula $RQ = 2^{-\Delta Ct}$ ($\Delta Ct = Ct_{16S} - Ct_{18S}$). Results are displayed as relative RQ (RQ*ic*) using the highest average RQ (RQ*max*) within each experiment as calibrator ($RQic = 100 \times RQi/RQmax$; RQ*i* is individual RQ) although all statistical testing (1- or 2-way ANOVA followed by Holm–Sidak post-hoc test as appropriate) were performed on ΔCt. Differences were considered significant at $p < 0.05$.

2.5. Fluorescence in situ hybridization (FISH)

The *L. lacteus* endobacteria probes were designed following the guidelines set by Behrens et al. (2003) and labeled with Cy3 (Table 1, Eurofins MWG Operon, Germany). The general *Bacteria* probe EUB338 (Amann et al., 1990) was used as a positive control and the NON338 probe (Wallner et al., 1993), an oligonucleotide complementary to the probe EUB338 served as a negative control for background autofluorescence (Table 1, Biomers, Germany).

Clam tissues were fixed in 3% formalin for 48 h before being dehydrated in an ascending ethanol series, embedded in paraffin blocks and cut into serial sections (5 μm thickness). Six consecutive sections were processed for standard hematoxylin–eosin staining (1 section) or for fluorescence in situ hybridization (5 sections) following methods adapted from Duperron et al. (2005). Briefly, the latter sections were deparaffinized in xylene, rehydrated through a descending ethanol series, and equilibrated in Tris 20 mM, pH 8. Two serial sections were separately hybridized with the two probes designed for *L. lacteus* symbionts (100 ng in 30 μl) in hybridization buffer (900 mM NaCl, 20 mM Tris pH 8, 0.01% SDS, 30% formamide; stringency was previously optimized for all the tested probes). In addition, three sections were used as controls and hybridized with the general probe EUB338 (positive control), the NON338 probe and nothing was added to the last section (negative controls).

Tissues were incubated for 3 h at 46 °C and then washed with preheated washing buffer (100 mM NaCl, 20 mM Tris pH 8, 0.0001% SDS, 5 mM EDTA) at 48 °C and finally washed with sterile water. Slides were covered with Citifluor (Citifluor Ltd, United Kingdom) or VectaShield (Vector Labs, California) and examined using confocal microscopes (Roscoff Biological Station: Confocal Leica TCS SP5 AOBS; Stony Brook University: Confocal Zeiss LSM 510 META NLO).

2.6. Transmission electron microscopy (TEM)

Clam gills were fixed in 4% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4 adjusted to 1100 mOsmol L^{-1} with sucrose and NaCl) for 6 h at 4 °C. After fixation, tissues were washed overnight in 0.2 M cacodylate buffer containing graded concentration of sucrose buffer. Samples were then postfixed in 1% osmium tetroxide in the same buffer for 1 h at 4 °C, rinsed with 0.2 M cacodylate buffer and dehydrated through a graded ethanol series and embedded in Epon 812. Polymerization was carried out at 60 °C for 24 h. Ultrathin serial sections were cut with a diamond knife on an ultramicrotome (Leica, ultracut UCT) and were mounted on copper grids. After staining with 2% uranyl acetate for 10 min and 2% lead citrate for 3 min, the grids were examined with a Jeol 1400 transmission electron microscope.

2.7. Effect of starvation on symbiont quantity

One hundred and eighty clams were randomly divided into four equal groups and submitted to the following treatments. Two groups were starved by placing them in 10-liter aquariums filled with aerated and filtrated seawater (FSW: 0.22 μm, salinity of 30) renewed twice a week. Two other groups were placed in 10-liter aquariums filled with aerated and partially filtrated seawater (20 μm) and were fed daily (15% dry weight) using fresh microalgae *Isochrysis* sp. Experiments were conducted at a temperature of 11 ± 1 °C. At the beginning of the experiment (control; 20 specimen) and after 1, 3, 5 and 8 weeks (10 specimen from each group), clams were sampled and gills were dissected, flash-frozen and conserved at −80 °C until processing for real-time PCR and in situ hybridization.

2.8. Re-acquisition of symbionts after starvation

Results of the starvation experiment were used to design an experiment assessing the ability of clams to re-acquire symbionts from their environment. Forty clams were starved for 5 weeks, randomly divided into two equal groups and placed in 10-liter aquariums filled with running (flow-through) but partially filtered seawater (20 μm). Daily, about 500 g of reduced sediments from sea grass beds harboring *L. lacteus* was collected and washed with seawater. The resulting supernatant (500 ml) was supplemented with sodium sulfide (1 μM Na2S, 9H2O, Gros et al., 1997) and added to the aquarium as well as fresh *Isochrysis* sp. (15% dry weight). In addition, gills from freshly collected clams were collected, ground, and added to the tanks (Gros et al., 2003) at about 10 mg wet weight L^{-1}. Running seawater was stopped for 16 h. The following day, running seawater was turned on again for 8 h but the settled sediment was not discarded. Experiment was conducted at 11 ± 1 °C and the different steps of the treatment were repeated daily for 3 weeks. At the beginning of the experiment (control) and after 3 weeks, 10 clams from each group were sampled and gills were dissected, flash-frozen and conserved at −80 °C until processing for DNA extraction and bacteria quantification by real-time PCR.

3. Results

3.1. Bacteriocytes and endobacteria

The gill filaments of *L. lacteus* present two short ciliated zones (frontal and abfrontal) separated by a long lateral zone (Fig. 1A) containing different types of cells including bacteriocytes, mucocytes (Fig. 2A and B) and intercalary cells (Fig. 3A). Bacteriocytes are large cells (about 20–30 μm) presenting a nucleus, often in a basal position, and a cytoplasm containing several different organelles such as mitochondria, granules of glycogen, globules of different sizes and numerous vacuoles enclosing only one symbiont (Fig. 3A, B and D). Coccoid endobacteria present various sizes and are numerous within bacteriocytes (Fig. 3B and D). The nucleoid, displaying filamentous appendices, is visible in the cytoplasm (Fig. 3C) as well as translucent granules which are thought to be sulfur granules (Dando et al., 1985) and several electron dense granules. Endobacteria present a double membrane, characteristic of Gram negative bacteria. Smallest bacteria (0.5 to 3 μm) are electron-translucent and often localized at the apical pole of the bacteriocytes (Fig. 3B). Larger bacteria (3 to 5 μm) are more electron-dense and are located at the basal pole of the cell. Most basal bacteria appear to be undergoing lysis and intracellular digestion as shown by loss of cellular structure (Fig. 3D, E). Resulting degenerative vacuoles fusion together to produce large inclusion bodies (round to oval shape, 10 to 15 μm)

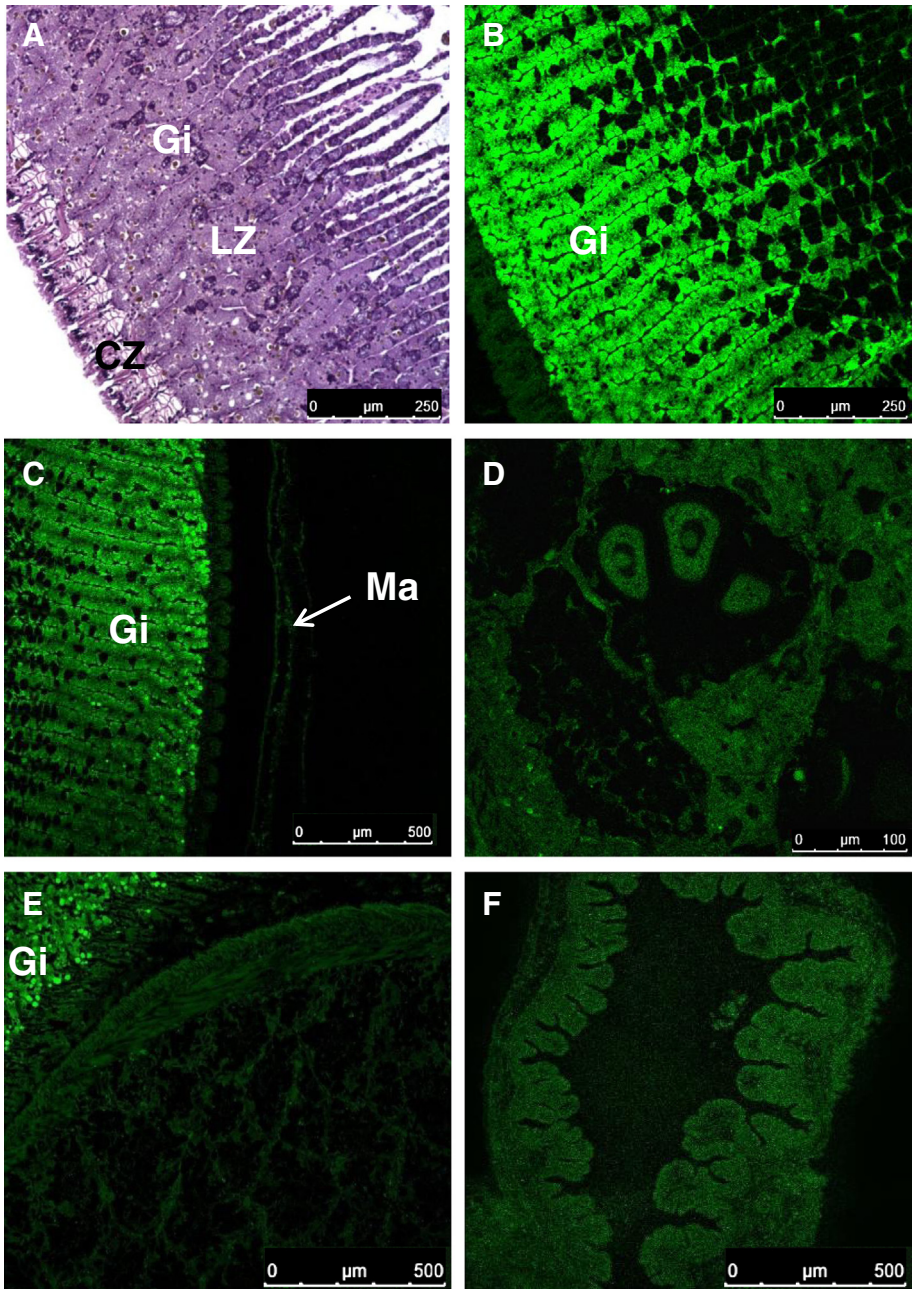

Fig. 1. Light and fluorescence micrographs of *Loripes lacteus*. (A) Hematoxylin–eosin staining of a transversal section of gill filaments (Gi) presenting a ciliated zone (CZ) and lateral zone (LZ) containing bacteriocytes (bright fluorescence). Fluorescence in situ hybridization (FISH, using LOR1 listed in Table 1) images of gill filaments (B) and mantle (Ma in C), gonad (female: D; male: E) and digestive tract (F).

that display a yellowish color in hematoxilin–eosin stained sections (Fig. 2C). In TEM, these bodies appeared as electron dense granules of various sizes (Fig. 3D, E and F).

3.2. 16S rRNA Phylogeny of *L. lacteus* endobacteria

Phylogenetic analysis of the endobacteria of *L. lacteus* based on the 16S rRNA gene (GenBank accession no. XXX00000 in progress) showed that the Roscoff clam population contains at least two phylotypes (named Clone 1-Roscoff and Clone 2-Roscoff) both being included in the subdivision of the γ-Proteobacteria with a high bootstrap support value (Fig. 4). One phylotype (Clone 1-Roscoff) formed a highly supported clade (91%) with previously sequenced *L. lacteus* symbionts (clones 1Aa, 2Aa, 1Bb, 2Bb, 1C, 2C) isolated from clams sampled in Rovinj seagrass bed, Croatia (unpublished results submitted to NCBI in 2009 by Mausz et al.) and with other thiotrophic symbionts of *Lucinidae*. The closest relatives of this group were found to be thiotrophic symbionts of several *Lucinidae* such as *Lucina floridana* (Distel et al., 1988) and *Myrtea spinifera* (Brissac et al., 2011; Dando et al., 1985). The second phylotype (clone 2-Roscoff) clustered outside the clade comprising mutualistic symbionts with a group of bacteria known for displaying non mutualistic symbiotic association, such as numerous γ-Proteobacteria found in marine sediments (Lenk et al., 2011). Interestingly, the Clone 1-Roscoff was found both in gills and sediment while clone 2-Roscoff was detected only in gills but not in sediment.

3.3. Distribution of *L. lacteus* endobacteria in different organs

The presence/absence and relative quantities of the two endobacterial phylotypes in clam organs (i.e. gills, gonad, and mantle) was determined

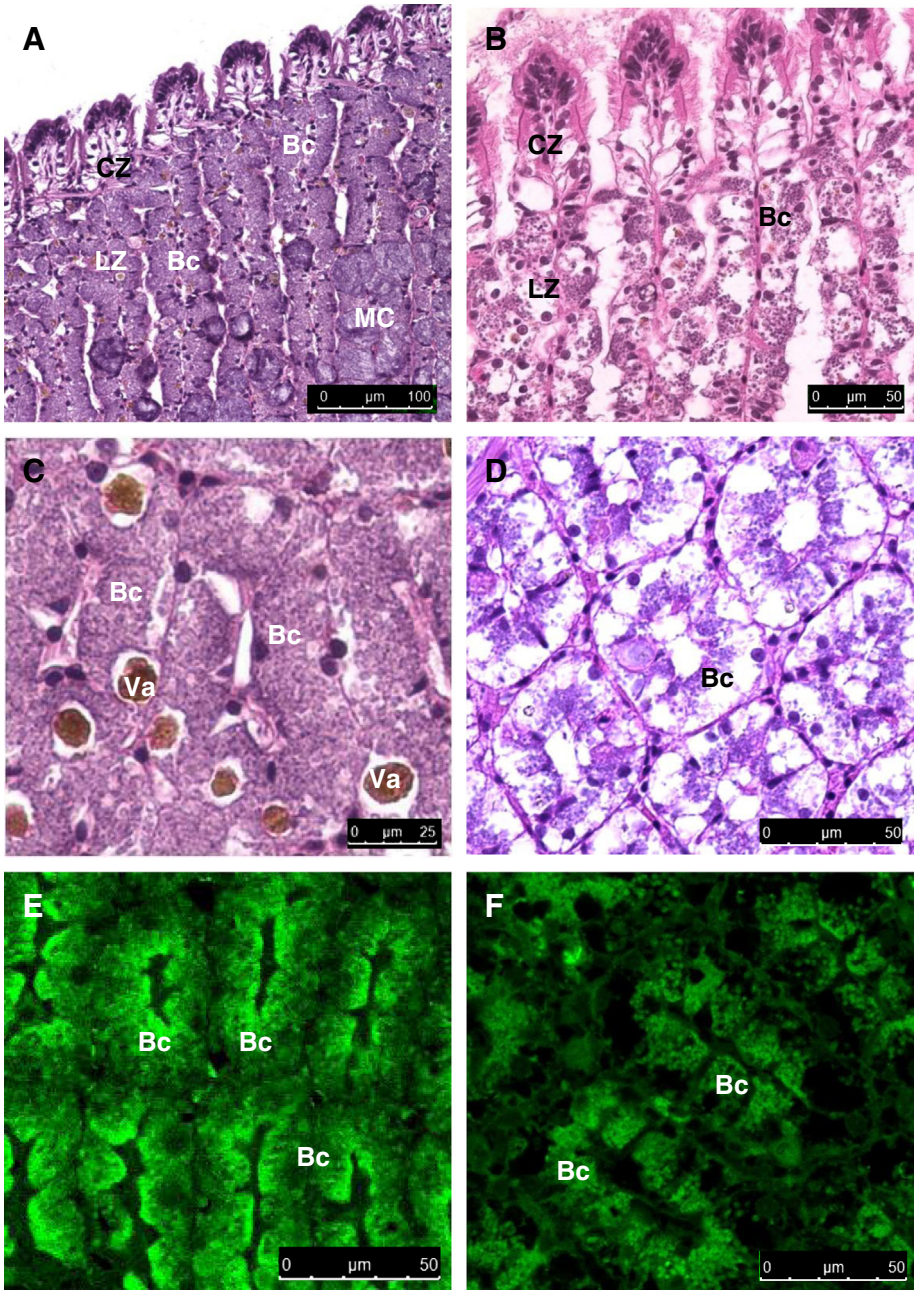

Fig. 2. Light micrographs and fluorescence in situ hybridization (FISH) images of freshly collected (A, C, E) and starved (B, D, F) *Loripes lacteus*. Transverse section of gill showed ciliated zone (CZ), lateral zone (LZ) and an overview of bacteriocytes (Bc), mucous cells (MC) and vacuoles (Va) containing lysed endobacteria. Starved clams displayed bacteriocytes depleted of endobacteria compared to freshly collected animals.

using fluorescence in situ hybridization (FISH) and quantitative Real-Time PCR (qPCR). Clone 1-Roscoff was significantly higher in gills compared to mantle (1500-fold, Holm-Sidak post-hoc test, $p < 0.001$) and gonad (28,115-fold, $p < 0.001$; Fig. 5). Clone 2-Roscoff was significantly less abundant in gills as compared to Clone 1-Roscoff (415-fold, $p < 0.001$). Levels of Clone 2-Roscoff in mantle and gonad were significantly lower than those measured in gills (1043- and 13,862-fold, $p < 0.001$).

As expected, FISH was less sensitive in detecting small quantities of the bacteria as compared to PCR. Thus, FISH allowed the detection of Clone 1-Roscoff but not the less abundant Clone 2-Roscoff, possibly because of low signal-to-background autofluorescence ratio. Positive Clone 1-Roscoff FISH signals (EUB338 and LOR1-192 probes) were exclusively detected in gills (Fig. 1B and C) but not in the mantle, the digestive tract, or in the female and male gonads (Fig. 1C, D, E and F respectively).

3.4. Effect of starvation on symbiont quantity

In this experiment, clams were maintained in the lab and either fed with natural seawater supplemented with cultured microalgae or submitted to complete starvation. Both treatments caused a decrease in the quantity of both bacterial clones in gill tissues as compared to fresh animals sampled at time 0 (ANOVA, $p < 0.01$, Fig. 6). The reduction of both clones was however faster in starved clams and significant differences were revealed between starved and fed clams at 3 weeks for Clone 1-Roscoff (2-way ANOVA followed by Holm–Sidak post hoc test, $p < 0.01$). Overall, the relative quantity of the two clones gradually

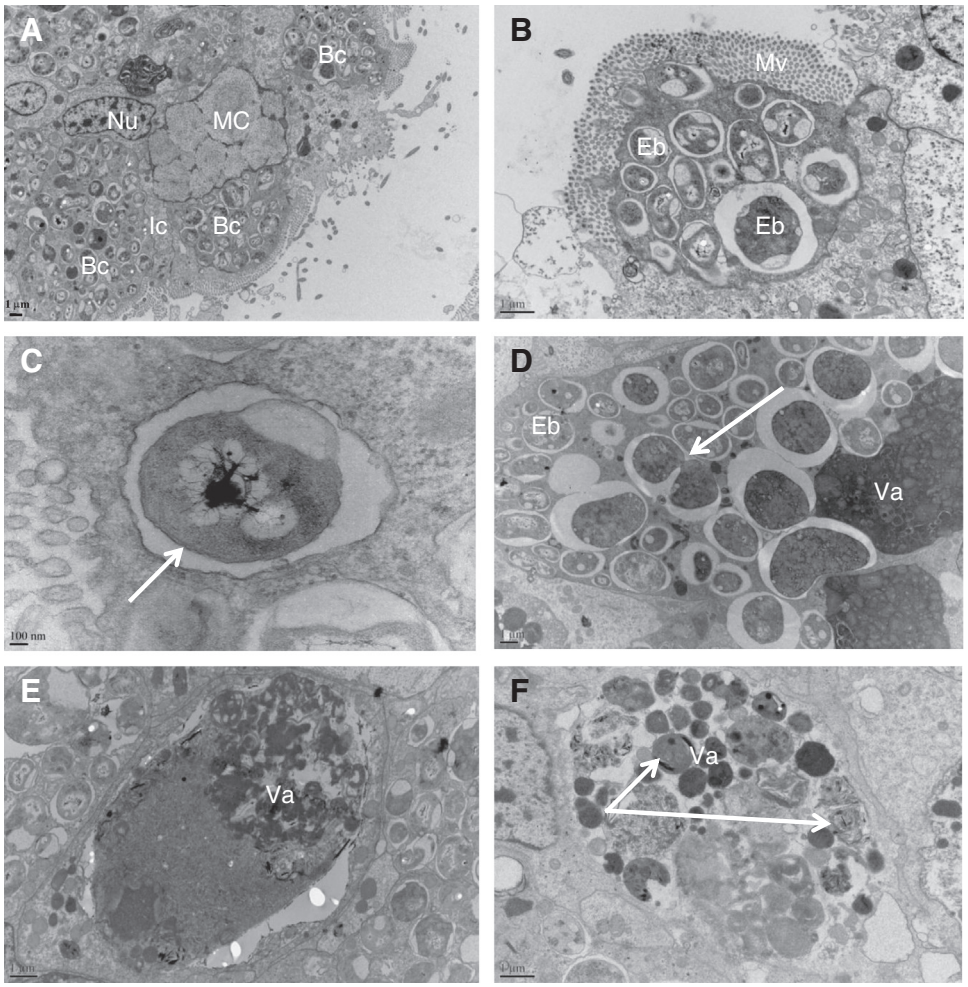

Fig. 3. Transmission electron micrographs of *Loripes lacteus* gill sections with endosymbiotic bacteria. (A) Transverse section of gill showing an overview of bacteriocytes (Bc), intercalary cells (Ic), nucleus (Nu) and mucous cells (MC). (B) Apical part of a bacteriocyte showing endobacteria (Eb) of different sizes (Mv: Microvilli). The smaller bacterial morphotype occupies the apical part of the bacteriocyte, while the larger morphotype is located more basally. (C) Endobacteria presenting a large electron-lucent vacuole, DNA strands and a double membrane (white arrow) characteristic of the Gram negative bacteria. The cytoplasm also contains electron dense or translucent granules. (D) Section of a bacteriocyte showing endobacteria and large electron dense vacuoles (Va) resulting in the lysis of one or several endobacteria (the white arrow shows the fusion of 2 vacuoles). (E) (F) Remains of endobacteria (white arrow) after lytic degradation.

decreased throughout the experiment and seems to have stabilized after 5 weeks of treatment. Microscopic observations revealed that gill filaments of starved clams were thinner and their bacteriocytes were partially depleted of bacteria compared to fresh clams (Fig. 2B and D). In situ hybridization analysis confirmed a decrease in fluorescence intensity (Fig. 2E and F).

3.5. Assay of re-acquisition of symbionts after starvation

Submitting clams starved for 5 weeks to "optimal" conditions (fresh sediments supplemented with sodium sulfide, algae, and endobacteria-ladden grounded fresh gills) for an additional 3 weeks did not increase the quantity of endosymbionts (both clones) in gill tissues (Fig. 7). For instance, endosymbiont depletion continued (significant for Clone 1-Roscoff, Holm-Sidak, $p < 0.01$) and no differences were detected between continuously starved clams and those maintained under optimal conditions for 3 weeks.

4. Disscussion

The biology of the clam *L. lacteus* and its symbiotic relationship with endobacteria were the focus of a few studies in the 1980s and 90s. Since that time, contemporary molecular tools and advances in the sequencing of 16S rRNA provide new valuable methods to precisely determine the diversity of endosymbiotic bacteria, their phylogeny, quantity and specific location in clams. In this study, we used a combination of molecular and microscopic methods to characterize this symbiotic association and determine factors potentially involved in endosymbiont acquisition and regulation.

Based on the 16S rRNA sequences of endobacteria of *L. lacteus*, two distinct phylogroups of bacteria were detected in gill of *L. lacteus* population from Roscoff. The most abundant endobacteria (Clone 1-Roscoff) present a phylogenetic relationship with other symbiotic bacteria and probably corresponds to the symbiont previously described in *L. lacteus* using microscopy techniques (Herry et al., 1989; Johnson and Fernandez, 2001). Interestingly, Clone 1-Roscoff 16S sequence is slightly different (95 to 97% similarity) from symbionts found in *L. lacteus* population from Adriatic Sea (Croatia). The identity and the role of Clone 2-Roscoff (commensalism, mutualism or parasitism) are not clear due to our inability to microscopically locate these cells in the gills likely because of their low abundance. Clone 1-Roscoff showed similar levels in mantle tissues as Clone 2-Roscoff in gills (Fig. 5), yet the former cells were not detected in mantle tissue using FISH neither. Overall, the two probes LOR1-192 and LOR2-196 were designed in the same part of the 16S rRNA that was previously shown to be readily accessible for hybridization (Behrens et al., 2003). It is however possible that our LOR2-196 probe was not efficient. Therefore, any hypothesis with regard to the nature or function of Clone 2-Roscoff bacteria remains

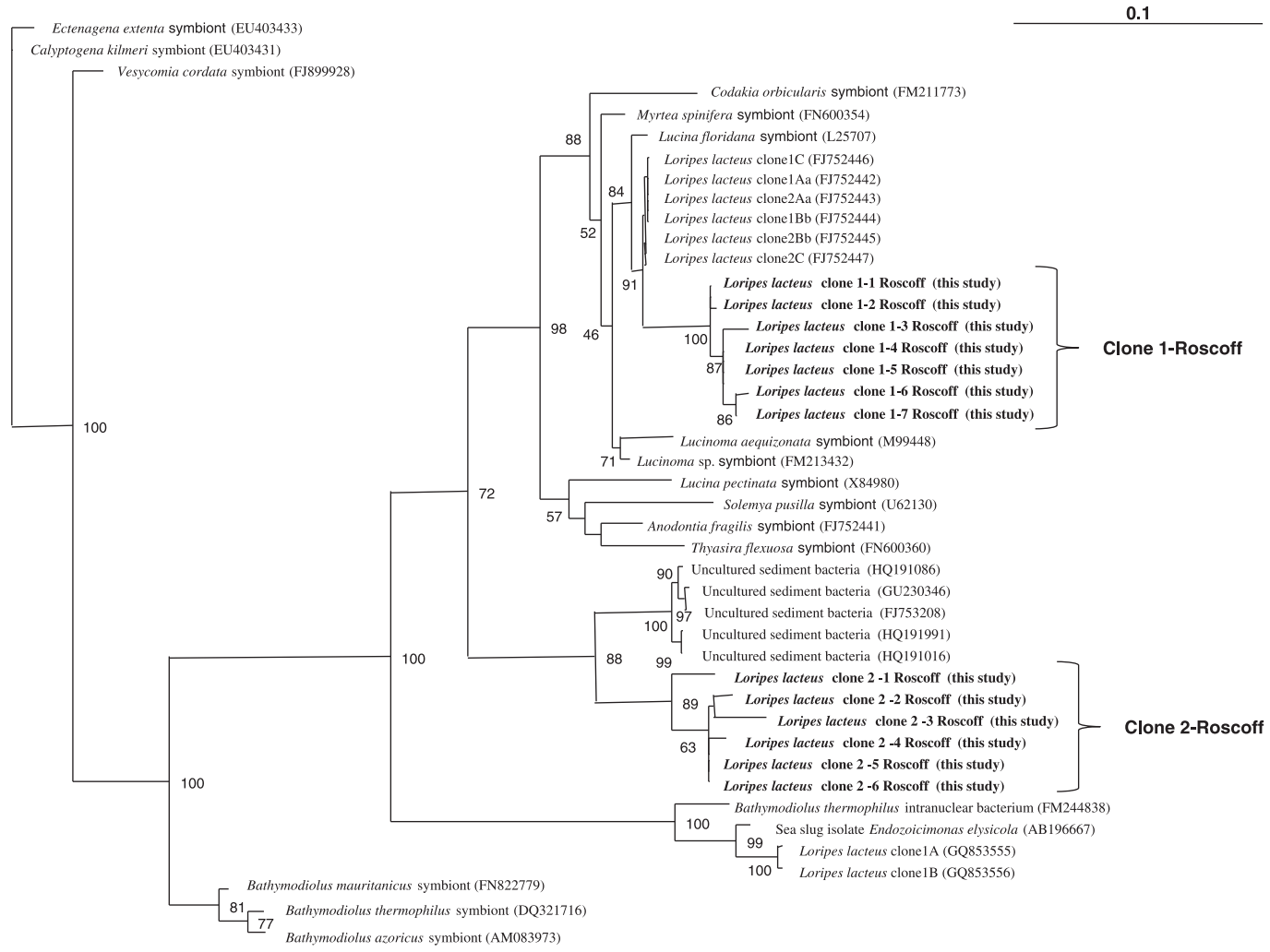

Fig. 4. Phylogenetic relationships, based on maximum-likelihood analyses (Phymlv2.4.4.) of 16S rRNA sequences (1220 bp average sequence length), between the two endobacteria strains found in *Loripes lacteus* from Roscoff and their closest relatives, including symbionts from *L. lacteus* from Croatia as well as other Lucinids, Mytilids, Solemyids, Thyasirids and Vesicomyids. The tree also included uncultured bacteria from sediment, parasitic bacteria of *B. thermophilus* and a sea slug endobacteria. The tree is unrooted and bootstrap values (%) are indicated at the nodes if greater than 50%. Scale bar: 0.1 nucleotide substitutions per sequence position.

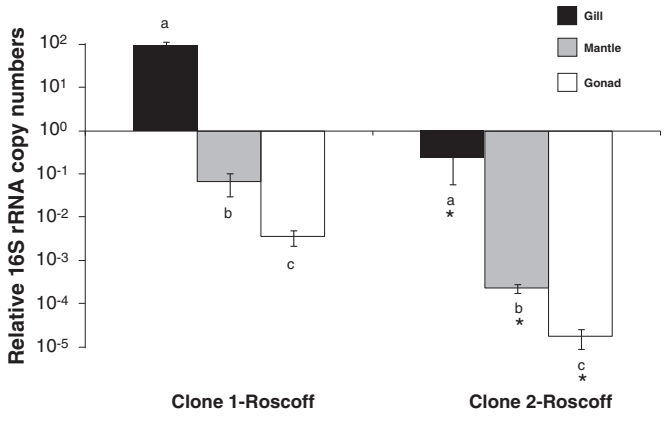

Fig. 5. Relative 16S rRNA copy numbers, as estimated by qPCR, of both bacterial clones in gill, gonad and mantle of *Loripes lacteus*. Values, in each organs, were normalized to a housekeeping gene (18S) then calibrated to average copy numbers of Clone 1-Roscoff in gill (highest levels, represented by 100) and results are presented as relative 16S rRNA copy numbers (mean, n = 10 clams). Letters (a, b and c) denote significant differences in ΔCt between different organs within each clone and * indicates significant differences between both clones for each organ (Holm–Sidak post-hoc test, $p < 0.001$).

speculative. They may be either endosymbiont or ectosymbiont. The possibility that these bacteria are parasitic cannot be ruled out since recent work demonstrated that mutualistic and parasitic endosymbionts can cohabitate in the gills of a symbiotic bivalve (Zielinski et al., 2009).

The major endobacteria (Clone 1-Roscoff) was precisely localized (qPCR and FISH) in gills but not in other organs including gonads confirming previous microscopy results (Herry et al., 1989; Johnson and Le Pennec, 1994; Johnson et al., 1996). In vertical transmission, symbionts usually live in specific host organs and have to migrate to the reproductive organs (see numerous examples in Bright and Bulgheresi, 2010). It is therefore possible that symbionts are detectable in gonads only temporarily. Our study was performed in November during the second spawning event in *L. lacteus* in Brittany (Johnson and Fernandez, 2001). Our microscopic observations revealed mature gonads (Fig. 1D) and consequently symbionts should have been present in gonads and detectable by qPCR and FISH if *L. lacteus* symbionts are vertically-transmitted. Based on these findings and its presence in sediments, it is reasonable to think that the mode of transmission of the symbionts in *L. lacteus* is horizontal and bacteria are acquired from the surrounding environment. Although vertical transmission of endosymbiotic bacteria from parents to descendants through the female gametes has been demonstrated in several bivalve species belonging to the

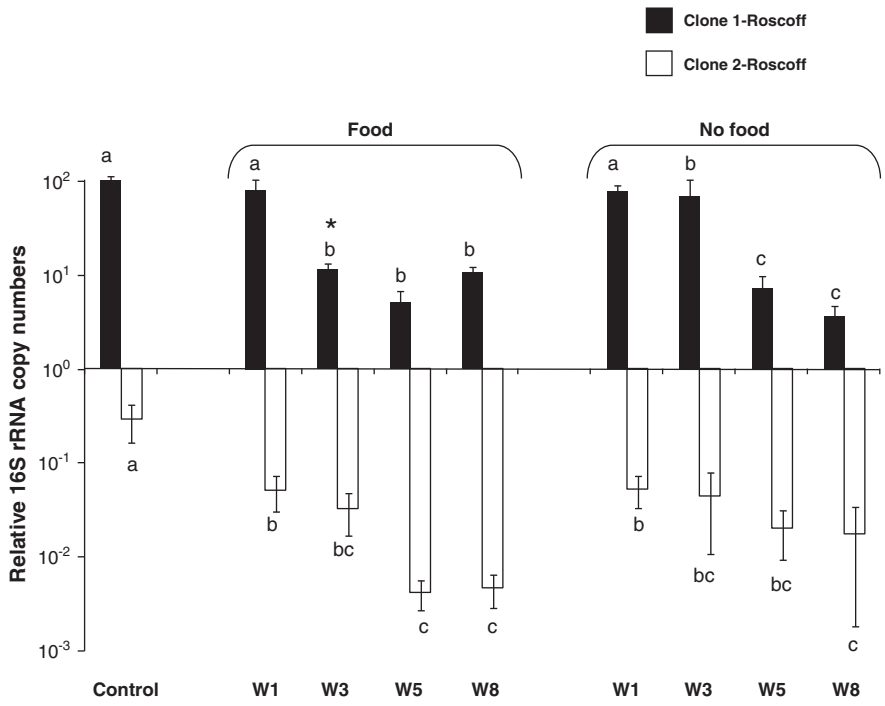

Fig. 6. Relative 16S rRNA copy numbers, as estimated by qPCR analysis, of both bacterial clones in gill of *Loripes lacteus* submitted to 8 weeks of starvation (W1 to W8). Values were normalized to a housekeeping gene (18S) then calibrated to average copy numbers of Clone 1-Roscoff in fresh control clams (highest levels, represented by 100) and results are presented as relative 16S rRNA copy numbers (mean, n = 10 clams). Letters (a, b and c) denote significant differences in ΔCt between different time points within each clone and * indicates significant differences between both clones for the same time point (Holm–Sidak post-hoc test, $p < 0.001$).

Vesicomyids and Solemyids families (Cary, 1994; Endow and Ohta, 1990; Krueger et al., 1996), horizontal transmission is more common in Mytilids, Thyasirids and Lucinids. Gros et al. (1998b) also reported horizontal transmission of endosymbiotic bacteria in *Codakia orbicularis*, another species of Lucinids. In this species, transmission happens when clams are in contact with sediments from clam's natural habitat (Gros et al., 1998a) but probably not from one clam to another since no bacterial release has been observed (Brissac et al., 2009). Symbiotic-competent bacteria penetrate by endocytosis into undifferentiated cells in the gills of aposymbiotic juvenile clams (Gros et al., 1998a). Newly engulfed bacteria are enclosed in host endocytic vacuoles and progressively, undifferentiated cells enlarge and are converted into bacteriocytes. Gros et al. (1998a) also determined that aposymbiotic clams remain receptive to symbiotic bacteria several months after their metamorphosis. Our study focused on adult of *L. lacteus* and therefore initial acquisition cannot be characterized although mechanisms similar to those described in *C. orbicularis* could be hypothesized to be similar.

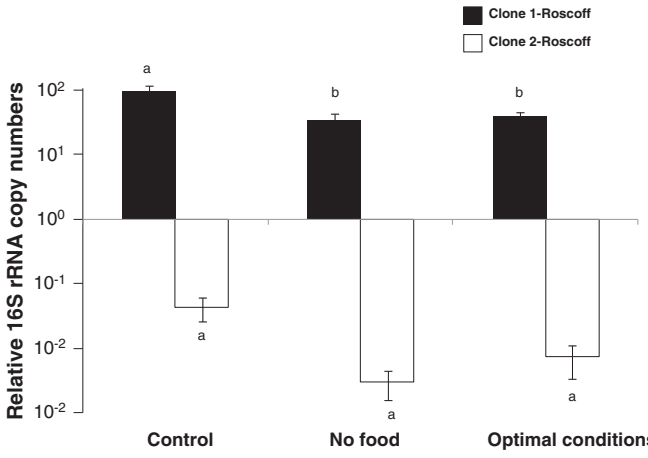

Fig. 7. Relative 16S rRNA copy numbers, as estimated by qPCR analysis, of both bacterial clones in gills of *Loripes lacteus* starved for 5 weeks (control) and subsequently submitted to optimal conditions for endosymbiont re-acquisition or to continuous starvation for another 3 weeks. Values were normalized to a housekeeping gene (18S) then calibrated to average copy numbers of Clone 1-Roscoff in control clams (highest levels, represented by 100) and results are presented as relative 16S rRNA copy numbers (mean, n = 10 clams). Letters (a and b) denote significant differences in ΔCt between different conditions within each clone (Holm–Sidak post-hoc test, $p < 0.001$).

While the initial acquisition of endosymbionts has been documented in the lucinids from the Codakia genus (Gros et al., 1998a), the characterization of their dynamics within host tissue and the potential continuous acquisition was uncertain until very recently (2012). Prior authors have however reported the lack of bacterial division inside bacteriocytes (Caro et al., 2007) and evidences of bacterial degradation in bacteriocytes (Frenkiel and Moueza, 1995; Herry et al., 1989; Le Pennec et al., 1988). In 2012, Gros et al. (2012) demonstrated that the reacquisition of bacteria in adult symbiont-depleted *Codakia orbiculata* was possible, but only when clams were placed back in their natural environment. Symbiont-depleted *C. orbiculata* maintained under "optimal" laboratory conditions close to those described in the current study failed to reacquire symbionts.

In this study, symbiont re-acquisition was not possible even though symbiotic bacteria were naturally present in sediments and experimentally supplemented to seawater using homogenates of fresh clam gills. Although experimental conditions were designed to match natural environment, it is possible that they were not optimal for symbiont acquisition in *L. lacteus*. For example, the duration of the experiment may have been too short to allow re-acquisition, although previous work showed that 2 weeks (in sulfur-enriched

seawater) were sufficient to produce an increase of endosymbiont loads (through re-acquisition or bacteria multiplication) in the bacteriocytes of previously endosymbiont-depleted hydrothermal mussels *B. azoricus* (Kadar et al., 2005). It is also possible that the sodium sulfide was quickly oxidized by the dissolved oxygen in seawater making it unavailable for symbionts. In addition, Gros et al. (2012) showed that symbiont-depleted *C. orbiculata* reacquired symbionts after 2 to 4 days in natural environment. Alternatively, symbionts derived from *L. lacteus* gill homogenates may not be competent for re-acquisition because of possible changes in their cell membrane epitopes causing alterations in the mechanisms of clam-symbiont recognition. Nevertheless, endosymbionts freshly extracted from *C. orbicularis* gills were able to infect aposymbiotic juveniles suggesting that endosymbiotic bacteria in this Lucinid species remain competent and are recognized by the host (Gros et al., 2003). Overall, our study failed to show an increase in endosymbiont loads despite the use of a combination of factors known to favor the re-acquisition of sulfur-oxidizing endosymbionts in bivalves (symbiont-ladden natural sediments and gill homogenates, sulfur-enriched seawater). It is also possible that symbiont acquisition in *L. lacteus* takes place in a precise window of the host life stage. This is actually the case for the insect *Riportus pedestris* that acquires the *Burkholderia* symbiont during a specific period of larval development (Kikuchi et al., 2011) or for hydrothermal tubeworms in which symbionts infect the skin of larvae just after settlement and for a limited period of time before the development of the symbiont housing organ (Nussbaumer et al., 2006). New experiments should be done in order to determine if clams placed back in their natural habitat after symbiont depletion would be able to reacquire symbionts.

Despite the apparent absence of bacterial cell division in *L. lacteus* bacteriocytes, endobacteria showed a high level of cell activity. Thus, some endobacteria displayed very large sizes (Fig. 3D) and modification of their density to electron, probably due to the accumulation of metabolites or substrates in their cytoplasm as described in other studies (Caro et al., 2007; Le Pennec et al., 1988). Some bacteriocytes contain large vacuoles enclosing several degraded bacteria (Fig. 3E and F). These large structures were described as the result of the degradation of bacteria by lytic enzyme contained in lysosomes (Le Pennec et al., 1988) and could be the result of the fusion of several small vacuoles (Fig. 3D).

The stressful conditions applied to *L. lacteus* (e.g. several weeks of starvation) led to a significant reduction of endobacteria loads as previously described in the lucinid *C. orbicularis* (Caro et al., 2009). It is possible that this decrease was the consequence of bacterial release in the environment although this hypothesis was clearly rejected for *C. orbicularis* by Brissac et al. (2009). The numerous vacuoles containing remains of endobacteria observed in *L. lacteus* gills, especially the starved clams, could indicate that bacteria could have been digested under lytic degradation. For example, lytic degradation was already reported in *L. lacteus* (Le Pennec et al., 1988), *B. azoricus* (Kádár et al., 2008) and *Codakia* sp. (Caro et al., 2009; Gros et al., 2012) under natural or starved conditions. This mechanism of symbiont degradation was suggested or shown to be a way to transfer organic material from endosymbiotic bacteria to hosts (Fiala-Medioni et al., 1986; Herry et al., 1989), especially under stressful conditions such as gametogenesis (Johnson and Fernandez, 2001). It can also represent a strategy to prevent against excessive bacterial proliferation as proposed by Le Pennec et al. (1988).

Overall, Herry et al. (1989) suggested that the symbiotic relationship between *L. lacteus* and its endobacteria seems to be more advantageous for the bivalve. If endobacteria take advantage of their location in gills (constant input of nutrients/sulfide/oxygen/carbon dioxide due to the ventilation of the pallial cavity), clams benefit from the products of bacterial metabolism and from the direct digestion of bacterial cells (Herry et al., 1989; Johnson and Fernandez, 2001). Lucinids hosts (*C. orbicularis* for instance) have also been suggested to tightly control gill colonization by bacteria since no division or release of endosymbionts was observed (Brissac et al., 2009). Interestingly, recent investigations in the mussel *B. thermophilus* showed that bacterial parasites are able to infect intercalary cells but not bacteriocytes, suggesting that mutualistic symbiosis may provide protection to bacteriocytes against infection by deleterious bacteria (Zielinski et al., 2009).

In conclusion, this study provided a molecular characterization of *L. lacteus* endosymbionts and determined symbiont dynamics under various experimental conditions. The lack of symbionts in reproductively-active gonads supports evidence for horizontal transfer of bacteria and is in agreement with previous studies in other Lucinid species. Starvation was associated with a significant reduction in symbiont loads in gills and resubmitting hosts to more favorable laboratory conditions did not increase symbiont abundance. The lack of symbiont re-acquisition following starvation may reflect the inappropriateness of our experimental conditions for host re-colonization rather than lack of symbiont uptake from the environment in adult clams. A better understanding of the molecular mechanisms that mediate acquisition, regulation and control of the bacteria by *L. lacteus* is needed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2013.07.015.

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