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Effects of Long-Term Starvation on a Host Bivalve (*Codakia orbicularis*, Lucinidae) and Its Symbiont Population▽

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The bivalve *Codakia orbicularis*, hosting sulfur-oxidizing gill endosymbionts, was starved (in artificial seawater filtered through a 0.22-μm-pore-size membrane) for a long-term experiment (4 months). The effects of starvation were observed using transmission electron microscopy, fluorescence in situ hybridization and catalyzed reporter deposition (CARD-FISH), and flow cytometry to monitor the anatomical and physiological modifications in the gill organization of the host and in the symbiotic population housed in bacteriocytes. The abundance of the symbiotic population decreased through starvation, with a loss of one-third of the bacterial population each month, as shown by CARD-FISH. At the same time, flow cytometry revealed significant changes in the physiology of symbiotic cells, with a decrease in cell size and modifications to the nucleic acid content, while most of the symbionts maintained a high respiratory activity (measured using the 5-cyano-2,3-ditolyl tetrazolium chloride method). Progressively, the number of symbiont subpopulations was reduced, and the subsequent multigenomic state, characteristic of this symbiont in freshly collected clams, turned into one and five equivalent genome copies for the two remaining subpopulations after 3 months. Concomitant structural modifications appeared in the gill organization. Lysosymes became visible in the bacteriocytes, while large symbionts disappeared, and bacteriocytes were gradually replaced by granule cells throughout the entire lateral zone. Those data suggested that host survival under these starvation conditions was linked to symbiont digestion as the main nutritional source.

The entire marine Lucinidae family, found in a wide range of sulfidic habitats, lives in association with chemoautotrophic sulfide-oxidizing bacterial symbionts, generally hosted in the gills of the bivalve. Lucinids are usually found in shallow water, such as intertidal mud or seagrasses (4, 53), in deeper water, e.g., *Bathyaustriella thionipta* (30), and in deep oceans at a 2,000-m depth, i.e., *Lucinoma kazani* (21, 55). The chemoautotrophic endosymbionts involved in such relationships are always localized inside specialized cells called bacteriocytes, and they have been found in several genera of the Lucinidae family, such as *Codakia* (4, 28), *Loripes* (39, 43), *Lucina*, and *Lucinoma* (17). Sulfur granules inside the symbiont cytoplasm have been demonstrated in most of the investigated species. The intracellular symbionts take energy from the oxidation of reduced sulfur compounds (27, 56, 59) and synthesize organic molecules by CO_2 fixation in a Calvin-Benson cycle, translocated to the host (18). This relationship between the host and its symbionts represents the autotrophic pathway for host nutrition (27). It has also been suggested that in symbiotic bivalves, intracellular digestion of the symbionts may be a nutrient source for the host, based on studies of hydrothermal vent and shallow water bivalves (6, 26, 39).

The relative importance of the autotrophic versus heterotrophic nutritional pathway can be estimated by measuring the carbon isotope (δ-^{13}C) ratios in the host tissue. Measuring this δ-^{13}C ratio on a wide range of invertebrates suggested that bivalves, including members of the Lucinidae, that live in reduced sediment may obtain a significant proportion of their organic carbon from chemoautotrophic endosymbionts (4, 10, 51, 52, 57). This suggestion was in agreement with the reduced functional digestive system previously described for the Lucinidae family (2, 53). Structural and morphological studies of gills of a few lucinids (belonging to the genera *Lucina* and *Lucinoma*) strongly suggested that symbionts play an important role in host nutrition since they occupy about 30% of the gill tissue and produce most of the host energy (17). Nevertheless, an alternative pathway for feeding, i.e., heterotrophic particulate feeding, could occur in some of the lucinid bivalves, since diatoms were found in the stomach of some lucinids (57). Duplessis et al. (22) showed that particulate feeding could be an important part of the nutritional strategy in symbiont-bearing *Lucinoma*, as opposed to the anatomical features that gave the impression that this bivalve relied only on symbiont nutrition.

In natural habitats, chemoautotrophic bivalves live at the interface between an anoxic sulfide-generating zone and water column oxygenated sediment. However, even if they are not close to a vent, these symbiotic organisms often have to deal with environments that are periodically depleted of oxygen (5, 12) and with extremely low sulfide concentration (13, 15, 16). These natural environmental variations lead to annual and seasonal changes in the δ-^{13}C ratio, as observed for some

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thyasirid species (13, 14). This δ-^{13}C ratio variation may be assumed to correspond to the capability of the host to rely on both autotrophic and heterotrophic pathways, and the preponderance of one pathway versus the other in the mixotrophic diet has been considered to be the way in which these organisms deal with changes in the chemical composition of their environment.

Apart from the decrease in symbiont abundance suggested by transmission electron microscopy (TEM) analysis and a decrease in sulfur and protein content in the gill tissue of thyasirids (20, 38, 40), little is known about the physiological status of these symbionts and the changes undergone by the symbiotic population of starved bivalves. A previous study of the population was carried out under natural conditions with *Codakia orbicularis*, a chemoautotrophic bivalve. This tropical bivalve lives in shallow-water sediment among the roots of seagrasses (*Thalassia testudinum*) (1). Like all lucinids studied so far, it is associated with sulfur-oxidizing symbionts (4, 27, 28) containing elemental sulfur in their cytoplasm (42). The bacterial symbiont of *C. orbicularis*, environmentally transmitted to the host (31), belongs to a single taxonomic group (*Gammaproteobacteria*) (25) and is shared by several other tropical lucinids (24, 25, 34, 35). Only a few data are available on the physiology of this symbiont. It was characterized by the presence of Rubisco and ATP sulfurylase enzymes and a δ-^{13}C ratio typical of chemoautotrophic bivalves (4). Unlike other related thyasirids tested for nitrate respiration even under oxygenated conditions (37), the symbionts of *C. orbicularis* use oxygen as the primary electron acceptor (23). Initial investigations of the population of *Codakia orbicularis*' symbiont revealed that the symbiotic population hosted by freshly collected individuals contained a high proportion of large bacterial cells containing multiple copies of their genome, typical of actively growing cells, despite the absence of dividing cells (11). It was assumed that the host could maintain a pure culture of the symbiont inside the bacteriocytes by regulating the entry and growth of newly recruited symbionts from sediment and probably regulating symbiont densities by host digestion (11).

This study was undertaken to investigate the dynamics of the symbiotic population hosted by *C. orbicularis* under experimental conditions based on long-term starvation of bivalves, i.e., incubated without planktonic food. We set out the ultrastructural, structural, and physiological changes that occurred in the symbiont population by examining the host gill sections using TEM and fluorescence in situ hybridization and catalyzed reporter deposition (CARD-FISH). A purified fraction of gill endosymbionts was analyzed by flow cytometry (FCM) to investigate the nucleic acid content and cell size of symbionts and by using the 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) method and epifluorescence microscopy to detect the respiratory activity of symbionts. The modifications induced by host starvation in the symbiotic population are described for the period of long-term starvation.

MATERIALS AND METHODS

Collection of bivalves. A set of 60 individuals of *Codakia orbicularis* was collected by hand at a depth of 5 to 10 cm in the sediment of a seagrass bed of *Thalassia testudinum* at "îlet Cochon" (16°12′53″N; 61°32′05″W, close to Pointe-à-Pitre, Guadeloupe, French West Indies). At the sampling site, water column depths varied from 0.3 to 1 m. Over the year, the water temperature varied between 25°C and 30°C.

Starvation experiment. The freshly collected specimens of *Codakia orbicularis* were divided into several 50-liter plastic containers filled with artificial seawater, prepared by dissolving sea salt (Sigma) in distilled water (salinity, ~35 ppt). The bivalves were starved for 4 months. The experiment was conducted at room temperature, between 25°C and 30°C. The artificial seawater was highly oxygenated using an aquarium pump and renewed twice a week to avoid any toxic effect from the excretion of nitrogen waste products, such as ammonia, released by the clams. At regular intervals (14 and 28 days and 3 and 4 months), three clams were selected at random for analysis. One gill was dissected and used to extract and purify the endosymbionts; the other gill was fixed and embedded as described below for CARD-FISH experiments.

Extraction and purification of gill endosymbionts. Gill endosymbionts were extracted and purified using the Percoll cushion method (19), with some modifications. To extract the endosymbiotic bacteria from the gill tissue, one gill was homogenized in 8 ml of sterile seawater (35 ppt) using a handheld Dounce homogenizer. The homogenized tissue was centrifuged ($30 \times g$ for 1 min). Four ml of the supernatant was then centrifuged at $400 \times g$ for 2 min to collect the bacteria in the pellet, which was resuspended in 1 ml of filtered (0.2 μm) seawater. The suspension was gently layered on a Percoll (Sigma) cushion (3 ml) diluted with imidazole-buffered saline (490 mM NaCl, 30 mM $MgSO_4$, 11 mM $CaCl_2$, 3 mM KCl, and 50 mM imidazole) and centrifuged at $1{,}000 \times g$ for 8 min at 4°C. Since the Percoll cushion method (19) is based on differences in density between the symbiont and the host organelles, the loss of elemental sulfur globules (S°) in the periplasmic space of the symbionts over a period of long-term starvation prevents the separation of the endosymbionts from the host debris. The final Percoll concentration, therefore, had to be adjusted from 50% to 30% throughout this study. The symbionts were finally collected under the cushion, washed once, and finally suspended in 1 ml of filtered (0.2 μm) artificial seawater (= purified symbiont suspension). The purification was performed at 4°C to avoid the growth of marine bacteria, initially present on the gills or between the gill filaments as contaminants (35). An aliquot of the purified symbiont suspension was fixed with formaldehyde (1%, final concentration) and immediately stored in liquid nitrogen for nucleic acid content analysis by FCM.

TEM preparation of the gill. Individuals were prefixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M pH 7.2 cacodylate buffer adjusted to 900 mosM with NaCl and $CaCl_2$ in order to improve membrane preservation. After a brief rinse, they were stored in the same buffer at 4°C until fixed. The gills were dissected, fixed for 45 min at room temperature in 1% osmium tetroxide in the same buffer, and then rinsed in distilled water and postfixed with 2% aqueous uranyl acetate for one more hour before embedding and observation as described already (33).

Histological preparation. An overall view and histological information were obtained from paraffin sections. The gill dissected from one individual was fixed in Bouin's fluid (29) for 24 h at room temperature and then embedded in Paraplast. Sections (7 μm thick) were stained by various techniques as described by Gabe (29). Goldner's trichrome staining was used for morphological information and periodic acid-Schiff staining for identifying glycoconjugates.

Fluorescence in situ hybridization. The gills were fixed for 1 to 3 h at 4°C in 2% paraformaldehyde in seawater filtered through a 0.2-μm-pore-size membrane. The specimens were then washed in seawater three times for 10 min each at room temperature and then dehydrated through an ascending series of ethanol concentrations and stored at −20°C before being embedded in Paraplast. Four-μm-thick sections were placed on precoated slides from Sigma before hybridization. Paraffin was removed prior to hybridization experiments using toluene, and sections were rehydrated in decreasing series of ethanol concentrations, finishing with distilled water. CARD-FISH experiments were performed as described by Pernthaler et al. (48, 49). Bacterial cell membranes were permeabilized using HCl (0.2 M, room temperature), Tris-HCl (20 mM, pH 8, room temperature), and proteinase K (0.5 μg/ml, 37°C). The *C. orbicularis* symbiont-specific probe Symco 2 (5′-ATGTCTCCCAGCGGTTGGGCAC-3′) (derived from reference 31) was used as a horseradish peroxidase-labeled probe. After cell membrane permeabilization, peroxidases present in the tissue were inhibited using HCl (0.01 M, room temperature). Hybridizations were performed using 50% formamide, and signals were amplified using a buffer containing carboxyfluorescein (fluorescein isothiocyanate). Slides were mounted with Cytomation fluorescent mounting medium (Dako, France) and viewed under an epifluorescence microscope, Eclipse 80i (Nikon, France).

Image recording. Images were acquired using a charge-coupled-device camera, DXM 1200F (Nikon, France), and stored as .jpg files. ImageJ for Windows software, a public-domain image analysis program (http://rsb.info.nih.gov/ij/), was used to process the images. The procedure involved a series of operations common to most image analysis software. The first step converted color images

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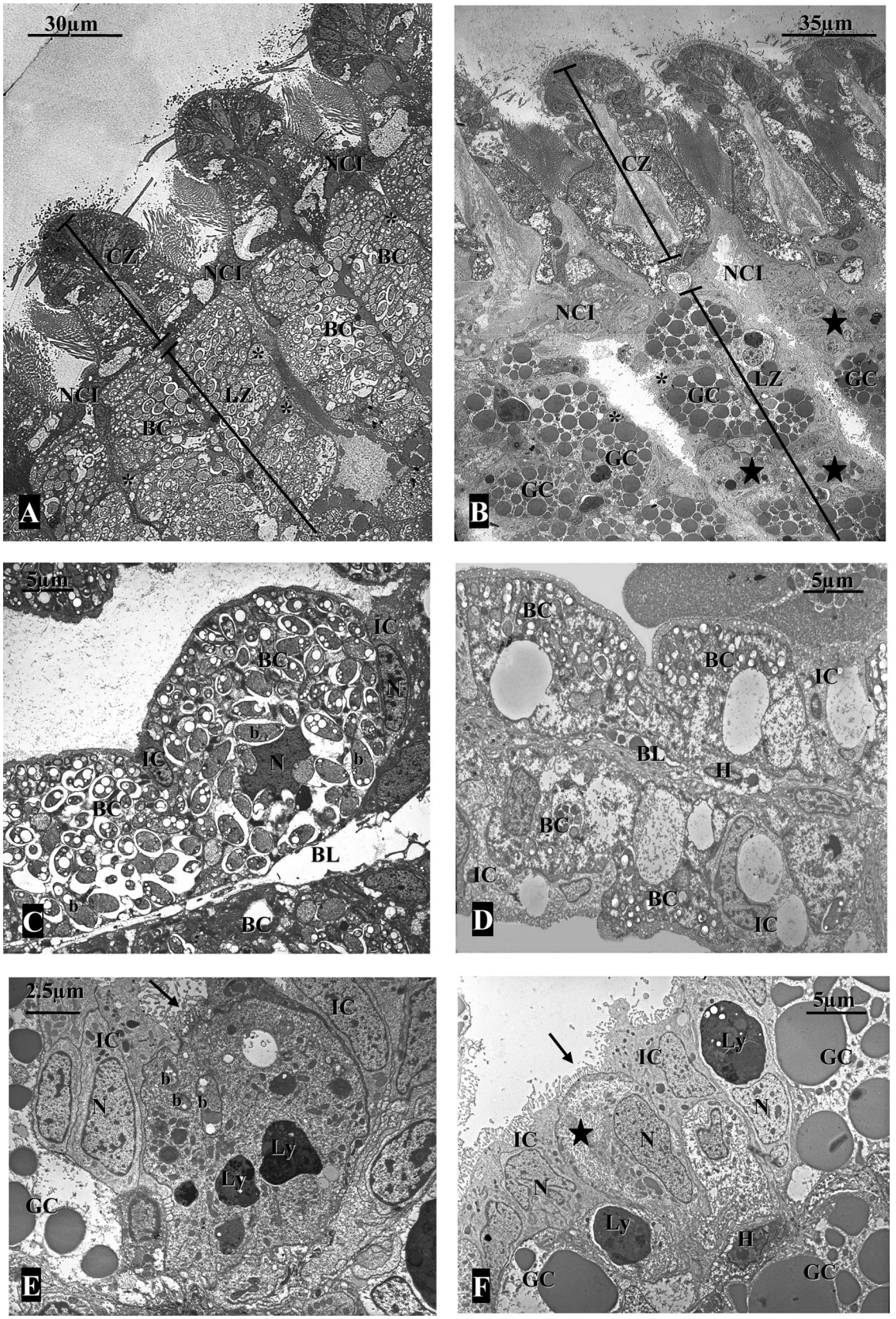

FIG. 1. Ultrastructural modifications in the *C. orbicularis* gills during starvation, as determined with ultrathin sections (TEM analysis). (A) Low magnification of gill filaments from adults dissected immediately after recovery. Each gill filament is characterized by a ciliated zone (CZ) separated from the lateral zone (LZ) by several nonciliated intermediary cells (NCI). Bacteriocytes (BC) filled with chemoautotrophic symbionts

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into 8-bit greyscale images. The greyscale image was then converted to binary format by defining a greyscale cutoff point. Greyscale values below the cutoff became black, and those above became white. The system manually adjusted to delimit clearly the zone with intracellular bacteria in bacteriocytes in order to optimize the computer detection and highlight objects below the machine threshold. Adjustment and background corrections were maintained constant during the operations. The regions of interest were then delimited for each image before processing. The analysis procedure calculated the relative area occupied by symbionts (percentage of fluorescent surface versus total surface) with selected objects which were outlined and numbered in a new window.

FCM analysis of relative nucleic acid content and cell size of symbionts in the whole population. Nucleic acid content analysis of the symbiont cells purified from *C. orbicularis* was performed using a FacsCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon laser (488 nm). The purified symbiont suspensions in 1% formaldehyde, stored in liquid nitrogen, were thawed and diluted (1/100) in saline water (30 ppt NaCl). Nucleic acids were stained for 15 min in the dark at 4°C with SYBR green I (Molecular Probes, Eugene, OR) according to the method described by Marie et al. (45) (1:10,000 [vol/vol]). Two-μm yellow-green fluorescent cytometry beads (Polysciences, Inc.) were added to the samples as an internal standard to normalize the symbiont fluorescence emission. The sheath fluid (NaCl solution, 30 ppt) was filtered through a 0.2-μm-pore-size membrane. Analyses were run at low speed (around 18 μl min^{-1}) and acquisition was performed for 2 min, corresponding to a total of 25,000 to 35,000 detected cells. Fluorescence from SYBR green-stained symbionts was collected in the green fluorescence channel FL1 (530 nm). Side-scattered light (SSC) was used as a proxy of cell size (7, 58). Both parameters were collected on a logarithmic scale. The relative nucleic acid content and cell size of symbionts were analyzed using samples from freshly collected bivalves (T_0) and starved bivalves.

Enumeration of respiring symbionts (CTC^+) by epifluorescence microscopy. The respiratory activity of the symbionts was measured using the CTC method, described by Rodriguez et al. (54). The purified symbiont suspension, adjusted to ~10^7 total cells ml^{-1}, was incubated with CTC at a final concentration of 4 mM in the dark at room temperature for 4 h. Incubation was stopped by addition of formaldehyde (4%, final concentration). The samples were stored at 4°C until they were examined. By microscopic observation, we checked that insoluble CTC formazan crystals had been formed inside the cells. A suspension of symbionts killed by using sodium azide (0.1%, final concentration) was also incubated as an abiotic control. Fixed samples, first incubated with CTC, were counterstained with 4′,6′-diamino-2-phenylindole (DAPI) at a final concentration of 2.5 μg ml^{-1} in Tris-HCl buffer (0.1 M, pH 7.1) for 10 min in the dark. CTC-DAPI-doubly stained symbionts were filtered through 0.2-μm-pore-size black polycarbonate membranes (Nuclepore) and counted using an epifluorescence microscope (Provis; Olympus). About 200 to 400 total DAPI-stained cells were examined to calculate the percentage of respiring cells (CTC^+).

RESULTS

Histological and cytomorphological changes. Each gill filament was composed of a ciliated zone and an extremely short intermediary zone, both without bacterial symbionts, and a lateral zone hosting the intracellular bacteria (Fig. 1A and 2A). The intermediary zone was so small in this lucinid species that it could not be detected using a light microscope in histological sections (Fig. 2A), although it can be seen in TEM images (Fig. 1A and B). Four different types of cells were observed in the lateral zone, such as mucocytes, granule cells, intercalary cells, and bacteriocytes (Fig. 1A and C and 2A). CARD-FISH and TEM data showed that the whole lateral zone of each gill filament seemed to be occupied with bacteriocytes full of bacteria (Fig. 1A and C and 2B). Each bacteriocyte corresponded to a large cell (up to 35 μm in length) characterized by a rounded apical pole in contact with circulating seawater and a cytoplasm filled with individually enclosed bacteria (Fig. 1C). Bacterial endosymbionts were characterized by numerous sulfur granules, which appeared as electron lucent vesicles after conventional TEM preparation (Fig. 1C). After 1 month of starvation, changes were noted in the gills, with a decrease of intracellular bacteria throughout the lateral zone shown by CARD-FISH hybridization (Fig. 2C). The intensity of the green fluorescent signal, proportional to the abundance of symbionts, decreased noticeably compared to that of individual signals at the beginning of the experiment (Fig. 2B). Each bacteriocyte in the lateral zone of the gill filament was hybridized, but the signal intensity from each bacteriocyte was weaker (Fig. 2C).

The structural changes in the gill filaments noticed after 1 month appeared more pronounced after 2 months of starvation. The CARD-FISH signal intensity, i.e., symbiont abundance, markedly decreased compared to that for gills from T_0 individuals (Fig. 2B to D) and was even more pronounced after 3 months of starvation, when only a few remaining bacteria could be observed (Fig. 2E). At an ultrastructural level, bacteriocytes contained fewer bacteria after 2 months of starvation, mostly located in the upper part of the bacteriocyte and which were smaller than at the beginning of the experiment (Fig. 1D). After 3 months of starvation, lysosome-like structures and numerous mitochondria could be seen in the bacteriocyte cytoplasm of the lateral zone, whereas only a very few intracellular bacteria, mostly without sulfur granules, could be observed on thin sections from TEM observations (Fig. 1E). Granule cells appeared in the lateral zone, while the gill filament did not change in length but became thinner (not shown),

are part of the lateral zone, with numerous intercalary cells characterized by their nucleus in an apical position (asterisks). (B) Low magnification of gill filaments from adults dissected after 4 months of starvation. While the organization of the ciliated zone (CZ) is fairly similar to that observed in field specimens, the lateral zone (LZ) is significantly different. The most prevalent cells are granule cells (GC), with no bacteria and few intercalary cells (asterisks). There are a few undifferentiated cells (stars) without bacterial endosymbionts evenly distributed through the lateral zone. (C) Bacteriocytes (BC), which are the most prevalent cells in the gill filament, have a basal nucleus (N) near the blood lacuna (BL) of the filament axis and a rounded apical pole developing broad contact with pallial seawater. The cytoplasm is crowded with envacuolated bacteria (b) which are individually enclosed inside the bacteriocyte vacuole. Intercalary cells (IC), characterized by a trumpet shape and an apical nucleus, are interspersed uniformly among the bacteriocytes. (D) The bacteriocytes (BC) within the lateral zone of a *C. orbicularis* specimen kept in starvation for 56 days (2 months). The larger symbionts have disappeared, and only small symbionts, located mainly at the periphery of the cell, can be observed. BL, blood lacuna; GC, granule cell; H, hemocyte; IC, intercalary cells. (E) In specimens that have been starved for 3 months, a few residual bacteriocytes (BC) with a small apical pole in contact with seawater (arrow) can be observed hosting very few bacteria (b) and large lysosomes (Ly) in their cytoplasm. Most of the cells in the lateral zone are devoid of bacteria. The small number of symbionts is below the detection limit of the other techniques used in this study (i.e., FISH and FCM). GC, granule cell; IC, intercalary cells; N, nucleus. (F) In specimens starved for 4 months, undifferentiated cells without any bacteria in their cytoplasm can be observed throughout the lateral zone. Such cells, with a rounded apical pole (arrow) in contact with the pallial seawater, could be considered putative bacteriocytes, as previously described for aposymbiotic juveniles of *C. orbicularis*. Some contain lysosome-like structures (Ly). GC, granule cells; H, hemocyte in the blood lacuna; N, nucleus.

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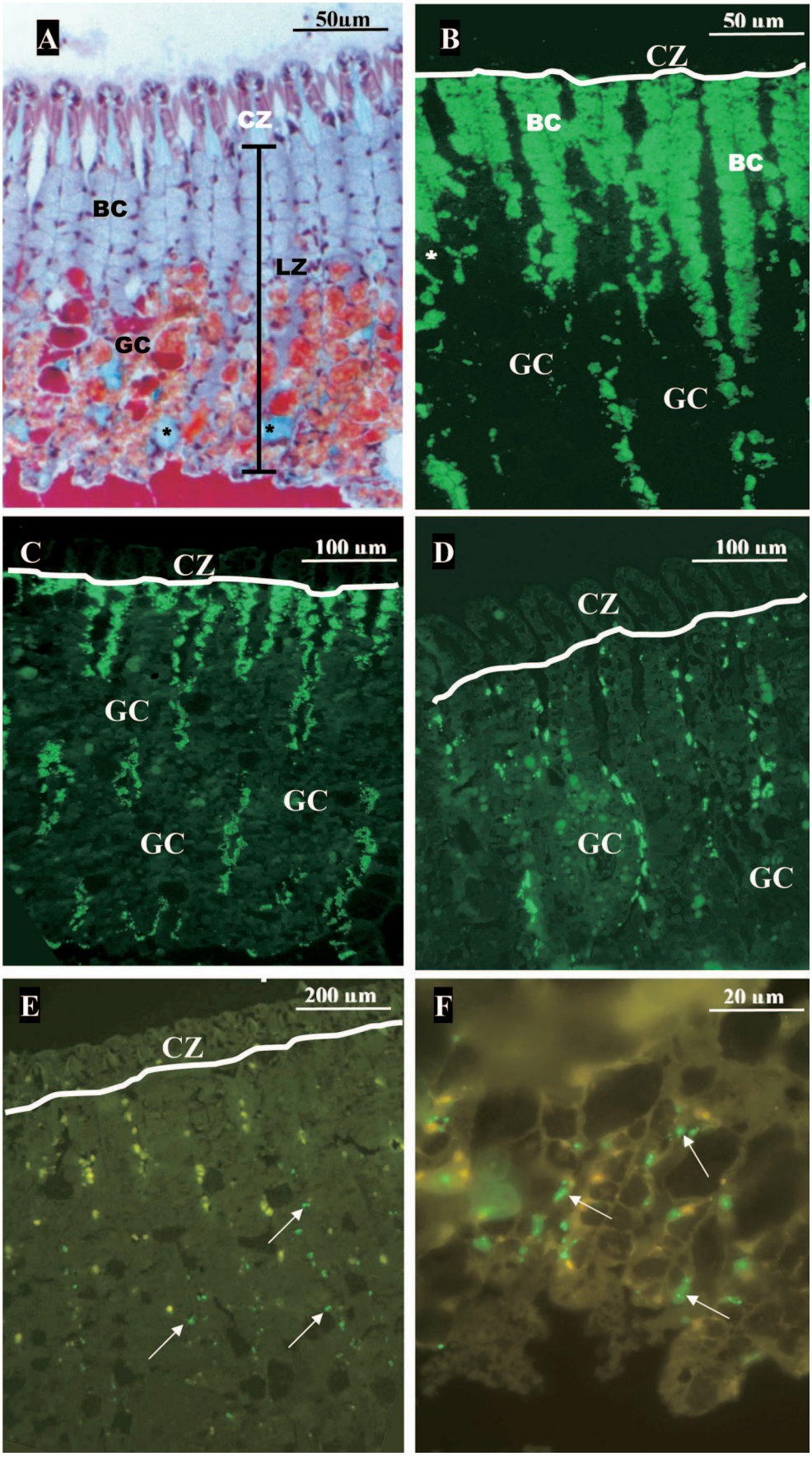

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suggesting that the gill structure had been significantly modified. This was confirmed after 4 months (115 days) of starvation. The cell organization of each filament had clearly changed, since granule cells then occupied the greater part of the lateral zone (Fig. 1B) and were in contact with the intermediary zone just below the ciliated zone (Fig. 1B). No bacteria could be detected using TEM (Fig. 1F). Small undifferentiated cells (up to 12 μm), similar in shape to the undifferentiated cells found in aposymbiotic juveniles of *C. orbicularis* (32) without any bacteria, were regularly interspersed between the granule cells throughout the lateral zone (Fig. 1F). Bacteria could not be detected in those bivalves by the techniques used in this study.

Quantifying the decrease in abundance of symbionts. To estimate the gradual disappearance of symbionts hosted in *C. orbicularis* gills through starvation, ImageJ software was used to measure the proportion of fluorescent area on the photos presented in Fig. 2B to E from histological sections (4 μm). The results are presented in Table 1. Fluorescence covered 32.4% of the gill at T_0. If this fluorescent area is considered to be a good proxy of the number of symbionts, then approximately one-third of the gill volume of freshly collected *C. orbicularis* (T_0) was occupied by symbionts. This fluorescent area regularly decreased with starvation (Table 1), confirming the decrease in the abundance of symbionts already revealed by TEM observations (Fig. 1). The host appeared to lose one-third of its symbionts in each month of starvation.

Percentage of respiring symbionts hosted by starved bivalves. The percentage of respiring symbionts (CTC^+) was measured for purified symbionts from freshly collected clams (T_0) and from clams kept in starvation for 1 and 2 months. The percentage of actively respiring symbionts (CTC^+) versus total symbionts remained high and constant throughout the experiment (Table 1). At T_0, 80.1% ± 6.6% of symbionts hosted in gills could be considered to be metabolically active, which supported the importance of symbionts in association with the host. After 2 months of starvation, the percentage of CTC^+ symbionts was still high (83.9% ± 10.5%) despite the decrease in the abundance of symbionts shown by CARD-FISH. No data were available after 3 months of starvation because the decrease in the abundance of symbionts in the gills led to a very low symbiont concentration in the purified suspension, undetectable by epifluorescence microscopy.

Physiological changes in the symbiont population investigated by FCM. The physiological changes undergone by symbionts through host starvation were investigated, first considering the SSC and FL1 (fluorescence in the green channel) values of the whole populations (Fig. 3); a second and more detailed analysis was performed, considering the SSC and FL1 values of each subpopulation discriminated by FCM along with its relative proportion of the whole population (Fig. 4).

Figure 3 shows that the first consequences of host starvation (14 days) were a decrease of the SSC value calculated for the whole population and an increase in the FL1 signal of the symbiotic population. The decrease in the SSC signal of the entire population (half that at T_0) might be caused by a reduction in the sulfur content and/or in the mean cell size, since the SSC signal was influenced by both parameters (11), or by the disappearance of larger cells. In both cases, there was an apparent reduction of the mean cell size for the whole population. At the same time, FL1 modifications showed an increase in the mean nucleic acid content of the population (twice as high as at T_0). Between 14 and 28 days of starvation, the SSC signal of the whole population continued to decrease whereas the FL1 signal (nucleic acid content) remained constant. After 3 months of host starvation, the population of symbionts was characterized by an FL1 signal similar to that at T_0 but with a dramatically reduced SSC (10 times lower).

Figure 4 shows the number of subpopulations and their relative importance as a percentage for each starvation time. In the initial population (Fig. 4A), four subpopulations of symbionts were discriminated (Fig. 4A and E), with a majority of cells (62.1%) with high SSC (1.14) and FL1 (0.088) values. If the lower FL1 value (0.014) is artificially converted into one equivalent genome (1n), cells at this stage were characterized by six equivalent genomes (6n). These cells were previously described as large cells with multiple copies of their genome, rich in sulfur granules (11). A few cells (6.5%) were characterized by a very low SSC value (<0.1) and FL1 signal, i.e., very small cells, and approximately one-third of the population had an SSC value close to 0.15. After 14 days of starvation, four subpopulations of symbionts were still discriminated but with modifications in their SSC and FL1 values. For all subpopulations, the SSC signal decreased and the FL1 value increased, resulting in 3n and up to 12n for the lowest and highest FL1 signals, respectively. Moreover, the relative percentages of the

FIG. 2. Changes in the bacterial population in *C. orbicularis* gills during starvation, shown by histological gill sections. (A) Goldner staining of gill filaments from a freshly collected *Codakia orbicularis* clam. The ciliated zone (CZ), characterized by numerous cilia, is devoid of bacteria. The lateral zone (LZ) contains mostly bacteriocytes (BC) hosting the bacteria, as well as mucocytes in blue (asterisks) and granule cells (GC) stained in orange. (B) In situ hybridization (CARD-FISH) with the *C. orbicularis* symbiont-specific probe, showing that bacterial symbionts fill the bacteriocytes (BC) found all along the lateral zone of gill filaments in a freshly collected *C. orbicularis* clam. The ciliated zone (CZ) is not hybridized by the *C. orbicularis*-specific probe owing to the lack of symbionts in this part of the gill filament. Asterisk, mucocyte; GC, granule cell. (C) In situ hybridization (CARD-FISH) with the *C. orbicularis*-specific probe of gill filaments from an individual starved for 30 days. CZ, ciliated zone; GC, granule cell. (D) In situ hybridization (CARD-FISH) with the specific probe Symco 2 for gill filaments in a *C. orbicularis* clam starved for 56 days. Only a few poorly hybridized bacteriocytes can be observed. GC, granule cell; CZ, ciliated zone. (E) In specimens starved for 3 months, a few residual bacteriocytes (arrows) can be observed, while large lysosomes, appearing in orange owing to their autofluorescence, can be observed throughout the lateral zone. Most of the cells in the lateral zone are not positively hybridized by the specific probe. The remaining bacteriocytes occupying the lateral zone are scattered and small (compared to the control in panel 2B), with a weak signal since there are very few gill endosymbionts per cell. (F) Higher-magnification focusing on the abfrontal zone of a gill filament of an individual clam starved for 3 months. At this magnification, only two to four bacteria (arrows) can be observed in each sectioned bacteriocyte. The large lysosome-like structures (orange dots) are widely distributed throughout the abfrontal zone, indicating strong cellular digestion of the bacteria inside the bacteriocytes or of the bacteriocytes themselves.

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TABLE 1. Fluorescent area measured by CARD-FISH on gill sections and percentages of respiring symbionts[a]

Time of measurement	Fluorescent area (%)	% Respiring symbionts (*n*)
T_0	32.4	80.1 ± 6.6 (14)
1 mo of starvation (28 days)	10.7	84.5 ± 7.7 (12)
2 mo of starvation	3.7	83.9 ± 10.5 (4)
3 mo of starvation	1.7	No data

[a] CARD-FISH experiments were performed with the Symco 2 probe on gill sections of *C. orbicularis* clams held under starvation. The percentage of respiring symbionts represents the amount of cells able to reduce the redox probe, CTC, relative to the total amount of cells counterstained by DAPI. Mean values are shown with associated 95% confidence intervals. *n*, number of clams examined.

subpopulations changed compared to those at T_0. The proportion of "large" cells (high SSC) decreased from 62.1% to 34.5%, leading to an increase in the proportion of smaller cells. Over a longer starvation period, there was a reduction in the cytometric pattern, with three and two subpopulations remaining after 28 days (up to 15n) and 3 months (up to 5n) of starvation, respectively (Fig. 4C and D). Larger cells progressively disappeared during host starvation, and after 3 months of starvation, taking the two remaining subpopulations together (Fig. 4F), the symbiotic population was totally (100%) composed of very small cells (with an SSC value of <0.1) as opposed to 6.5% at T_0 (Fig. 4D).

DISCUSSION

This study used ultrastructural and physiological investigations to monitor the changes in the symbiotic population hosted by *C. orbicularis* clams kept under starvation conditions for a long period (4 months). The methods used to understand the effect of host starvation on its symbiotic population revealed significant changes in the symbiotic cells at different levels.

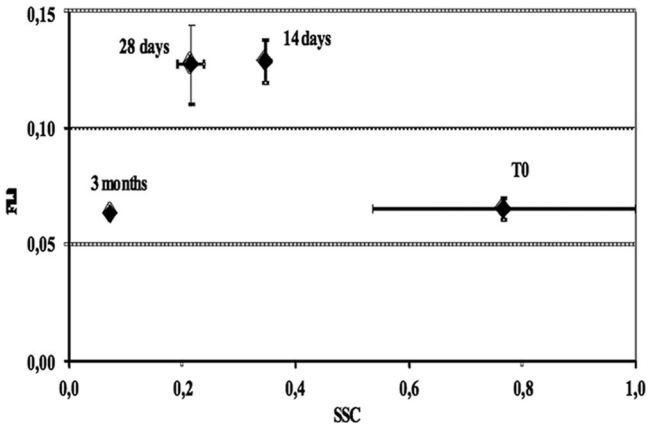

FIG. 3. FCM analyses of the sulfur-oxidizing population hosted by *Codakia orbicularis* during host starvation. The cells were stained with SYBR green I, and green fluorescent cytometry beads (2 μm) were added to the samples as an internal standard. For each period of starvation, one plot represents the whole population of symbionts, characterized by SSC and FL1 mean values normalized with respect to 2-μm beads. Horizontal and vertical bars represent 95% confidence intervals for the means, calculated for three distinct symbiotic populations, with the exception of the 3-month starvation period owing to the high clam mortality rate (30%).

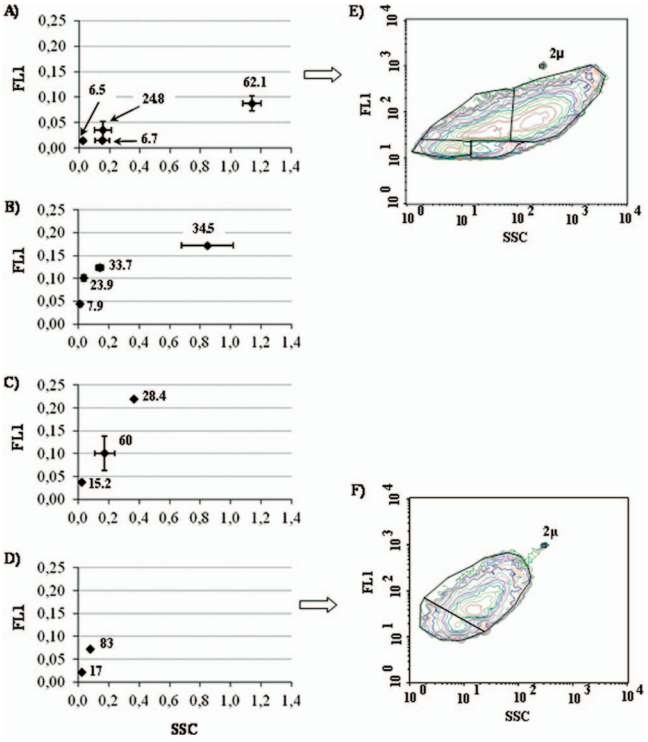

FIG. 4. SSC and FL1 mean normalized values for the different symbiotic subpopulations, discriminated by FCM after SYBR green I staining. Analyses of the subpopulations of symbionts were performed in triplicate at T_0 (A) or after host starvation for 14 days (B) or 28 days (C). For the 3-month starvation period (D), only one clam was analyzed. SSC and FL1 values were normalized with respect to 2-μm green fluorescent cytometry beads. Horizontal and vertical bars represent 95% confidence intervals of the SSC and FL1 means. Each plot corresponds to a subpopulation discriminated by FCM analysis. The percentage of each subpopulation is shown beside the plots. Contour plot presentations of the subpopulations discriminated by FCM are shown for T_0 (E) and for the 3-month starvation period (F).

A gradual disappearance of symbionts with time and significant modifications in the gill structure of *C. orbicularis* were shown by TEM and in situ hybridization. There was also a similar symbiont decline in thyasirid species and bathymodiolines after starvation in seawater devoid of sulfide or while kept under controlled conditions along with particle feeding (20, 40). The conditions in this study were more drastic for the host and its symbionts because no particle feeding was allowed for the host and no sulfide was available as an energy source for the symbionts. Nevertheless, the bivalve *C. orbicularis* was able to survive for up to 4 months under extreme starvation conditions. A similar survival time was observed for another lucinid (*Lucinoma aequizonata*) in a long-term experiment (3).

The decrease in the fluorescence signal observed by using CARD-FISH in the gill sections of *C. orbicularis* during starvation may be due either to a decrease in the bacterial symbiont abundance per bacteriocyte or to the decrease in ribosomes per bacterial cell caused by a drop in their physiological activity. Data on respiring (CTC^+) symbionts seemed to indicate that a high percentage (~80%) of the remaining symbiotic

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population in the gills exhibited respiratory activity during the starvation experiment, indicating that most FISH-detected symbionts were active cells rather than physiologically damaged bacteria. Using CARD-FISH and ImageJ analysis, it was estimated that symbionts occupied 32.4% of the gill tissue of freshly collected *C. orbicularis* clams, which is in agreement with the estimate for the *Riftia* trophosome (8, 50). It was therefore estimated that one-third of the symbiont population was lost in each month of starvation, with very few symbionts being detected after 3 months and none being detected after 4 months. The disappearance of the symbiont cells was clearly caused by the lysis of bacterial cells, possibly the result of bacterial autolytic processes or host-driven lysis. In both cases, lysis of bacterial cells was the main nutritional resource for the clam's survival. The importance of this trophic pathway has already been shown for hydrothermal-vent and shallow-water bivalves (6, 26, 39, 41) and for vestimentiferan tubeworms (9). Morphological data from TEM corroborated the hypothesis of host-driven lysis of bacterial cells since the lysosomes were larger and more numerous in the cytoplasm of bacteriocytes after 3 months of starvation. Lysosomes are found in the gills of freshly collected clams (28) but rarely observed by TEM owing to a low abundance. These intracellular structures can be observed when host starvation causes increased lysosomal activity to digest the symbionts and recover carbon or nitrogen for the host's own metabolism. This study did not, unfortunately, attempt any cytochemical detection of arylsulfatase or acid phosphatase (the main lysosomal enzymes) to confirm this process of degradation of the symbionts by the host. The gradual disappearance of symbionts was also related to the increased incidence of lysosomes in experimentally manipulated *Bathymodiolus azoricus* (40, 41). The possibility that the host *Codakia* could survive on symbiont digestion under these starvation conditions is consistent with the δ-^{13}C ratios measured in *C. orbicularis* by Berg and Alatalo (4). Although this ratio does not distinguish between nutrition based on translocation and that based on symbiont digestion, it is possible that this clam relies mainly on symbionts for its nutrition under natural conditions. Moreover, during the decline in the symbiont population, the proportion of bacteriocytes in the gill decreased while the number of granule cells appeared to increase. This indicates some evidence of plasticity within each gill filament during starvation, with competition between bacteriocytes and granule cells. Bacteriocytes are gradually and partially replaced by granule cells, which do not host bacteria and which become predominant in the lateral zone.

FCM analysis of the symbiotic population during the starvation experiment showed significant physiological modifications at the cellular level according to symbiont size and nucleic acid content. The results obtained in this study from freshly collected *C. orbicularis* clams were in agreement with those described previously (11). The symbiotic population of *C. orbicularis* at the onset of the experiment had a multiple subpopulation pattern, with some variations in the number of subpopulations of symbionts isolated from freshly collected clams. This multiple subpopulation pattern also revealed heterogeneity in the nucleic acid content (up to six equivalent genome copies for the highest FL1 fluorescence level) and a wide range of SSC signals related to symbiont size and sulfur content. The multigenomic state, typical of fast-growing cells, is not restricted to symbionts hosted by bivalves. This has already been demonstrated in symbiosis between *Rhizobium* and leguminous plants, where the host plant controlled its bacterial symbionts (*Rhizobium*), blocking bacterial division, triggering endoreplication cycles of DNA, and leading to polyploid bacterial cells (46). A comparison of the symbiont subpopulation pattern of *Codakia orbicularis* and the symbiotic population hosted by *Riftia pachyptila* (8) strongly suggests similarities in the population structure of sulfur-oxidizing symbionts hosted in the trophosome of *R. pachyptila* and in the gills of *C. orbicularis.* Bright and Sorgo (8) proposed a life cycle of symbionts through the trophosome section, where rod-shaped morphotypes (central) progressively changed their form into large cocci while being pushed outward from the central zone of the trophosome to the peripheral zone. Finally, lysis of large cocci occurred at the periphery of the trophosome to balance frequent symbiont division in the trophosome lobules to regulate symbiotic population.

Previous work (11) and data from this study suggest analogies between the hypothetical life cycle of *C. orbicularis*' symbiont and that of *R. pachyptila*. The symbiont of *Codakia*, which is environmentally transmitted (31), as is the case for that of *R. pachyptila* (47), probably enters the bacteriocyte at the apical pole, as described for juveniles (33). The bacteria then gradually increase in size and in nucleic acid content while migrating toward the basal pole of the bacteriocytes. Large symbionts in this study, representing high biomass, localized in the basal pole of the bacteriocytes, could be the first target for host digestion, which might partially explain the progressive disappearance from FCM data of the subpopulation with high SSC during the first month of starvation. The lysosomes seem to "empty" the bacteriocytes from the basal to apical poles, causing first large symbiont cells and then all cells to disappear. At that point, the bacteriocytes themselves disappeared, being replaced by granule cells in the gill organization. The role of these granule cells remains unclear, but this plasticity is noteworthy since it occurs in adult clams (28).

The effects of host starvation on the bacterial symbiotic population, in addition to the decrease in the number of symbionts, are consistent with this proposed "life cycle." Host starvation caused a subsequent symbiont starvation, since the symbiont itself was deprived of sulfides. Under these experimental conditions, the general pattern of the symbiotic population changed significantly, with a final reduction in the number of subpopulations and a progressive decrease in the cell size. Modifications in the nucleic acid content were also detected, since it increased (up to 15n) during the first month of starvation and dropped to 1n and 5n after 3 months for the two remaining subpopulations. This physiological change may be the reverse of the process mentioned above for natural conditions (11). Over a long starvation period, the symbionts underwent a degradation-like process, unlike the differentiation process described for *R. pachyptila* (8), possibly culminating with host digestion with the appearance of lysosomes. However, during the first 2 weeks, there was an increase in the fluorescence levels (FL1) of the clams analyzed (up to 12n), whereas the proportion of large cells decreased. The increase in the fluorescence level could be caused by the completion, under stress, of chromosome replication rounds starting at various points if most symbiotic cells in the bacteriocyte of *C. orbicu-*

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laris are considered to be multigenomic (11). The undefined host mechanism that controls symbiont cell division may also be altered during host starvation. Maldonado et al. (44) demonstrated that the nucleic acid content of *Azotobacter vinelandii* cells held in culture increased from the late exponential phase to the stationary phase prior to cyst differentiation. In both cases, starvation conditions seem to be responsible for the increase in the nucleic acid content per cell. For the *C. orbicularis* symbiont, the energy required for DNA replication could come from the oxidation into sulfate of the sulfur granules that are present in large symbiotic cells under natural conditions (11, 42), since "large" cells, according to the SSC signal, disappeared rapidly in the starvation experiment. This could explain why an apparent reduction in the SSC signal could be concomitant with an increase in the nucleic acid content.

The decrease in the Lucinidae symbiont population during a long starvation experiment has already been described for *Lucinoma aequizonata*, whose symbiont respires nitrate (36). This was confirmed in this study for another member of the Lucinidae, *C. orbicularis*, whose symbiont respires oxygen (23). The rates at which the symbionts disappeared were similar in the two cases. As far as we are aware, this is the first time that the physiological changes undergone by the symbiont population of a starved host have been studied using single-cell analysis. The change in symbiont size and the modifications to the nucleic acid content and the population structure, also confirmed by CARD-FISH and TEM, strongly suggest that all of these ultrastructural and physiological changes were a consequence of host digestion, but the possibility that senescent symbionts were enzymatically autolysed independently of lysosomes cannot be excluded (41). Direct evidence of host digestion should be further investigated using cytoenzymatic techniques. It can be assumed that under starvation conditions, the autotrophic pathway remains the main source of nutrition, allowing the host to survive for long periods with a lack of dissolved or particulate material.

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