

Characterization of the Population of the Sulfur-Oxidizing Symbiont of *Codakia orbicularis* (Bivalvia, Lucinidae) by Single-Cell Analyses^{∇†}

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We investigated the characteristics of the sulfur-oxidizing symbiont hosted in the gills of *Codakia orbicularis*, a bivalve living in shallow marine tropical environments. Special attention was paid to describing the heterogeneity of the population by using single-cell approaches including flow cytometry (FCM) and different microscopic techniques and by analyzing a cell size fractionation experiment. Up to seven different subpopulations were distinguished by FCM based on nucleic acid content and light side scattering of the cells. The cell size analysis of symbionts showed that the symbiotic population was very heterogeneous in size, i.e., ranging from 0.5 to 5 μm in length, with variable amounts of intracellular sulfur. The side-scatter signal analyzed by FCM, which is often taken as a proxy of cell size, was greatly influenced by the sulfur content of the symbionts. FCM revealed an important heterogeneity in the relative nucleic acid content among the subclasses. The larger cells contained exceptionally high levels of nucleic acids, suggesting that these cells contained multiple copies of their genome, i.e., ranging from one copy for the smaller cells to more than four copies for the larger cells. The proportion of respiring symbionts (5-cyano-2,3-ditolyl-terazolium chloride positive) in the bacteriocytes of *Codakia* revealed that around 80% of the symbionts hosted by *Codakia* maintain respiratory activity throughout the year. These data allowed us to gain insight into the functioning of the symbionts within the host and to propose some hypotheses on how the growth of the symbionts is controlled by the host.

Thioautotrophic symbioses in marine invertebrates have been studied for the last 25 years. Most of those studies were focused on hydrothermal vent environments. Nevertheless, similar types of symbiosis occur in sulfide-rich environments in the coastal zone with invertebrate hosts belonging to annelids, bivalves, gastropods, and protozoans (19, 42). These coastal organisms may represent attractive models to study thioautotrophic symbiosis, since these animals are much easier to sample and can often be kept alive in the laboratory with their symbionts for some extended period. This is an advantage for studies aiming to unravel the functioning of the symbionts within the host tissues and their interactions with the host.

Codakia orbicularis, a tropical bivalve, has been studied with respect to the symbiont-bearing host tissue (1, 18), its larval development (4, 25), and the mode of transmission of the symbionts to the new host generation (24, 26). The endosymbionts are hosted in specialized animal cells, i.e., the bacteriocytes, which in *C. orbicularis* are part of the gill tissue. Phylogenetic analyses demonstrated that the bacteria hosted by *C. orbicularis* belong to the same species (16S rRNA gene sequence for the strain from Guadeloupe; EMBL accession number X84979) (14), which is affiliated with a monophyletic group comprising sulfur-oxidizing symbionts of the bivalve su-

perfamily Lucinacea (10, 11, 14) within the *Gammaproteobacteria*. Studies of the physiology of these endosymbiotic bacteria have, however, been quite limited in scope and so far comprised the detection of enzymes such as Rubisco and ATP sulfurylase in the gill tissue (4) and, more recently, an analysis of their respiration strategies using microrespirometry with O_2 and H_2S microelectrodes (13). Therefore, the physiological status of these symbionts remains poorly characterized.

In general, metabolic exchanges between the thioautotrophic symbionts and their hosts have been inferred from enzyme assays (3, 44, 45), measurements of stable carbon isotope ratios (7, 41, 44), autoradiography (9, 15), or measurements of symbiont respiratory metabolism (2, 13, 31, 32). All these techniques have either no or only very limited potential to study the heterogeneity at the population level. Knowledge of the variability of the physiological status within symbiont populations is most important for an understanding of the functioning of these populations and their interactions with the host. Therefore, we have chosen to study symbionts with single-cell approaches based mainly on flow cytometry (FCM).

The introduction of FCM to the field of microbial ecology during the last 20 years has greatly improved the understanding of the functional role of bacteria in natural ecosystems. This method allows the rapid generation of data on large numbers of individual cells, recording several different parameters that can be linked to a variety of cellular characteristics for each single cell (49). This promoted numerous studies on the structure of natural microbial communities (reviewed in reference 20). Hence, many investigations of marine bacterioplankton by FCM revealed the presence of two major groups based on cell genomic content: high-nucleic-acid-content

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(HNA) and low-nucleic-acid-content (LNA) cells. It was demonstrated that cells with HNA were the most active cells in microbial communities (22, 36, 37, 48). Among the methods available to distinguish active from inactive cells with single-cell resolution, the reduction of the fluorogenic tetrazolium dye 5-cyano-2,3-ditolyl-terazolium chloride (CTC) to monitor respiring bacteria (43) is one of the most commonly used methods. It has been applied successfully in various types of environments including seawater (43, 47, 50), freshwater (8, 54), and soil samples (29, 40, 56) to assess respiring bacteria as a good indicator of active cells.

By combining several single-cell analysis methods, we aimed to characterize the physiological status of the thioautotrophic symbionts in *C. orbicularis* in order to gain insight into the functioning of the bacteria at the population level in the bacteriocyte. Therefore, we used fluorescent dyes in combination with FCM and epifluorescence microscopy. We characterized the structure of the symbiont population on the basis of their cell sizes, nucleic acid contents, and respiration activities; we paid special attention to describing the heterogeneity of the population. In addition, the sulfur content of the population was also investigated but not with a single-cell approach. To our knowledge, this is the first time that such single-cell approaches have been applied to endosymbiotic sulfur-oxidizing bacteria.

MATERIALS AND METHODS

Collection of bivalves. From May 2003 to April 2004, four to seven individual (a total of 56 over the year) *C. orbicularis* bivalves were collected by hand monthly at a depth of 5 to 10 cm in the sediment of the sea grass bed of *Thalassia testudinum* from Ilet Cochon (close to Pointe-à-Pitre, Guadeloupe, French West Indies). At the sample site, water column depths varied from 0.3 to 1 m. Over the year, the water temperature fluctuated between 25°C and 30°C. Samples were processed in the laboratory within 1 h after collection.

Five bivalves (bivalves G1 to G5) were collected simultaneously in August 2003 to investigate the interindividual variation in symbiont genomic content, cell size, and sulfur content, and the others were studied for comparison.

Transmission electron microscopy (TEM) preparation of gills. Individual bivalves were prefixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) adjusted to 900 mosM with NaCl and CaCl₂ in order to improve membrane preservation. After a brief rinse, they were stored in the same buffer at 4°C until fixed. 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 previously (26).

Extraction and purification of gill endosymbionts. Extraction and purification were performed by the Percoll cushion method (9), with modifications. To extract the endosymbiotic bacteria from gill tissue, one gill was homogenized in 8 ml of sterile seawater (35 ppt) using a hand-held Dounce homogenizer. The homogenized tissue was centrifuged (30 × g for 1 min). A volume of 4 ml of the supernatant was then centrifuged at 400 × 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 top of a Percoll (Sigma) cushion (3 ml) diluted with imidazole-buffered saline (490 mM NaCl, 30 mM MgSO₄, 11 mM CaCl₂, 3 mM KCl, 50 mM imidazole) and centrifuged at 1,000 × g for 8 min at 4°C. The symbionts were finally collected under the cushion, washed once, and suspended in 1 ml of filtered (0.2 μm) seawater (equals purified symbiont suspension). The purification was performed at 4°C to avoid the multiplication of marine bacteria initially present on the gills or between the gill filaments as contaminants (27). 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.

Incubation of symbionts with CTC. A 50 mM stock solution of CTC (Tébu) was freshly prepared just before experiment by dissolving the fluorogenic ester in sterile distilled water; this stock solution was kept at 4°C for no more than 5 days.

The purified symbiont suspension was diluted with filtered (0.2 μm) natural

seawater to ~10⁷ total cells ml⁻¹ and incubated with CTC at a final concentration of 4 mM in the dark at room temperature for 4 h, according to a modification of the method described previously by Rodriguez et al. (43). Incubation was ended by the addition of formaldehyde (4% final concentration); the sample was stored at 4°C until microscopic observation. We checked that the insoluble CTC formazan crystals were formed inside the cells by microscopy. Moreover, we also incubated a suspension of sodium azide-killed symbiont cells (0.1% final concentration) as an abiotic control.

Enumeration of CTC-positive (CTC⁺) cells by epifluorescence microscopy. Fixed samples already incubated with CTC were counterstained with DAPI (4',6'-diamino-2-phenylindole) at a final concentration of 2.5 μg ml⁻¹ in 0.1 M Tris-HCl buffer (pH 7.1) for 10 min in the dark. CTC-DAPI double-stained symbionts were filtered through 0.2-μm-pore-size black polycarbonate membranes (25-mm diameter; Nuclepore), mounted onto slides, and immediately observed at a magnification of ×1,000 using an epifluorescence microscope (BH2; Olympus). About 200 to 400 total cells (DAPI-stained cells) were enumerated using filter set U (UG-1, DM400 + L-420). The same microscopic fields as those examined with DAPI were enumerated using filter set G (BP545, DM580 + O-590) to count symbionts with respiring activity. These data are presented as percentages, so the ratio of CTC-active to total cells was multiplied by 100.

FCM analysis of relative nucleic acid contents and cell sizes of the symbionts in the whole population. Nucleic acid content analysis of the symbiont cells purified from *C. orbicularis* was performed with 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 a method described previously by Marie et al. (39) (1:10,000, vol/vol). As an internal standard for the normalization of symbiont fluorescence emission, 1-μm and 2-μm yellow-green fluorescent cytometry beads (Polysciences, Inc.) were added to the samples. True Count beads (Becton Dickinson Biosciences) were also added to the samples to determine the total cell concentration of each sample analyzed. The sheath fluid (NaCl solution, 30 ppt) was filtered through 0.2-μm-pore-size membrane. Analyses were run at low speed (around 18 μl min⁻¹), and acquisition was done for 2 min, corresponding to a total of 25,000 to 35,000 detected cells. Fluorescence of SYBR green-stained symbionts was collected in the green fluorescence channel FL1 (530 nm). Side-scattered light was used as a proxy of cell size (5, 53). Both parameters were collected on a logarithmic scale. The analysis of relative nucleic acid and cell sizes of symbionts was made on samples from five bivalves (gills G1 to G5). For comparison, the nucleic acid content (FL1) and side scatter (SSC) of marine bacteria sampled from the same location were analyzed in triplicate (SW1, SW2, and SW3).

Cell size-based fractionation of the symbiotic population by filtrations. The purified symbiont suspensions extracted from gills G1 to G5 were mixed and diluted in 30 ppt sterile NaCl (pool W) to get sufficient volume to perform cell size fractionation from the symbiont population. This sample was filtered successively through 3-μm-, 2-μm-, and 1-μm-pore-size polycarbonate membranes (Nuclepore; Whatman).

Aliquots of pool W and of each fraction were collected for FCM analysis; to determine the concentration of total cells, cell size, and genomic content (see above); and for cellular sulfur content measurements (see below).

Analysis of S⁰. Filtrates corresponding to the different size fractions were freeze-dried and subsequently extracted in methanol. The sulfur was separated from other UV-absorbing compounds and quantified by high-performance liquid chromatography on a C₈ column (Sunfire C₈ 3.5-μm-grain-size, 4.6-mm by 150-mm column; Waters Corporation, Milford, MA) using a binary gradient from A (70% methanol mixed with 30% of an aqueous solution of 1 M ammonium acetate) to B (100% methanol) with a Waters (Milford, MA) multisolute delivery system. A Waters PDA2996 photo diode array detector was used to monitor the online absorption spectra from 200 to 340 nm. A solution of rhombic sulfur (FLUKA, Buchs, Switzerland) in methanol was used as a standard. Using this system, the elemental sulfur (S⁰) eluted at a retention time of 8.4 min and showed the characteristic absorption maximum at 260 nm with a shoulder at 277 nm. Hence, sulfur was quantified from the area of the absorption peak at 260 nm (response factor = 75,138 μV s nmol⁻¹). The calibration curve was linear from 10 to 100 nmol of sulfur injected. The purity of the sulfur peaks in the filtrate extracts was checked by comparing their 200- to 340-nm absorption peaks with the standard, which were fully coincident. This protocol successfully separated the elemental sulfur from some unknown UV-absorbing compounds that were present in the extracts.

The average specific sulfur content of the different size fractions was calculated

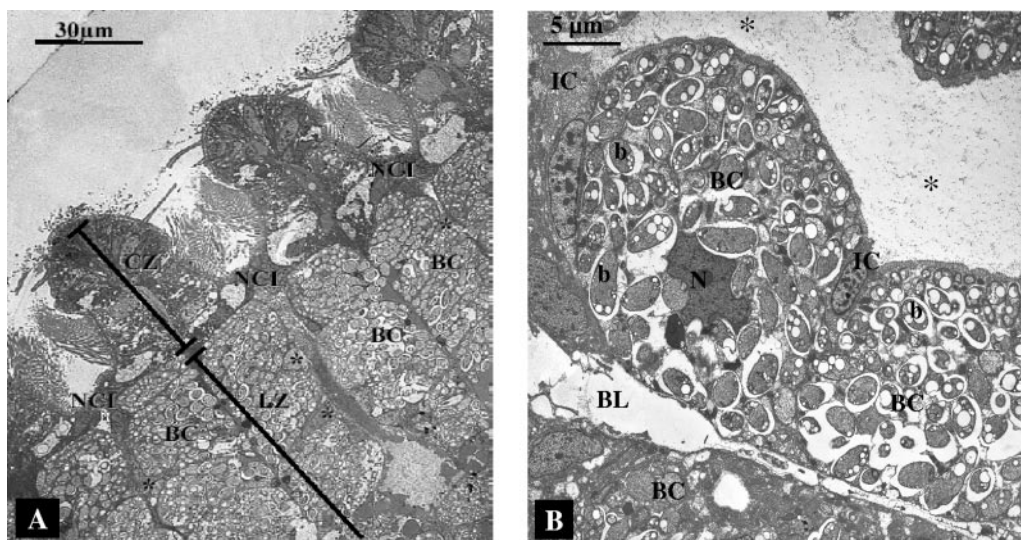


FIG. 1. TEM of ultrathin sections of *C. orbicularis* gill and bacteriocyte. (A) Low magnification of gill filaments from adult individuals dissected immediately upon recovery. Each gill filament was characterized by a ciliated zone (CZ) separated from the lateral zone (LZ) by several nonciliated intermediary cells (NCI). Bacteriocytes (BC) filled with chemoautotrophic symbionts were part of the lateral zone with numerous intercalary cells characterized by their nucleus in an apical position (asterisks). (B) Bacteriocytes (BC), which were the most prevalent cells in the gill filament, had a basal nucleus (N) and a rounded apical pole developing broad contact with pallial seawater (asterisks). The cytoplasm was crowded by envacuolated bacteria (b), which were usually individually enclosed inside bacteriocyte vacuoles. IC, intercalary cells; BL, blood lacuna. Sulfur globules appeared as electron-lucent bodies within the symbiont cells.

by dividing the sulfur concentration by the cell concentration determined by FCM. We calculated the corresponding values for selected size fractions by subtraction of the values for the appropriate filtrates; e.g., the values for the 2- to 3- μm size fractions were calculated from the difference between the values in the filtrate at $<3\ \mu\text{m}$ and the filtrate at $<2\ \mu\text{m}$.

RESULTS

TEM. Figure 1 shows TEM observations of bacteriocytes in the gills. As previously described by Frenkiel and Mouëza (18), each gill filament was composed of three zones, i.e., a ciliated zone, an intermediary zone (both devoid of bacterial symbionts), and a third lateral zone harboring the intracellular bacteria (Fig. 1A). The whole lateral zone of each gill filament was occupied with bacteriocytes full of bacteria, intercalary cells (Fig. 1A and B), granule cells, and mucocytes (not shown). 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. 1B). The bacterial endosymbionts were very heterogeneous in size (Fig. 1B). Bacteria were mostly small at the apical pole of the bacteriocyte (around 1 μm in length), while some of them reached up to 5 μm in length near the basal pole (Fig. 1B). Similar patterns have been systematically observed in the TEM cross sections studied previously by Gros (23). The smallest bacteria usually presented a single S^0 globule (located in the periplasmic space and appearing as electron-translucent bodies after conventional TEM preparation). The larger cells near the basal pole showed a wide variety of sulfur content, including some that were heavily loaded with numerous (up to 10) sulfur globules and others that comprised mainly cytoplasm and no visible or a very limited amount of sulfur (Fig. 1B).

Annual variability of symbiont respiration. From May 2003 to April 2004, individual bivalves (varying from four to seven bivalves) were collected monthly to measure the percentage of respiring symbionts harbored by the host. Special attention was paid to the spawning period, from June to October. A total of 56 individual bivalves were sampled over the year. Over the sampling period, respiring symbionts (CTC^+ cells) ranged between 67.9% and 85% of the total bacterial count, with an average value of $77.6\% \pm 3.7\%$ and with no clear tendency regarding to the spawning period. Moreover, no apparent relation with the water temperature (25 to 30°C) was observed.

FCM analysis of nucleic acid content and light scattering of symbionts in the whole populations. Figure 2A and C show a cytogram (SSC versus FL1) for a whole population of symbionts extracted from the gills of a single bivalve (bivalve G4). According to SSC and FL1 signals, the symbiotic population exhibited a great heterogeneity, and up to seven subpopulations (P1 to P7) have been distinguished. Similar cytograms were obtained for the symbiont populations extracted from the four other bivalves (bivalves G1, G2, G3, and G5) in which we have distinguished six or seven equivalents of the subpopulations observed for bivalve G4. Subpopulation P5 was observed in only two of the five bivalves (see Table S1 in the supplemental material). The percentages of the different subpopulations P1 to P7 in the gills of the five bivalves are shown in Fig. 3, and the normalized average FL1 and SSC values that characterize these subpopulations are shown in Fig. 4. Subpopulation P7, which comprised cells with the highest FL1 and SSC signals, was the most abundant subpopulation, with a proportion accounting for 55 to 79% of the total population. As subpopulation P6 was the second most abundant subpopulation

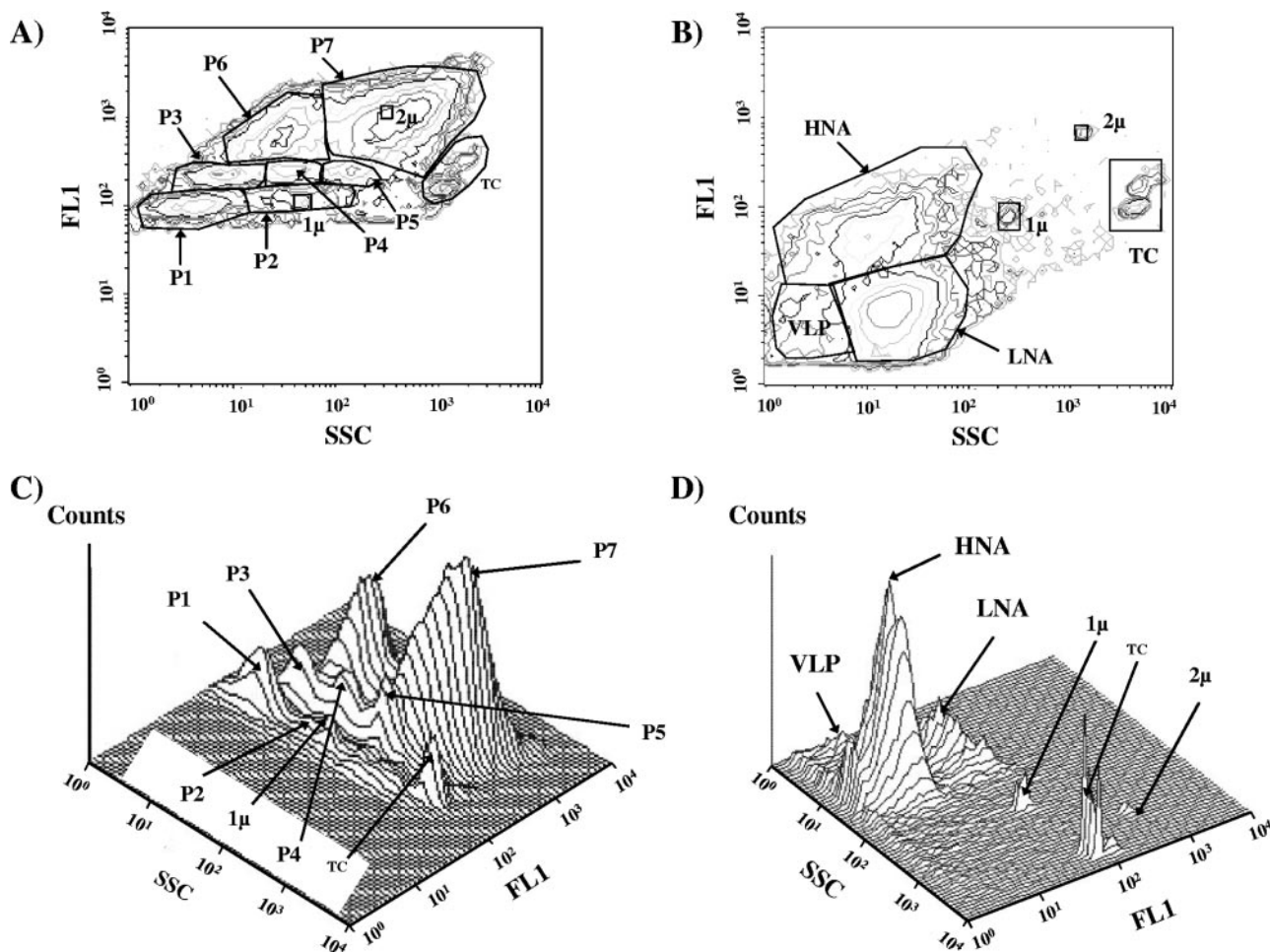


FIG. 2. FCM analyses of a sulfur-oxidizing population hosted by *C. orbicularis* (A and C) and of seawater (B and D) sampled above the sampling site of *C. orbicularis*. (A and B) Contour plot presentations. Gray lines represent the contour plots that are isopleths of the number of events and are scaled proportionally to the maximum values. Note that the innermost contour plots describe the peaks of the different subpopulations. Black lines delimit the identified subpopulations. (C and D) Three-dimensional projections of the A and B cytograms. The cells were stained with SYBR green I. Fluorescent beads (1 μm and 2 μm) were added to the samples as an internal standard. True-count (TC) beads were used to determine total cell concentrations. Seven subpopulations (P1 to P7) were discriminated according to SSC and FL1 (green fluorescence) signals in the symbiotic population (A and C). Three different subpopulations, HNA and LNA cells and virus-like particles (VLP), were identified according to SSC and FL1 signals in the seawater sample (B and D).

in four of the five bivalves, these two subpopulations (P6 and P7) accounted for approximately 80% of the total symbionts.

For comparison, we also analyzed the cell characteristics of the free-living bacteria sampled in triplicate (SW1, SW2, and

SW3) from the overlaying water column above the *Thalassia* sea grass bed. Figure 2B and D show the cytogram for one of those samples (SW1). The SSC-versus-FL1 cytometric signature of this sample is markedly different from the cytograms obtained for the symbiont populations. Thus, in the overlying water, only three subpopulations that corresponded to (i) HNA bacteria, (ii) LNA bacteria, and (iii) a minor group characterized by low SSC and FL1 values probably comprising virus-like particles were distinguished. The other two replicates also showed the same pattern, and the normalized average FL1 and SSC values for the subpopulations distinguished in the seawater samples are also depicted in Fig. 4.

In general, it was observed that subpopulations P2 to P7 were clearly separated from the LNA and HNA free-living bacteria (Fig. 4A). Nevertheless, certain confusion was possible for subpopulation P1 in the bacteriocyte and the HNA bacteria of the overlying seawater (Fig. 4B); i.e., the mean value of SSC of subpopulation P1 was significantly different from the corresponding value for HNA bacteria, and both

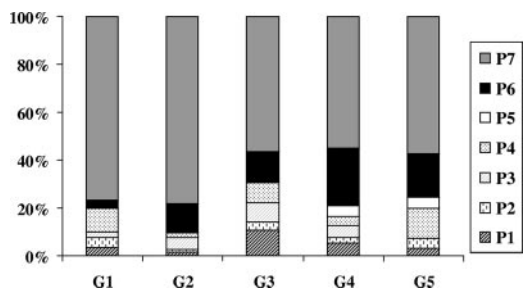


FIG. 3. Analysis of the percentages of the different subpopulations (P1 to P7) (Fig. 2A and C) (see Table S1 in supplemental material) among the five different individual bivalves collected in August 2003 (gills G1 to G5).

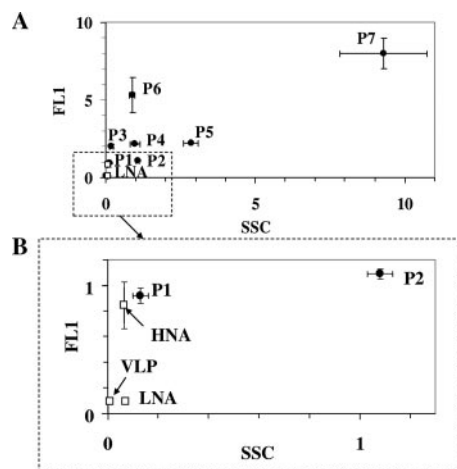


FIG. 4. SSC and FL1 mean normalized values for the different subpopulations discriminated by FCM analysis of the populations of symbionts (●) and the seawater samples (□) (A). FL1 and SSC values were normalized with respect to 1- μm and 2- μm green fluorescent cytometry beads. A focus of low SSC and FL1 values in A is shown (B). Horizontal and vertical bars represent 95% confidence intervals of the means. The mean values were calculated from the five endosymbiotic populations (G1 to G5) and from the three seawater samples (SW1 to SW3).

subpopulations had comparable (not significantly different) mean FL1 values (Fig. 4B). An overlap of P1 and HNA populations is therefore possible. This means that free-living HNA bacteria could in principle be present in the symbiont populations extracted from the gills, more precisely in subpopulation P1, and that free-living HNA bacteria could comprise the symbiont species with characteristics of subpopulation P1. Nevertheless, because the distinguished P1 subpopulations were always less than 10% of the total symbionts (mean value, 4.8%), we can conclude that the cytograms are not significantly influenced by marine bacteria. Hence, the FCM analysis based on fluorescence levels (FL1) and light-scattering characteristics (SSC) of SYBR green-stained cells showed that the endosymbiotic population extracted from host gill was different from the free-living marine bacteria sampled from the habitat of the bivalves.

The analysis of FL1 values for SYBR green-stained cells thus clearly demonstrated that the symbiotic population of *C. orbicularis* was characterized by a largely heterogeneous nucleic acid content, with a multimodal distribution pattern and with some subpopulations (P6 and P7) characterized by a very HNA (Fig. 2A and C). As shown in Fig. 4 (see also Table S1 in the supplemental material), among the seven symbiont subpopulations, we distinguished four different mean levels of FL1. Subpopulations P1 and P2 were characterized by mean FL1 levels close to 1 (normalized). Three subpopulations, namely, P3, P4, and P5, were characterized by mean FL1 levels close to 2 (normalized). Subpopulations P6 and P7 were characterized by mean normalized FL1 values of 5.31 and 7.98, respectively.

The light-scattering properties of the symbiotic cells were very heterogeneous (Fig. 4A) (see Table S1 in supplemental material), and accordingly, up to four different average levels of SSC could be distinguished among the seven subpopula-

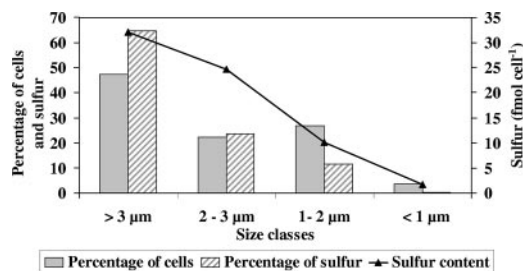


FIG. 5. Frequency distribution of symbiont cell abundances and sulfur amounts among the different size classes (see Materials and Methods and Results). The average cell-specific sulfur content is also given for each size class.

tions. Thus, subpopulations P1 and P3 were characterized by normalized SSC values close to 0.1 and 0.2, respectively. Subpopulations P2, P4, and P6 were characterized by normalized SSC values close to 1, while subpopulations P5 and P7 were characterized by normalized SSC values of 2.8 and 9.3, respectively. In general, the SSC values are taken as a proxy for the cell size of the microbes. However, the symbionts of *C. orbicularis* carried a variable amount of elemental sulfur as intracellular globules that are highly refracting. Hence, intracellular sulfur globules as shown in Fig. 1B may have a great influence on the light-scattering properties of the cells.

FCM analysis of the relative nucleic acid content and cell size and sulfur content determination in the size-fractionated symbiont's populations. We performed a size fractionation experiment of a pooled sample (pool W) of symbiont populations to study the link between cell size, fluorescence level (FL1), light scatter (SSC), and sulfur content of the cells. The endosymbiotic suspension (pool W) was successively filtered through 3- μm -pore-size, 2- μm -pore-size, and 1- μm -pore-size membranes. By calculating the differences in cell and sulfur concentrations between subsequent filtration steps (3, 2, and 1 μm), we determined the proportion of the population and sulfur amounts in the different size classes, as depicted in Fig. 5. Thus, we found that 47.2% of the population comprised cells larger than 3 μm , and 22.3% of the cells ranged between 2 and 3 μm . Another fraction between 2 and 1 μm represents 26.8% of the cells, and the smallest fraction, <1 μm , comprised 3.7% of the cells. The analyses of the elemental sulfur content in the different size fractions showed that 65% of the sulfur was carried by cells that were >3 μm , which thus had an average specific sulfur content of 32 fmol cell^{-1} (Fig. 5). A significant proportion of the sulfur was carried by cells of 2 to 3 μm and 1 to 2 μm , which represented 23.5% and 11.6% of the total sulfur, respectively. An unequivocal S signal was detected in the filtrate after filtration on 1- μm -pore-size filters, although this size fraction represented only less than 0.3% of the total sulfur and 3.7% of the cells. The average S content for this <1- μm size fraction was 1.6 fmol cell^{-1} ; this low value is in agreement with the microscopic observations that smallest bacteria usually carried a single S° globule (Fig. 1B). The average specific S content clearly increased with cell size (Fig. 5), which reflects the fact that larger cell volumes allow the storage of a larger quantity of sulfur (Fig. 1B). Unfortunately, S content is the only parameter studied that is not available on a single-cell

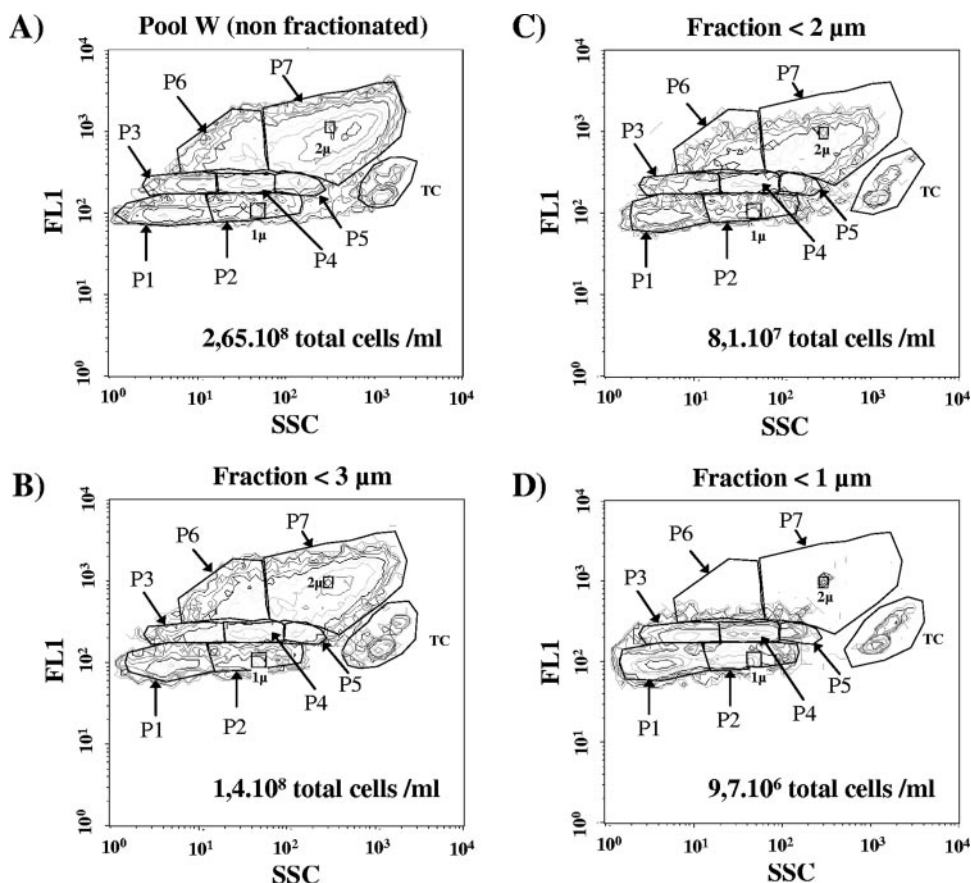


FIG. 6. Contour plot representation of the total population of the initial nonfractionated pooled sample W (A) and of the cell-size-fractionated populations after successive filtrations through 3- μm (B)-, 2- μm (C)-, and 1- μm (D)-pore-size membranes of symbionts in *C. orbicularis*. Gray lines represent the contour plots that are isopleths of number of events and are scaled proportionally to the maximum values. Also see the legend of Fig. 2. Black lines delimit the subpopulations identified in the nonfractionated sample. Bacterial cells were stained with SYBR green I, and fluorescent beads (1 μm and 2 μm) were added to the sample as an internal standard. True-count (TC) beads were used to determine the total cell concentration, which is mentioned at the bottom within each cytogram (see the legend of Fig. 2).

basis. SSC can be taken as an indication of S content, although that signal is also influenced by cell size.

Aliquots of the different size fractions were also stained with SYBR green and analyzed by FCM for their FL1 and SSC values; the results are shown in Fig. 6. The dominant subpopulations P6 and P7 virtually disappeared only after filtration on the 1- μm -pore-size membrane (Fig. 6D). Hence, these subpopulations comprised cells that are $>3 \mu\text{m}$ and cells ranging from 2 to 3 and from 1 to 3 μm , as subsequent filtrations removed a part of each subpopulation. The latter phenomenon was also observed for the other subpopulations. Hence, while subpopulations P6 and P7 were heterogeneous in size but mainly larger than 1 μm , subpopulations P1 to P5 seemed to include cells from all different size classes. Moreover, the analysis of the remaining suspension after filtration on 1- μm -pore-size membranes clearly showed that cells that were $<1 \mu\text{m}$ were widespread among these five subpopulations, which covered two fluorescence levels. Thus, subpopulations P3, P4, and P5, which are characterized by a normalized FL1 value of about 2, are clearly separated by their three distinct SSC values (Fig. 4). Based on these observations, we suggest that subpopulations P3, P4, and P5 differ mainly by their sulfur contents

rather than by their sizes. Similarly, subpopulations P1 and P2 are cells ranging from 0 to 1 μm , with comparable nucleic acid contents but are likely different with regard to their sulfur contents. The range for SSC values in the predominant subpopulations P6 and P7 is much wider than could be expected from their size ranges, which thus indicates a variability of S contents within these subpopulations. It thus appears that subpopulations P6 and P7, with cells that are $>1 \mu\text{m}$, are differentiated mainly by their sulfur content related to their strongly different SSC values.

DISCUSSION

The comparison of FCM signatures of the symbiotic population hosted by *C. orbicularis* and that of free-living marine bacteria sampled from the habitat of the bivalves showed that they were significantly different (Fig. 2). Previous phylogenetic studies have shown that the symbiont population isolated by our extraction protocol from the host bivalve *C. orbicularis* corresponded to a single bacterial species characterized by a unique 16S rRNA gene sequence that was found in various individuals sampled from different environments ranging from

Bermuda to Guadeloupe (10, 14). These data indicate that the host maintains a monospecific culture within the bacteriocytes and is able to prevent the entry and the development of other bacterial species in the gill.

Fluorescence intensity analysis of the SYBR green-stained endosymbiotic population to determine the nucleic acid content of individual cells revealed very high levels of fluorescence, with a multimodal distribution comprising up to four distinct nucleic acid contents. Heterogeneity of genomic content has been observed previously in FCM studies of bacteria and has been described for pure cultures of *Aleromonas haloplanktis* (34) and *Yersinia ruckeri* (52), with up to four and five DNA fluorescence levels. In such cultures, the lowest fluorescence level has been interpreted as cells with one genome copy (1n), and the additional higher fluorescence levels reflect additional genome copies (6). Because the symbiont population is monospecific and comparable to the pure cultures, the FL1 levels can therefore be interpreted in terms of genome equivalents. However, we consider that SYBR green I stains both DNA and RNA, although the quantum yield for DNA is double that of RNA (35). With some caution, we can interpret the different mean FL1 levels observed for the different subpopulations as being indicative of multiples of genome copies, while some variation in FL1 can also be attributed to varying RNA levels. Accordingly, subpopulations P1 and P2 corresponded to cells with a single genome (1n), and subpopulations P3, P4, and P5 probably corresponded to 2n cells, while subpopulations P6 and P7 were probably more than 4n, respectively. In general, the normalized FL1 values observed for the symbionts are much higher than the values observed for the free-living marine bacteria (Fig. 4) and also higher than the values generally described for free-living bacteria in freshwater, brackish water, and seawater samples (36, 37), which also shows an exceptionally high genomic content of these endosymbiotic bacteria.

Multiple genome copies are a characteristic of fast-growing bacteria. This is related to the fact that the bacterial genome can contain more than one pair of replication forks. The presence of multiple forks allows cells to double at a faster rate than the time needed for a single fork to traverse the entire genome. Thus, an ultrafast-growing *Escherichia coli* cell may comprise up to eight chromosome copies prior to division (30). Therefore, the multigenomic state of bacteria has been considered in the literature to be characteristic of cells that are capable of rapid cell division when nutrients become available (52). In addition, it has been suggested that cells with HNA are highly active, as reflected by high rates of leucine incorporation compared to cells with LNA (36, 46, 48).

The substrates for the growth of sulfur-oxidizing chemoautotrophic endosymbiotic bacteria (i.e., H_2S or $\text{S}_2\text{O}_3^{2-}$, CO_2 , and O_2) are provided by the host (17). It seems unlikely that the host could deliver these substrates and other essential nutrients at a rate sustaining ultrafast growth of the symbionts, as suggested by the multigenomic state of the symbiont cells, without controlling cell division. Indeed, dividing cells are rarely observed in TEM photographs (Fig. 1B) (see the many examples in reference 23). An undetectable proportion of dividing cells obviously cannot sustain the rate of cell multiplication that is characteristic of ultrafast growth. We rather suggest that cell division was inhibited within the bacteriocyte while maintaining a high level of activity in symbiont cells. It

has been shown that a multigenomic state and large cell sizes can also be induced by drugs preventing bacterial division. Accordingly, Boye and Løbner-Olesen (6) succeeded in artificially obtaining *E. coli* K-12 cells with seven or eight fully replicated chromosomes by adding rifampicin and cephalixin to their log-phase cultures. Therefore, we hypothesize that the host bivalve may excrete bacteriostatic molecules to control endosymbiotic bacterial division in the bacteriocyte while allowing ongoing rounds of DNA replication to the termini. Alternatively, it is possible that the bacteria themselves inhibit cell division, after genome replication is completed, as a response to high cell densities via a quorum-sensing system. Under both circumstances, a multigenomic stage is achieved without a need for multiple replication forks. While within a population, a continuum of DNA levels may occur under such circumstances, a multimodal pattern will be generated when the chromosome replication periods occupy only a fraction of the total life span. The more frequent cell states will then correspond to cells with fully replicated chromosomes, which may explain the patterns depicted in Fig. 2A and C and 4.

If endosymbiotic bacterial division is indeed inhibited in the bacteriocytes by the host, it appears that the metabolic potential of the cells remains high. This is shown by the detection of respiratory activity of symbionts extracted from bivalves collected throughout a whole year. The proportion of respiring bacteria (cells with an active electron transport system) was very high and showed little variation throughout the year. The data available in the literature on the percentages of actively respiring bacteria are provided mainly for marine and freshwater samples and generally indicated that no more than 10% of total bacteria would be considered active in reference to CTC reduction (21, 33, 50). Higher values (maximum of 45%) of CTC⁺ cells have been reported for bacteria attached to particles (51) or for bacteria living in anoxic sediments (40).

We have already highlighted that the metabolic rates of the intracellular symbionts could be controlled by the host, supplying limited amounts of nutrients to the symbionts. As a result, growth and cell division of the bacterial symbionts can be limited by nutrient supply. Such a control mechanism has been documented for alga-cnidaria symbioses, where growth of the symbionts was found to be limited by nitrogen or carbon (12).

In most thiotrophic bacteria, sulfur is the intermediate product of the oxidation of different reduced sulfur compounds including hydrogen sulfide, thiosulfate, and polysulfides. In this paper, we observed the S globules in the cells as typical electron-translucent bodies in TEM photographs (Fig. 1) and quantified the amount of sulfur extracted from the cells by high-performance liquid chromatography (Fig. 5). Recently, it was confirmed by cryo-energy-filtered TEM and parallel electron energy loss spectroscopy analysis that the symbionts in the gills of *C. orbicularis* indeed harbor S⁰, which occurs as granules in the periplasmic space (J.-P. Lechère, G. Frébourg, F. Gaill, and O. Gros, unpublished data). The presence of these intracellular sulfur globules shows that the symbionts were supplied with a reduced sulfur compound, probably by the hosts. The in vivo primary electron donor for the symbionts of *C. orbicularis* is not known and could be either H_2S or $\text{S}_2\text{O}_3^{2-}$. The intracellular elemental sulfur can be further oxidized to sulfate when the supply of the primary electron donor is stopped by

the host. In addition, *in vitro*, it has been shown that under anoxic conditions, the intracellular sulfur can be used as an electron acceptor by the symbionts and reduced to hydrogen sulfide (13). However, the reduction of sulfur to sulfide seems to be unfavorable for the host under *in vivo* conditions.

Generally, the SSC values are taken as a proxy for the cell sizes of the microbes. However, the symbionts of *C. orbicularis* carry a variable amount of S⁰ as intracellular globules that are highly refracting. Thus, the SSC values of the symbionts are strongly influenced by their sulfur contents; therefore, we expect that the SSC value represents a mixed signal influenced by both the cell size and sulfur content of this thiotrophic symbiont. For the larger cells, it is most likely that the sulfur-rich cells belong to subpopulation P7, while the sulfur-poor cells are expected to belong to subpopulation P6. However, a straightforward quantification is difficult because it is not possible to deconvolute the SSC signal into components corresponding to cell size and sulfur content, respectively. The use of new-generation flow cytometers with improved cell-sorting capacities and laser excitation provides an interesting future perspective to obtain more detailed information about the characteristics of the seven different subpopulations.

The large cells with very low or no sulfur are concentrated mainly along the basal pole of the bacteriocyte, while the sulfur-containing cells of smaller size are located near the apical pole. In the center of the bacteriocyte, cells are generally large with a variable amount of sulfur globules (Fig. 1B). We suggest two different mechanisms to explain this spatial organization in the bacteriocyte. The first suggested mechanism relies on a dynamic in time. Thus, recently recruited symbionts are located near the apical pole and abundantly supplied with growth substrates by the host, while their division is inhibited. As new symbionts are recruited, the older ones migrate toward the basal pole. During this process, they are progressively deprived of primary electron donor supply and therefore oxidize their intracellular sulfur globule. The second mechanism assumes a spatial variation in the supply of the primary electron donor due to a diffusion gradient within the bacteriocyte. Thus, we expect that a high level of primary electron donor is available in the apical part of the bacteriocyte, while the concentrations are close to zero in the basal part.

An interesting picture emerges from the single-cell analyses of the thiotrophic symbionts living in the bacteriocytes of *C. orbicularis*. The population in the bacteriocytes is monospecific (10, 14), and the symbionts are horizontally transmitted (24, 26) and thus recruited from the external environment. Nevertheless, the cytograms indicate that only subpopulation P1 (<10% of the total) may be confounded with a signal characteristic of the free-living HNA population in the seawater. Therefore, the symbiont population living in the bacteriocyte of *C. orbicularis* can be compared to a pure laboratory culture that is virtually devoid of contaminants. This implies that the host is capable of controlling the population dynamics of the symbionts by regulating the entry to the bacteriocyte and by regulating growth in the bacteriocyte. Growth of the symbionts in the bacteriocytes can be controlled by the host by regulating the supply of the major substrates for thiotrophic chemosynthesis (i.e., H₂S, S₂O₃²⁻, CO₂, and O₂), and in return, symbionts provide their host with organic compounds (55). We suggest that the host controls growth to obtain larger cells without

sulfur close to the basal part within the bacteriocyte and that this is a very useful strategy, which allows the host to harvest biomass without the need to evacuate large quantities of sulfur. Thus, symbiont densities could be regulated by host digestion that allows the control of population size and harvesting of symbiont biomass production. This hypothesis is supported by the observation of lysosomes inside the bacteriocytes (16, 38) and by studies of carbon isotope ratios (4). In this paper, we clearly show that the symbiont population comprises a large proportion of large cell sizes and a multigenomic state together with an undetectable proportion of dividing cells (Fig. 1) (23). This clearly indicates that the host can also regulate the densities of the symbionts by inhibiting cell division. We suggest that this phenomenon is based on the excretion, by the host, of specific cell division-inhibitory compounds into the bacteriocyte. The identification of such compounds is an important research perspective for this symbiosis, as is the study of a potential role of a quorum-sensing-like mechanism for controlling cell division in the bacteriocyte.

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