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Respiration Strategies Utilized by the Gill Endosymbiont from the Host Lucinid *Codakia orbicularis* (Bivalvia: Lucinidae)

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The large tropical lucinid clam *Codakia orbicularis* has a symbiotic relationship with intracellular, sulfide-oxidizing chemoautotrophic bacteria. The respiration strategies utilized by the symbiont were explored using integrative techniques on mechanically purified symbionts and intact clam-symbiont associations along with habitat analysis. Previous work on a related symbiont species found in the host lucinid *Lucinoma aequizonata* showed that the symbionts obligately used nitrate as an electron acceptor, even under oxygenated conditions. In contrast, the symbionts of *C. orbicularis* use oxygen as the primary electron acceptor while evidence for nitrate respiration was lacking. Direct measurements obtained by using microelectrodes in purified symbiont suspensions showed that the symbionts consumed oxygen; this intracellular respiration was confirmed by using the redox dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride). In the few intact chemosymbioses tested in previous studies, hydrogen sulfide production was shown to occur when the animal-symbiont association was exposed to anoxia and elemental sulfur stored in the thioautotrophic symbionts was proposed to serve as an electron sink in the absence of oxygen and nitrate. However, this is the first study to show by direct measurements using sulfide microelectrodes in enriched symbiont suspensions that the symbionts are the actual source of sulfide under anoxic conditions.

Nutritional symbiotic associations between marine invertebrates and intracellular sulfur-oxidizing bacteria have been characterized for over 2 decades (15, 18). Chemoautotrophic symbiosis was first described in invertebrates living at the hydrothermal vents but is now known to occur in invertebrates from more accessible habitats, such as shallow-water sediments (4, 43). Since their discovery, only one chemosymbiotic animal, the shallow water lucinid *Codakia orbicularis* (20, 22), has been successfully reared to the juvenile stage without symbionts. This species belongs to the Lucinidae, one of five bivalve families known to have a symbiotic relationship with intracellular chemoautotrophic bacteria (reviewed in references 18 and 44).

Shallow-water lucinid species are found among the sea grasses *Thalassia* and *Zostera* (2). *C. orbicularis* is specifically associated with the roots of *Thalassia testudinum* and is distributed from Bermuda throughout the Caribbean and southward to Brazil (1, 2). Comparative analysis of bacterial 16S rRNA gene (rDNA) sequences derived from *C. orbicularis* gill tissue revealed that the symbiotic gamma proteobacterium is shared between two *C. orbicularis* populations, one from the northern Caribbean islands of the Bahamas and the other from the French West Indies (Guadeloupe) located north of the lesser Antilles in the Caribbean (12, 13). In addition, the symbiont species is shared among six tropical lucinid species: *Anodontia alba*, *C. orbicularis, C. orbiculata, C. pectinella*, *Divaricella quadrisulcata*, and *Linga pensylvanica* (13, 14, 21, 24). Molecular and ultrastructural studies have shown that the symbiosis is established after *C. orbicularis* reaches the juvenile stage and is acquired from the sediment of its natural habitat (20, 23).

Phylogenetic analysis of 16S rDNA sequences derived from lucinid symbionts indicates that they form a monophyletic lineage (11–13), although the habitats of lucinids range from highly oxygenated water to hypoxic overlying water (7, 30). It has been shown that competition between the host lucinid *Lucinoma aequizonata* and its symbiont for the scarcer electron acceptor oxygen is avoided through the obligate use of nitrate by the symbiont as an electron acceptor, even under oxygenated conditions (25, 26, 28). *L. aequizonata* lives in the Santa Barbara Basin at a water depth of 500 $\pm$ 10 m, where the oxygen concentrations are $\leq$20 μM (7, 25) and nitrate is present at a concentration of 30 μM (25) in the overlying water. In similar studies, nitrate respiration has also been reported in the endosymbionts from the vestimentiferan *Riftia pachyptila* (27) and the solemyid *Solemya reidi* (47). Because the symbionts found in these three systems have been shown to respire nitrate, it has been proposed that nitrate may be an important electron acceptor among chemoautotrophic bacteria involved in this type of symbiosis (25). Previous studies have reported the presence of nitrate reductase activity in the gill tissue of *C. orbicularis* as well (4, 16). Unlike with the other chemosymbioses tested for nitrate respiration, *C. orbicularis* inhabits sea grass beds which are typically nitrate-poor environments (reviewed in reference 45). *T. testudinum* beds are characterized by high organic and sulfide contents, with most of the inorganic nitrogen being present in the form of ammo-

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nia (17, 45). Nitrification at the root surface of sea grasses occurs to a small extent where oxygen leaks from the roots (29), while oxygen in the overlying seawater can reach concentrations as high as 200% saturation due to photosynthesis from the sea grass (30). Thus, it is unclear whether the nitrate reductase activity observed in the gill tissue of *C. orbicularis* is due to assimilatory nitrate reductase or to nitrate respiration by the symbionts.

In the absence of oxygen and nitrate, elemental sulfur (S^0) stored as globules in the periplasmic space of thioautotrophic chemosymbionts has been proposed to serve as an electron sink (3). In intact chemosymbiotic associations, hydrogen sulfide production was found to occur when *Bathymodiolus thermophilus* (Mytilidae), *R. pachyptila* (Vestimentiferan), *Calyptogena magnifica* (Vesicomyidae), and *L. aequizonata* (Lucinidae) were held under anoxic conditions (3, 8). A further study using excised tissues from these animals showed that sulfide originated from symbiont-containing tissues (3). By using $^{35}SO_4^{2-}$, it was determined that free-living sulfate reducers were not responsible for the production of hydrogen sulfide and that the sulfide must originate from intracellular sulfur stores in the symbiont and not from sulfate in the seawater (3). However, hydrogen sulfide was not produced when purified symbionts from *L. aequizonata* were incubated under anoxic conditions (the symbionts from the other chemosymbiotic associations were not tested) (3).

Most studies on *C. orbicularis* symbiosis have focused on describing the development of the animal (4, 22), the symbiont transmission mode, and the colonization process of the gills (23) and on determining the phylogeny of the symbionts (13, 14, 21, 24). Very little is known about the environmental requirements and respiration strategies of this model organism and its symbiont. To learn more about the biology of the symbiont in this chemosynthetic-based association, the following questions were addressed. (i) Does nitrate play a role in symbiont respiration; is it abundant in the environment and metabolized by the symbionts? (ii) Do the symbionts respire oxygen? (iii) Does sulfide production occur in symbionts under anoxic conditions?

MATERIALS AND METHODS

Collection and maintenance of *C. orbicularis*. Adult specimens of *C. orbicularis* were collected from *Thalassia* sea grass beds from Ilet Cochon and Petit Havre, Guadeloupe. Specimens collected from the field were used immediately for assays to determine NO_3^- and NO_2^- concentrations in the hemolymph. Live adult clams were wrapped in moist newspaper and transported back to Scripps Institution of Oceanography (October 2000 and April 2002). The clams were held in a recirculating water bath maintained at 28°C and a salinity of 37 ppt. The tank was aerated using an aquarium pump.

Nitrate and nitrite measurements in porewater and overlying water. Cores were taken within 25 mm from the location of a live clam to a depth of 23 cm from the sea grass beds of Ilet Cochon. Clear acrylic coring tubes (diameter, 56 mm; length, 30 cm) were used for sediment sampling and subsequent extraction of the porewater. The tubes had vertically aligned silicone-filled ports in 1-cm intervals that allowed direct sampling of the pore water. Samples were taken with 3-ml Vacutainers (Becton Dickinson and Company) closed with silicone stoppers. Using two connected syringes (21 gauge; Venoject), porewater was drawn into the Vacutainers by inserting one end of the double syringe through the ports into the sediment while the other end penetrated through the silicone stopper into the vial. The porewater was kept on ice and in the dark for transport back to the laboratory (within 3 h). The porewater was centrifuged at 4,200 × *g* for 5 min and then stored frozen at −80°C until analysis.

Nitrate levels in the porewater and overlying water were measured through the reduction of nitrate to nitrite by using a nitrite reductase-deficient strain of *Escherichia coli* strain JCB606C (38). An older culture of JCB606C (36 h) was used for the conversion of nitrate to nitrite since nitrate reduction is inhibited in younger cultures (38). Nitrite concentrations were determined spectrophotometrically at 640 nm after the addition of sulfanilamide (2- and 8-min waiting times) and naphthylethylenediamine. Porewater was diluted 1:2 with M9 minimal medium, while undiluted porewater was used for the nitrite assay. Standard curves were made for measurements of both nitrate and nitrite concentrations in porewater. The detection limit for this assay for biological fluids and seawater is 0.5 μM (38).

Nitrate and nitrite concentrations in hemolymph. The hemolymph was removed from *C. orbicularis* ($n = 16$) by using a 1-ml syringe with a 27-gauge needle to pierce the ligament and enter the visceral mass. Clams measuring less than 3 cm in length were used so that the needle could easily enter the visceral mass. The hemolymph was stored on ice until transport back to the laboratory. Three criteria were used to verify that hemolymph, not seawater, was withdrawn. (i) A vacuum was present during withdrawal of blood. (ii) Hemocytes were visible under microscopic observation at 1,000× magnification. (iii) A white pellet (hemocytes) was visible to the naked eye after the blood was centrifuged (13,100 × *g*) for 10 min.

For measurements of nitrate and nitrite concentrations, undiluted hemolymph was denatured at 95°C for 10 min, cooled on ice, and centrifuged at 13,100 × *g* for 2 min to remove denatured proteins and other interfering substances (38). The hemolymph was diluted 1:5 and 1:10 with M9 minimal medium for the nitrate assay and diluted 1:2 and 1:4 with Milli-Q water for the nitrite assay. For each set of assays with hemolymph, standard curves were generated by adding a known concentration of either nitrate or nitrite. All measurements took place within 5 h of collection.

Nitrate reductase activity in gill tissue. Measurements for nitrate reductase assays were done within 5 days of collection by using adult clams. Three animals were maintained in aerobic seawater with 30 μM nitrate for 20 h, and six additional animals were maintained in nitrate-free seawater. Tissues from *C. orbicularis* were pulverized in a handheld homogenizer containing 10 to 20 volumes of ice-cold extraction buffer, and the nitrate reductase activity was assayed according to the method of Lee et al. (34). The reaction was allowed to proceed for 30 min or 1 h, after which it was terminated. Gill tissue from a frozen specimen of *L. aequizonata* was used as a positive control.

Isolation of symbiotic bacteria from *C. orbicularis*. Because the symbionts had not been cultured, purified symbiont preparations were made from the gill tissue of *C. orbicularis* for the respiration experiments. Symbiotic bacteria from *C. orbicularis* were isolated by the Percoll cushion method (10) and maintained at 4°C throughout the purification process. To minimize contamination from outside sources, all equipment and solutions were either autoclaved or filter sterilized with a 0.2-μm-pore-size filter (Acrodisc). Gill tissue was removed from the clam, rinsed in imidazole-buffered saline (IBS buffer) containing 490 mM NaCl, 30 mM $MgSO_4$, 11 mM $CaCl_2$, 3 mM KCl, and 50 mM imidazole (pH 7.5) at 23°C, and blotted dry on kimwipe tissue. The gill tissue was homogenized in IBS buffer (1:10, wt/vol) by using a handheld Dounce homogenizer. The homogenized tissue was filtered through a 200-μm-mesh Nitex screen to remove large particles. The tissue extract (1.5 ml) was gently layered over an 8-ml cushion of 60% Percoll in IBS and centrifuged to pellet the bacteria. The bacteria were washed three times and resuspended in 37-ppt seawater buffered with 10 mM HEPES, pH 8.0, stored at room temperature. No external energy source was supplied to the symbiont suspensions. Under a fluorescence microscope, symbiont cells stained with DAPI (4′,6′-diamidino-2-phenylindole) could be easily distinguished from marine bacteria based on the size difference (5 to 6 μm in length for the symbiont and 1 to 2 μm for marine bacteria), and under a light microscope, they could be distinguished based on the presence of intracellular sulfur granules in the symbiont cell (19).

CTC reduction assay method. To ensure that the symbiont cells were responsible for oxygen consumption, the fluorescent redox dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride; Polysciences Europe, Eppelheim, Germany) was used along with direct microscopic visualization of stained cells to verify respiration. CTC was used to confirm that oxygen consumption was due to symbiont respiration and not to potential free-living marine bacteria contaminants in the symbiont suspension. A symbiont suspension, prepared as described above, was adjusted to 10^7 cells/ml in filtered (0.2 μm pore size) seawater. Streptomycin and chloramphenicol were added to the symbiont suspension (respective final concentrations, 60 and 30 mg/liter) to inhibit the growth of free-living marine bacteria after purification. The respiration activity of the symbionts was checked immediately after purification (T_0) and after storage for 24 h in the dark at room temperature. CTC was added to a final concentration of 2.5 mM and incubated in the dark for 2 h at room temperature. CTC reduction was stopped by the addition of formaldehyde (final concentration, 4%). Symbionts previously treated with CTC were counterstained with DAPI in Tris-HCl, pH 7.1, for 15 min in the dark at a final concentration of 2.5 mg/ml (37). The analysis of the stained cells was performed with an Olympus BH2 epifluorescence microscope. The stained cells were captured by microfiltration through a 0.2-μm-pore-size Nuclepore black polycarbonate filter (47-mm diameter). The filters were air dried and mounted with immersion oil on glass microscope slides. The preparations were examined under immersion with a 100× objective lens. A total of approximately 500 bacterial cells were counted. Respiring bacterial symbionts were counted with excitation of 420 nm, and total bacterial symbionts were counted at 357 nm. The fraction of CTC^+ cells was calculated as the ratio of the CTC^+ cell count to the DAPI cell count.

Microelectrode measurements. Several preliminary measurements for oxygen and sulfide levels in symbiont suspensions isolated within 48 h from gill tissue were made independently. These initial experiments were used to define the final conditions for oxygen respiration and sulfide measurements made with symbionts resuspended in a total of 4 ml of seawater buffered with 10 mM HEPES (filter sterilized, 0.2-μm pore size; pH 8) to a density of 1×10^8 to 5×10^8 cells/ml. The measurements were started within 2 h of isolation and completed within 24 h. The symbiont suspensions were stirred slowly in an open vessel by using a microflea bar to keep the cells in suspension. The oxygen- and hydrogen sulfide-measuring microelectrodes (Unisense, Aarhus, Denmark) as well as the long-needle combination pH electrode (Diamond General) were attached to micromanipulators (Maerzhaueser) mounted on a heavy stand and were simultaneously submerged in the cell suspensions. Oxygen and hydrogen sulfide values were directly recorded on a personal computer, and pH values were read manually. In a different series of experiments, a symbiont suspension was split into two aliquots (2 ml each). After 28 min, symbiont suspension A was poisoned by using 0.1% sodium azide (final concentration) while symbiont suspension B remained unpoisoned. The data were logged at intervals of 30 s over a time period of 90 min.

Oxygen and hydrogen sulfide concentrations were measured continuously using amperometric microsensors connected to a high-sensitivity picoammeter (PA 2000; Unisense). The output currents were amplified and converted to millivolt signals by the picoammeter and digitized by an A/D converter for data acquisition on a personal computer. The Clark-type oxygen microelectrode was equipped with a built-in reference and a guard cathode (32, 39). The oxygen electrodes had a sensing tip of 20 to 50 μm, a stirring sensitivity of <2%, and a 90% response time of ≤1 s. Electrode currents had a linear response to 0 and 100% air saturation with O_2. Linear calibration was done at 20°C in 100% saturated seawater (35 ppt) and nitrogen-purged seawater with 0% oxygen saturation.

The H_2S microelectrode was a miniaturized amperometric sensor (31, 33) with a sensing tip of 50 μm and a linear response over a defined range of H_2S concentrations. For the calibration, a stock solution of S^{2-} (100 mM) was prepared from dissolving Na_2S in N_2-flushed 0.1 M NaOH in a closed container. The final concentration of stock solution was determined by standard spectrophotometric analysis (9). Further calibration points were prepared by using a microsyringe to inject defined amounts of the S^{2-} stock solution into closed vials containing a calibration solution (100 mM phosphate buffer, pH 7). The pH was measured with a long-needle combination pH electrode (Diamond General).

To check for contamination (i.e., cell debris and nonsymbiotic bacteria) and to perform counts on the symbionts, the symbiont suspensions were stained with DAPI (37). Aliquots of cells were serially diluted in seawater (total volume, 2 ml) with 2% formaldehyde. The cells were stained with 1 μg of DAPI/ml overnight and then filtered on 0.2-μm-pore-size black polycarbonate filters (Nuclepore) by vacuum using a Millipore filtration system. The filter was affixed on microscope slides by using paraffin oil and stored at −20°C until the cells were counted. A minimum of 30 fields or 300 bacteria was counted for each sample.

Anaerobic incubations with live *C. orbicularis*. Live specimens of *C. orbicularis* ($n = 3$) kept in captivity for 5 days were held in individual jars containing 400 ml of seawater at 23°C. The jars were sealed with polyvinylchloride lids and several layers of electrical tape to prevent oxygen exchange with the atmosphere (3). The jars were opened after 8 days, and water was immediately removed for sulfide and oxygen analyses. Sulfide was precipitated as ZnS and frozen at −20°C until analysis (9). The oxygen concentration was measured using a Vacu-Vial kit with self-filling ampoules for photometric analysis (Chemetrics) (3).

RESULTS

Nitrate and nitrite concentrations in the environment and hemolymph. During the sampling periods in March, the burrowing depth of the clams was 3 to 4 cm below the surface of the sediment. Nitrate was detectable in the porewater samples, but in variable quantities, with most samples having no detectable nitrate. Of 32 porewater samples obtained from a depth of 1 to 22 cm in the sediment, only seven sporadic samples had detectable levels of nitrate in the four cores analyzed. In the samples with detectable nitrate, the concentration was 3.5 ± 1.16 μM (mean ± standard deviation [SD]), with a maximum concentration of 5.6 μM. Nitrite was not detectable in any of the porewater samples examined. Nitrate and nitrite were not detectable in the overlying water by the methods used in this study.

Nitrate and nitrite were both undetectable by our methods in the hemolymph of freshly collected animals. A standard curve made, with the addition of hemolymph, in conjunction with the nitrate and nitrite measurements ruled out the presence of inhibitory substances in the blood. Using this same technique, we determined the nitrate concentration to be 42 ± 13.6 μM (mean ± SD) and the nitrite concentration to be 0.76 ± 0.42 μM (mean ± SD) in the hemolymph of *L. aequizonata* 20 h after collection ($n = 10$).

Activity for nitrate reductase was present in *C. orbicularis* but was too low to quantify ($n = 9$). Even after the addition of 30 μM nitrate to seawater for 20 h, the nitrate reductase activity in the tissues of *C. orbicularis* did not increase to quantifiable levels.

Preliminary oxygen and sulfide measurements. After purification, a single morphotype was the predominant bacterium in the symbiont suspension. Approximately 20 h after purification, other morphotypes (bacterial contaminants) began to become more common. Because of contamination from free-living marine bacteria, symbiont respiration experiments were completed within 24 h for sulfide and 15 h for oxygen. During periods of anoxia, the pH of unbuffered symbiont suspensions in seawater decreased quickly. In an attempt to stabilize the pH of the medium, the symbionts were resuspended in 10 mM HEPES (pH 8).

Oxygen respiration in purified symbionts. Oxygen respiration rates were measured in symbionts purified from clams held in captivity for less than 2 weeks. Measurements taken from freshly purified symbiont preparations showed an oxygen consumption rate of 0.9 ± 0.279 fmol of oxygen $cell^{-1}$ day^{-1} ($n = 3$) in open vessels stirred slowly with a microflea bar. To test the viability and oxygen tolerance of the symbionts, the oxygen level was allowed to drop repeatedly and brought back up to 100% saturation once the medium was anoxic. The symbionts continued to respire oxygen, even after periods of anoxia. Oxygen respiration was inhibited in a purified suspension by using sodium azide (Fig. 1), while a duplicate unpoisoned suspension continued to consume oxygen. The increase in oxygen seen in the poisoned suspension came from the introduction of oxygen into the medium by stirring. In the unpoisoned suspension, the oxygen level continued to decrease due to respiration.

To confirm that the symbiont cells were responsible for oxygen consumption, CTC was employed as a cellular redox indicator to visualize the respiratory activity of the symbiont cells. All cells in the symbiont suspension (10^7 cells/ml) immediately exposed to CTC after isolation exhibited an active respiratory function. The number of cells respiring dropped to

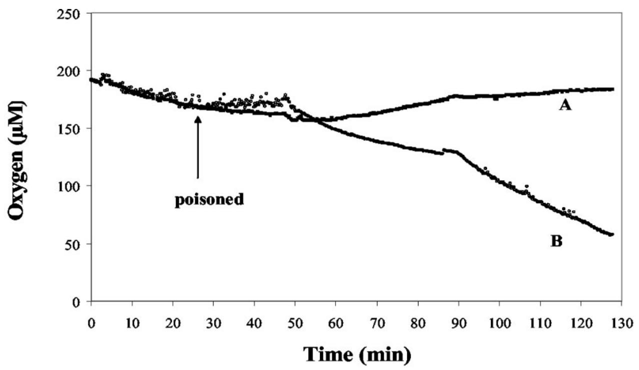

FIG. 1. Oxygen consumption in replicate *C. orbicularis* symbiont suspensions. After 28 min, suspension A was poisoned with 0.1% sodium azide and suspension B was left unpoisoned.

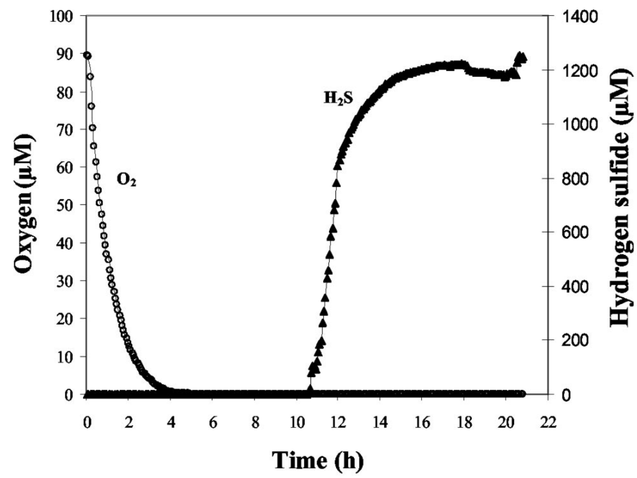

FIG. 2. Time course of oxygen respiration (○) and sulfide production (▲) measured simultaneously in a purified preparation of *C. orbicularis* symbionts. The symbionts were resuspended at a density of 5.23×10^8 cells/ml in 10 mM HEPES-buffered seawater. At the start and the end of the experiment, the pHs were 7.74 and 4.46, respectively.

82.9% after the symbionts were maintained in the dark for 24 h with antibiotics. Formazan crystals did not form in the surrounding medium. The bacterial contaminants did not form intracellular fluorescent crystals of formazan. One to three formazan crystals were observed in each respiring symbiont cell.

Anaerobic sulfide production in purified symbionts. After approximately 6 h of anoxia, freshly prepared symbiont suspensions began to release hydrogen sulfide (Fig. 2 and 3). From this point on, there was a continuous increase in hydrogen sulfide levels until the maximum concentration of sulfide was reached in both cell suspensions, ~1,200 μM (Fig. 2) and ~600 μM (Fig. 3), leading us to make the assumption that sulfide production in the cell suspensions was a function of the cell number. The initial sulfide release occurred at a pH value of 7.3 (Fig. 3 and 4), and when the sulfide concentration reached 180 μM, the pH value was ~7. During continuous exposure to anoxia in the course of the 22- to 24-h experiment, the pH went from 7.74 to an end value of 4.46 in the first experiment and dropped to a final value of 4.5 in the presence of 10 mM HEPES, pH 8 (Fig. 3). The compounds which caused the drop in the pH were not identified.

Anoxic clam incubations. Two of the three clams held in sealed jars had high levels of sulfide in the seawater without 10 mM HEPES (260 and 323 μM). The oxygen concentration in the seawater for the remaining clam was 7.5 μM; sulfide was not detectable. The pH of the seawater dropped slightly to 7.38 ± 0.1, with the hypoxic clam having a slightly higher pH, 7.49.

DISCUSSION

Unlike what is seen with *L. aequizonata* symbionts, nitrate respiration does not appear to sustain the oxidative requirements of the symbionts found in *C. orbicularis*. Evidence against nitrate respiration includes low nitrate reductase activity in the gill tissue of the clam and nondetectable levels of nitrate in the hemolymph of *C. orbicularis* taken directly from its native habitat. Chemosymbionts shown to respire nitrate or use nitrate as a nitrogen source obtain nitrate through its transport via the coelomic fluid or hemolymph of the animal (38). Providing further supporting evidence against nitrate respiration is the observation that low nitrate reductase activity in the gill tissue remained unstimulated when the clams were exposed to relatively high nitrate levels (30 μM) for 20 h. These results are not unexpected when the *C. orbicularis* symbiosis is viewed in an ecological context. Analysis of core samples taken next to the burrow of live clams during daylight hours showed that nitrate levels ranged from nondetectable to a maximum concentration of 5.6 μM with no detectable nitrate in the overlying seawater.

Oxygen is abundant in the habitat where *C. orbicularis* is found and from this study appears to be preferably used over nitrate by the symbiont at least during the daylight hours. Microelectrode and CTC studies showed that oxygen is rapidly consumed from the seawater medium by the symbionts. After the addition of sodium azide, oxygen consumption was inhibited, demonstrating that the removal of oxygen was due to respiration and was not caused abiotically. Direct visualization of the respiratory activity of individual symbiont cells was obtained by using the redox indicator CTC, which competes with molecular oxygen as an electron acceptor. The CTC-formazan granules formed inside the symbiont cells verified that the symbionts were alive and responsible for the oxygen consumption measured. The symbionts formed two to three intracellular formazan crystals per cell when exposed to CTC. This may reflect the presence of an active transport chain (40) since generally no more than one formazan crystal per cell is observed with CTC experiments (5, 6, 41). Purified symbionts did not appear to be sensitive to oxygen levels but continued aerobic respiration once oxic conditions returned.

The physiological and environmental data available suggest that the respiration strategies used by lucinid symbionts may be more reflective of the respective habitat than a characteristic trait of the chemosymbioses. According to 16S rDNA data, the tropical lucinids *A. alba*, *C. orbicularis*, *C. orbiculata*, *C. pectinella*, *D. quadrisulcata*, and *L. pensylvanica* appear to share the same symbiont species and live in or near sea grass beds (13, 14, 21, 24). Further studies are needed to confirm whether the

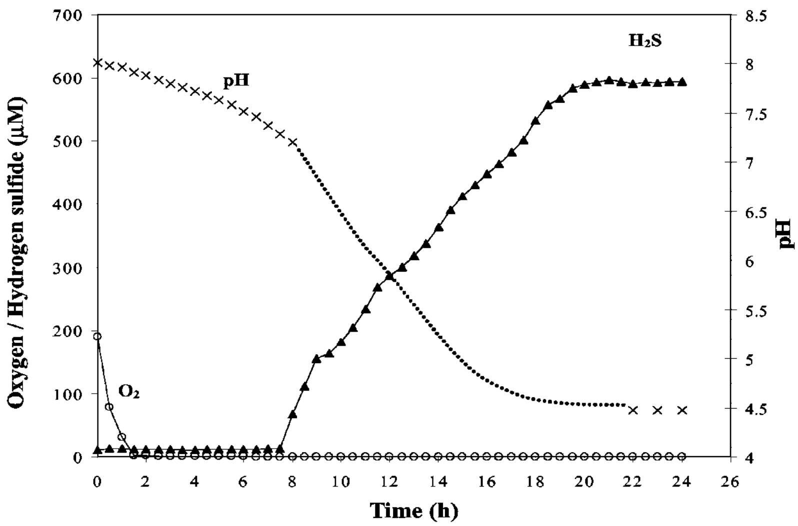

FIG. 3. Time course of oxygen respiration (○), sulfide production (▲), and pH (×) measured simultaneously in a purified preparation of *C. orbicularis* symbionts in 10 mM HEPES-buffered seawater, pH 8.0. The symbionts were resuspended in the medium to a density of approximately 2.56×10^8 cells/ml. Data points represent single values.

symbionts from these lucinids use aerobic respiration, which seems likely.

During periods of anoxia, sulfide is present in seawater medium containing both *C. orbicularis* and its purified bacteria. Previous attempts to demonstrate sulfide production by purified symbionts from *L. aequizonata* have failed (3), and the production was speculated to originate from either *L. aequizonata* or its intracellular symbionts (3). The hydrogen sulfide generated in the purified symbiont preparations from *C. orbicularis* was measured directly with microelectrodes submerged into the suspensions. From these measurements, it is evident that the symbiont, and not the clam, is the source of the sulfide. Sulfide was produced only in live symbiont suspensions while medium containing heat-killed or poisoned symbionts did not contain hydrogen sulfide. These data indicate that some metabolic product or process is involved in the liberation of sulfide.

Chemoautotrophic bacteria (free living and symbiotic) have been hypothesized to use sulfur as an electron sink during temporary periods of anoxia (3, 36, 42). Experiments directly testing this hypothesis are lacking due to the inability to culture chemosymbionts. Even with cultured chemoautotrophs, sulfur respiration has been difficult to show. To date, anaerobic sulfide production has been shown to occur in the sulfur-oxidizers *Beggiatoa* sp. (36) and *Beggiatoa alba* (42). Enhanced short-term growth was observed in *Beggiatoa* sp. cells with S^0 granules when the cells were exposed to acetate compared to what was seen in cells without internal S^0 stores, but the metabolic pathway involved in this process is still unknown. Internal sulfur stores are a possible source for the hydrogen sulfide produced in the purified symbiont suspensions. Previous work done on *L. aequizonata* showed that there was a relationship between sulfide production and the S^0 stores in the gill tissue of the clam (3, 46). In our experiments, sulfide levels in the medium appeared to be linked to an undetermined storage compound in the symbiont cell; cell suspensions containing 2.56×10^8 cells/ml and 5.23×10^8 cells/ml yielded 600 and 1,200 μM concentrations of sulfide, respectively, at the end of the two time series. These data suggest that the number of cells present in the suspension determines the final concentration of sulfide found in the medium. Because S^0 stores were not measured before and after sulfide production, it is unclear whether the sulfide originates from S^0 stores or from the acidification of internal sulfide in the symbiont. But initial release due to acidification is unlikely because sulfide production occurred at pH values of 7.3 in the cell suspension in two separate experiments. When the pH began to approach 7, the sulfide levels were >100 μM in the external medium (Fig. 3 and 4).

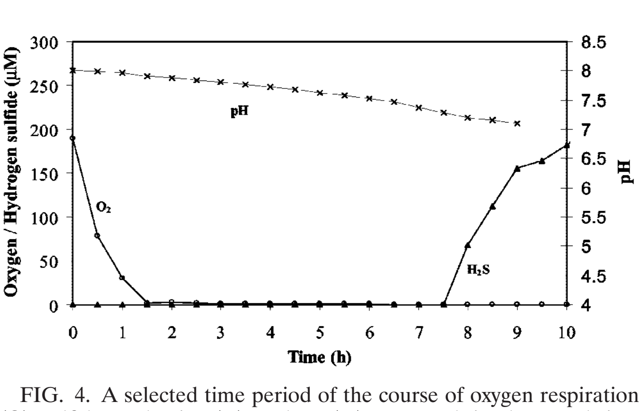

FIG. 4. A selected time period of the course of oxygen respiration (○), sulfide production (▲), and pH (×) measured simultaneously in a purified preparation of *C. orbicularis* symbionts in 10 mM HEPES-buffered seawater, pH 8.0.

Another possible source for the sulfide production observed is the fermentation of sulfur-containing amino acids such as methionine, cysteine, cystine, and taurine. The sulfide production in gut flora from the large intestine in humans has been attributed to this mechanism (35). This may be the case in our experiments after the symbionts are exposed to long periods of anoxia and with the possible growth of bacterial contaminants in the purified symbiont suspension towards the end of the experiment. The pH drop in anoxic sulfidic seawater containing intact clams was less severe, from 8 to 7.38 ± 0.1, while the pH of the cell suspension in HEPES-buffered seawater dropped from 8 to ~4.5. The drop in the pH was not due exclusively to the production of sulfuric acid from sulfide oxidation. To test this possibility, a titration experiment with HEPES-buffered seawater was done. By titrating 10 mM HEPES (pH 8.0) with sulfuric acid, it was found that it would take nine times more sulfide than was present in the medium to bring the pH down to the levels observed. Moreover, a pH drop to 5.3 was observed in two symbiont suspensions maintained under anoxic conditions. The cause of the pH drop in the purified suspensions was not determined; possibilities include compounds generated from either fermentative or nonfermentative pathways. Further experiments based on our findings and characterization of excreted compounds in the symbiont suspensions may provide insight for understanding the metabolic pathways involved in hydrogen sulfide generation.

We provide evidence that the symbiont from the lucinid *C. orbicularis* carries out oxic respiration and that exposure to more than 6 h of anoxia leads to the production of sulfide, presumably from internal S^0 stores acting as an electron sink in the symbiont. Our study demonstrates the need for further investigations to achieve an understanding of the relationship between respiration strategies utilized in animal-symbiont associations and their respective environmental conditions.

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The experiments comply with the present laws of the countries (United States and France) and institutions in which they were performed.

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