Thioautotrophic Bacterial Endosymbionts Are Degraded by Enzymatic Digestion During Starvation: Case Study of Two Lucinids *Codakia orbicularis* and *C. orbiculata*

STEN KÖNIG,[1]* HERVÉ LE GUYADER,[2] AND OLIVIER GROS[1,3]
[1]*Institut de Biologie Paris-Seine, UMR 7138 - Evolution Paris-Seine, Equipe Biologie de la Mangrove, Université des Antilles et de la Guyane, UFR des Sciences Exactes et Naturelles, Département de Biologie, BP 592. 97159 Pointe-à-Pitre Cedex, Guadeloupe, France*
[2]*Sorbonne Universités Paris VI, Institut de Biologie Paris-Seine, UMR 7138 - Evolution Paris-Seine, Equipe Phylogénie, Anatomie, Evolution, C.N.R.S, Institut de Biologie Paris-Seine, UMR 7138 - Evolution Paris-Seine, Equipe Biologie de la Mangrove*
[3]*C3MAG, UFR des Sciences Exactes et Naturelles, Université des Antilles et de la Guyane, BP 592 - 97159 Pointe-à-Pitre, Guadeloupe, French West Indies*

KEY WORDS chemoautotrophic symbiosis; cytochemistry; EDXS analysis; TEM; bacterial degradation

ABSTRACT The Caribbean bivalves *Codakia orbicularis* (Linné, 1758) and *C. orbiculata* (Montagu, 1808) live in seagrass beds of *Thalassia testudinum* and harbor intracellular sulfur-oxidizing gamma-proteobacteria. These bacterial symbionts fix CO_2 via the Calvin Benson cycle and provide organic compounds to the bivalve. During experimentally induced starvation, no reduced sulfur compounds and no organic particle food are available; the symbionts could be considered as the sole nutrient source of the host bivalve. A previous study has shown that the intracellular bacterial population decreased considerably during starvation and that bacterial endosymbionts were not released by the bivalves. In this study, the activity of two lysosomal marker enzymes (acid phosphatase and arylsulfatase) was detected using cytochemical experiments coupled with energy-dispersive X-ray transmission electron microscopy during sulfide and organic particle starvation. The degradation of bacterial endosymbionts began after 2 weeks of starvation in *C. orbiculata* and after 3 weeks in *C. orbicularis*. Degradation processes seem to be continuous over several months and could be responsible for the disappearance of the bacterial endosymbionts within the gills during starvation. These data suggest that the host use symbionts as a nutrient source to survive a hunger crisis. The carbon transfer from the symbionts to the host could be flexible and could consist in transfer of organic matter, "milking," under normal feeding conditions and digestion of the symbionts under starved conditions. *Microsc. Res. Tech. 78:173–179, 2015*.

INTRODUCTION

The association between chemoautotrophic bacterial symbionts and marine invertebrates was first discovered in the deep sea at hydrothermal vents (Felbeck, 1981). In shallow-water environments, similar symbiotic relationships were described involving bivalves (Felbeck, 1983; Reid, 1990), nematodes (Ott et al., 1982), and annelids (Cavanaugh et al., 1981; Felbeck, 1981). To date it is known that the entire bivalve family Lucinidae harbors chemoautotrophic gamma-proteobacteria in specialized gill cells called bacteriocytes (Taylor and Glover, 1997). Many lucinid bivalves inhabit seagrass beds, where they dominate the sedimentary infauna (Reynolds et al., 2007). The seagrass ecosystem is a very productive system and is enriched in organic matter (Duarte and Chiscano, 1999). The anaerobic degradation of organic matter in seagrass bed sediments by sulfate reducers produces reduced sulfur compounds such as hydrogen sulfide (Jørgensen, 1982). Reduced sulfur compounds (herein called sulfides) are toxic to most plants and animals (Dorman et al., 2002). Lucinid bivalves live at the interface of oxic and anoxic sediment zones. They build burrows in sulfide-rich anaerobic sediments and are connected to the surface water, which is saturated with oxygen. The bacterial endosymbionts oxidize sulfides, produce organic matter through the Calvin Benson Cycle, and supply these organic compounds to the host (Van Dover, 2000). In a direct carbon transfer called "milking," the symbiont permanently translocates organic compounds to the host soon after carbon fixation (Schweimanns and Felbeck, 1985; Streams et al., 1997; Van Dover, 2000). Therefore, the digestion system and filtering capacity of lucinids are reduced (Reid, 1990) compared with other bivalves without

*Correspondence to: Sten König, University Greifswald, Institute of Pharmacy, Friedrich-Ludwig-Jahn Straße 17, 17489 Greifswald, Germany. E-mail: sten.koenig@gmail.com

REVIEW EDITOR: Prof. George Perry
Received 31 July 2014; accepted in revised form 10 November 2014
Contract grant sponsor: Ph.D. grant from the EU Marie Curie Initial Training Network (ITN) "Symbiomics" (to S.K.).
DOI 10.1002/jemt.22458
Published online 28 November 2014 in Wiley Online Library (wileyonlinelibrary.com).

endosymbionts. Lucinids provide through permament water pumping sulfide and oxygen to their endosymbiont, oxygen act as terminal electron acceptor for the endosymbiont.

Codakia orbicularis (Linné, 1758) and *C. orbiculata* (Montagu, 1802) inhabit the shallow water seagrass bed of *Thalassium testudinum* in the Caribbean Sea. Both these lucinids have been studied with regard to the interaction between host and symbionts in the last years, and these systems represent an attractive model to investigate symbiosis. According to 16S rRNA sequence analysis, both the *Codakia* species harbor the same single bacterial symbiont phylotype inside the gills (Durand et al., 1996). Cultivation of aposymbiotic juveniles of *C. orbicularis* in controlled conditions and in natural seagrass bed sediment demonstrate that the transmission of the gill-endosymbionts is environmental (Gros et al., 1996). Free-living symbionts were detected in seagrass bed sediments (Gros et al., 1996, 2003). No gill-endosymbionts were detectable in either larvae or juveniles of *C. orbicularis*, suggesting that the bivalve acquires symbiosis-competent bacteria once their gill tissue is sufficiently developed (Gros et al., 1998). *C. orbiculata* can acquire bacteria during their whole life by phagocytosis at the apical pole of the bacteriocytes (Elisabeth et al., 2012). The symbionts of both the lucinids are not released by the host; so, the intracellular life of the symbionts seems to be a dead end for the bacteria (Brissac et al., 2009). Caro et al. (2007) suggested that *C. orbicularis* could control bacterial growth to harvest bacterial biomass. Interestingly, it was shown that there are up to seven subpopulations of this symbiont phylotype within a single bivalve. These bacterial subpopulations differ in size, number of chromosome copies, and content of sulfur granules (Caro et al., 2007, 2009).

The gills of lucinids are divided in three zones (i) the ciliated zone, (ii) the intermediary zone, and (iii) the lateral zone, which takes up most of the volume of the gills (Frenkiel and Mouëza, 1995). The ciliated and intermediary zones are free of symbiotic bacteria. The upper two thirds of the lateral zone are occupied by bacteriocytes (which harbor bacterial symbionts) and by intercalary cells. The lower third is mainly occupied by granule cells, considered to be important for storage and metabolic conversion of sulfur compounds (Frenkiel and Mouëza, 1995). In case of starvation, the organization of the gills in *C. orbicularis* changes, and the bacterial population decreases by about one third per month (Caro et al., 2009). After 6 months of starvation, no symbiont is detectable in the gills, the main part of which is then occupied by granule cells. The reduction in bacteriocyte volume occurs parallel to the reduction in the number of bacterial symbionts (Caro et al., 2009). Because no bacteria were detectable in the seawater tanks used during lucinid starvation experiments, Brissac et al. (2009) concluded that there was no release of the bacterial symbionts. The decrease in size of the symbiont population could be either due to bacterial autolysis or due to host-driven lysis, as a means of obtaining nutrients from these bacteria. Indeed, bivalves with chemoautotrophic symbionts are known to digest and use symbionts as a nutrient source (Boetius and Felbeck, 1995; Fiala et al., 1994; Herry et al., 1989; Liberge et al., 2001). The direct digestion of the symbiont and the subsequent nutrient transfer to the host is called "farming" (Streams et al., 1997). Symbiont autolysis has been described in *Bathymodiolus azoricus* (Kádár et al., 2007). In this case, the authors hypothesized that the autolysis of the bacteria is part of the cell cycle and that a bacterial acid phosphatase is involved in the process of the autolysis. Acid phosphatase and arylsulfatase are common enzymes in gram-negative bacteria (Chandramohan et al., 1974; Rogers, 1960). In eukaryotes, acid phosphatase and arylsulfatase are lysosomal markers that have been detected in the Mollusca (Liberge et al., 2001; Patel and Patel, 1985; Pipe and Moore, 1985). Lysosomes are eukaryotic membrane-bound cell organelles with hydrolytic enzymes to chemically deteriorate polymers and bacteria. Acid phosphatase and arylsulfatase are the two marker enzymes for lysosomal bodies (Löffler et al., 1984). Here, we document changes in acid phosphatase and arylsulfatase activity in the gills of *C. orbicularis* and *C. orbiculata* during host starvation. We suggest that the host can use this large amount of bacteria as a nutrient source through lysosomal degradation.

MATERIAL AND METHODS

Sampling of Bivalves

Adult individuals of *Codakia orbicularis* and *C. orbiculata* were manually collected in *Thalassium testudinum* seagrass beds on "îlet cochon" (16°12′53″N; 61°32′05″W) in Guadeloupe (French West-Indies, Caribbean).

Laboratory Maintenance of the Bivalves

Lucinids ($N = 80$ *C. orbiculata* and $N = 40$ *C. orbicularis*) were kept in separate 50-L plastic tanks with 0.22 μm filtered seawater at 26°C up to 6 months. The water was oxygenated with an aquarium air pump. No food (organic particles) or reduced sulfur compounds were added to the water throughout the starvation experiment. The water in the tanks was changed every 7 days. At specific times, three bivalves were randomly sampled from the tanks for analysis; for *C. orbiculata*, sampling took place upon collection and then every 2 weeks throughout the experiment (i.e., 5 months), whereas for *C. orbicularis*, sampling took place upon collection and then every 3 weeks.

Cytochemistry

The gills of the bivalves were dissected and fixed (2% glutaraldehyde, 1% paraformaldehyde in 0.1 M cacodylate adjusted to 900 mOsm,) for 2 h at 4°C. The fixed gills were washed overnight at 4°C in 0.2 M sodium cacodylate buffer with an adjusted osmolarity at 1,800 mOsm with NaCl and $CaCl_2$. The gills were sliced in sodium cacodylate buffer into 100-μm sections using a Vibratome OCT Slicer at 4°C. The activity of only one enzyme can be detected per gill section; therefore, for each enzyme reaction, three replicate sections were prepared. Arylsulfatase activity was detected according to a method from Lewis and Knight (1992). Briefly, the 100-μm sections were transferred directly to the enzyme reaction solution (60 mM sodium acetate, 15 mM acetic acid, 8 mg/mL sulfate *p*-nitrocatechol, 60 mM barium chloride) and incubated for 45

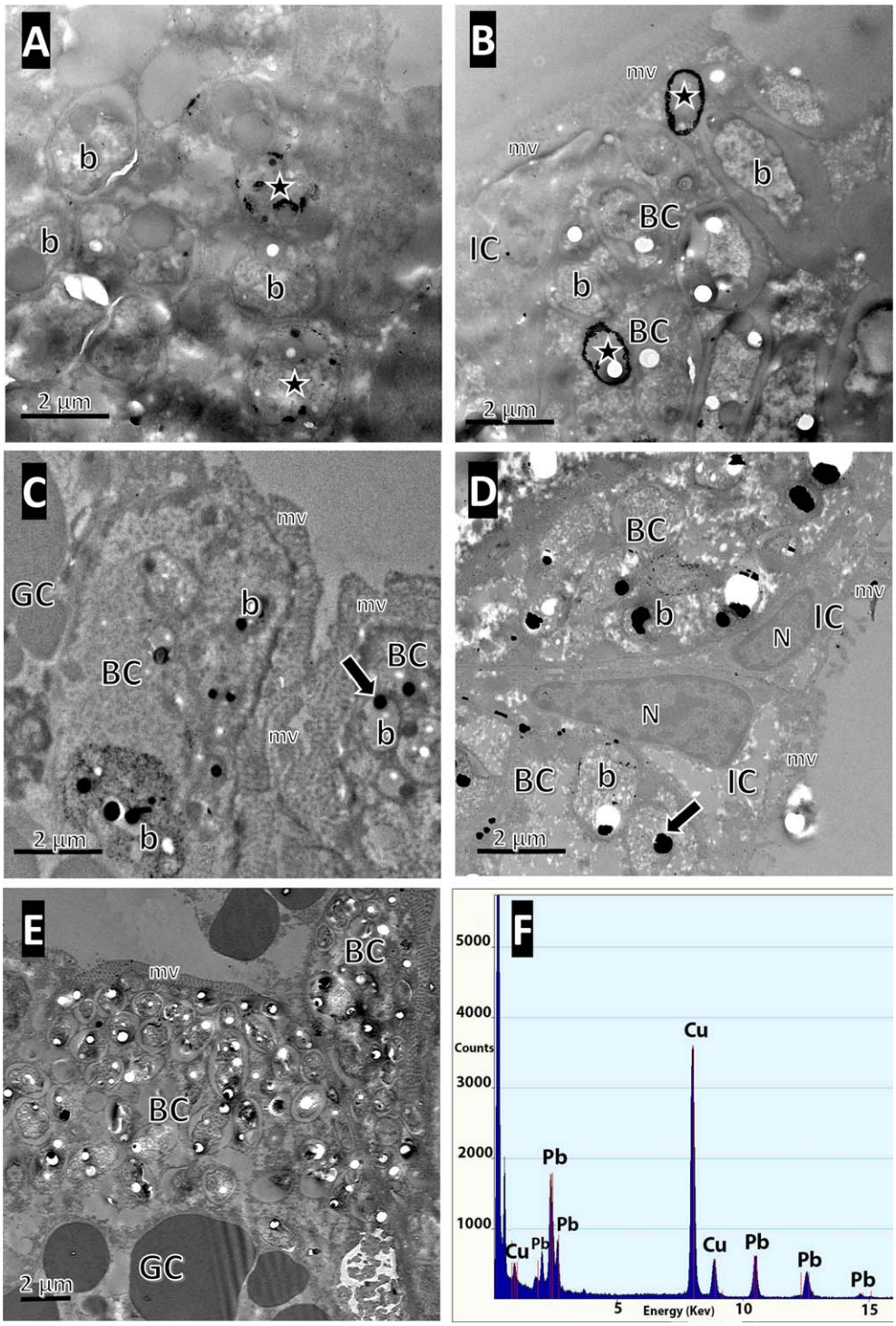

Fig. 1. Cytochemical detection of arylsulfatase and acid phosphatase in thin gill sections of *Codakia orbiculata*. **A,B:** Very few bacteriocytes from freshly collected individuals contained low numbers of bacterial endosymbionts (*) that were partially degraded because of the activity of arylsulfatase (A) and acid phosphatase (B). **C,D:** In individuals starved for 2 weeks, the number of bacteria degraded by acid phosphatase increased, with numerous bacteriocytes affected (results for arylsulfatase were similar, but are not shown). In each bacteriocyte (BC), several bacteria (b) were degraded by these enzymes. At the same time, other gill cells, such as intercalary cell (IC) and granule cell (GC), devoid of bacterial symbionts presented no lysosomal activity. **E:** In bivalves starved for 2 months, most of the bacteria inside the bacteriocytes seemed to be affected by arylsulfatase degradation. **F:** EDX spectrum obtained from cytochemical detection of acid phosphatase by lead precipitation from black areas located inside bacterial endosymbionts are indicated by black arrows in (C) and (D). Copper originated from the grid bars supporting the thin sections (mv: microvilli; N: nucleus). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

min at 37°C. After the incubation, the samples were washed in cacodylate buffer for several changes of sodium cacodylate buffer overnight.

Acid phosphatase was detected by a method from Barka and Anderson (1962). The sections were pre-incubated for 10–20 min at 37°C in 50 mM acetate buffer with 15 mM acetic acid. The enzymatic reaction took place in the pre-incubation buffer containing an additional 0.1 M sodium β-glycerophosphate substrate, saturated with lead-(II)-nitrate. The sections were incubated for 45 min at 37°C. After incubation, the sections were washed in acetate buffer over a period of 60 min with several changes of the washing buffer.

For both acid phosphatase and arylsulfatase assays, a precipitate containing the heavy metal formed as a result of enzyme activity during the incubation stage. For both enzymes, a negative control was prepared: 100 μm gill sections were incubated in the particular enzyme reaction solution without the enzyme substrate but with the heavy metal.

Following incubations, 100-μm sections were processed for embedding. Samples were dehydrated through a graded acetone series and embedded in Epon-araldite resin according to Glauert (1991). Ultrathin (100 nm) sections were made using an ultracut E microtome, and two grids containing four to five sections were observed with a Tecnai G2 TEM at 200 kv. The barium or lead precipitates were detected using a 30-mm^2 energy-dispersive X-ray (EDX) detector. The EDX spectroscopy analysis corresponds to an analytical method used for elemental characterization. Micrographs presented in this study are representative of the three replicate bivalves examined at each sampling time during starvation.

RESULTS

During starvation, the gills of the bivalves changed visibly from thick and beige to thin and dark brown. The mortality of the bivalves in the sterile sea water tanks was low: around 1%–5% per week. The mortality was observed to be greater during the first 3 weeks compared with the last 5 months of starvation. On transmission electron micrographs, positive enzymatic reactions appeared as black areas, resulting from the reaction of acid phosphatase and arylsulfatase with lead and barium, respectively.

In freshly collected *C. orbiculata*, gill filaments showed a weak positive reaction: only a few black areas were visible in a couple of bacteriocytes (Figs. 1A and 1B), whereas most of the bacteriocytes throughout the gill filaments are free of enzyme activity (data not shown). These data suggest that adult individuals in their natural habitat can digest symbionts, probably in the frame of resorption of dead or living bacteria. After 2 weeks of starvation, the acid phosphatase activity was detected close to symbionts, whereas other gill cells were free of enzyme activity (Figs. 1C and 1D). Likewise, the activity of arylsulfatase increased during starvation, and after 2 months, most of the symbionts inside the bacteriocytes were affected by arylsulfatase activity (Fig. 1E). After the beginning of bacterial degradation in the gills, the enzymatic activity was observed in all bacteriocytes of the lateral zone. It seems that it is a global process in the bacteriocytes of the gills in case of starvation. The further the course of starvation in both bivalves went, the amount of black areas increased until most of the bacteria were degraded. Nevertheless, for long periods of starvation (5 months and more), the enzymatic activity globally decreases as fewer bacteriocytes are present through the lateral zone, which now contains mostly granule cells. However, in the few remaining bacteriocytes that contain only a few bacterial symbionts, digestion is still occurring. No black areas were observed in granule cells and intercalary cells. For *C. orbicularis*, the story looked similar. After 3 weeks of starvation, no positive signal was detected for arylsulfatase and acid phosphatase in the gills of *C. orbicularis*, indicating very weak to no enzymatic activity (Figs. 2A and 2B). In *C. orbicularis*, the activity of both enzymes was detected after 6 weeks of starvation (Figs. 2C–2E). The longer the starvation period, the more extensive was the area corresponding to the enzyme activity, until most symbionts seemed affected (data not shown). Similarly to *C. orbiculata*, the enzymatic activity in *C. orbicularis* increased during the first months of starvation, then progressively decreased after 4–5 months of starvation when the number of bacteriocytes and bacterial gill-endosymbionts have been strongly reduced through the lateral zone. The EDX analysis confirmed that the black areas contained barium and lead, demonstrating that arylsulfatase and acid phosphatase enzymes were efficient (Figs. 1F and 2F). In the negative controls, no barium or lead was detectable (not shown).

DISCUSSION AND CONCLUSION

This study used cytochemical methods to explain how lucinid bivalves survive when no organic particulate food or sulfides (needed for symbiont thiotrophic metabolism) are available. The symbionts occupy around 34% of the gills of freshly collected *C. orbicularis* (Caro et al., 2009). During starvation, the bacterial population decreases by one third per month (Caro et al., 2009), and no released bacteria are detectable using CARD-FISH (Brissac et al., 2009). Lysosomes were rarely observed in freshly collected gills of *C. orbicularis* (Frenkiel and Mouëza, 1995). In our study, symbiont digestion was rarely observed in freshly collected *Codakia* bivalves. These data suggest that adult individuals in their natural habitat can digest symbionts, probably in the frame of resorption of dead or living bacteria. In deep sea bivalves, it was suggested that lysosomal symbiont digestion is a way of controlling symbiont populations (Fiala-Medioni et al., 1990). During starvation, lysosomes in the gills of both *Codakia* species become larger and more abundant (Caro et al., 2009). Moreover, Elisabeth et al. (2012) described a massive decrease in the area occupied by bacteriocytes in *C. orbiculata* during starvation. *C. orbiculata* can acquire its symbionts in all life stages, from a free living stock of symbionts in the seagrass sediment (Gros et al., 2003) probably through phagocytosis at the apical pole of bacteriocytes (Gros et al., 2012). Inside the gills, the proliferation of the bacteria is inhibited by an unknown mechanism (Caro et al., 2007). The molecular mechanisms that regulate symbiont proliferation likely involve complex symbiont–host signaling processes (Stewart and Cavanaugh, 2006). In the laboratory, it is possible to maintain adult bivalves under starvation conditions for up to 6

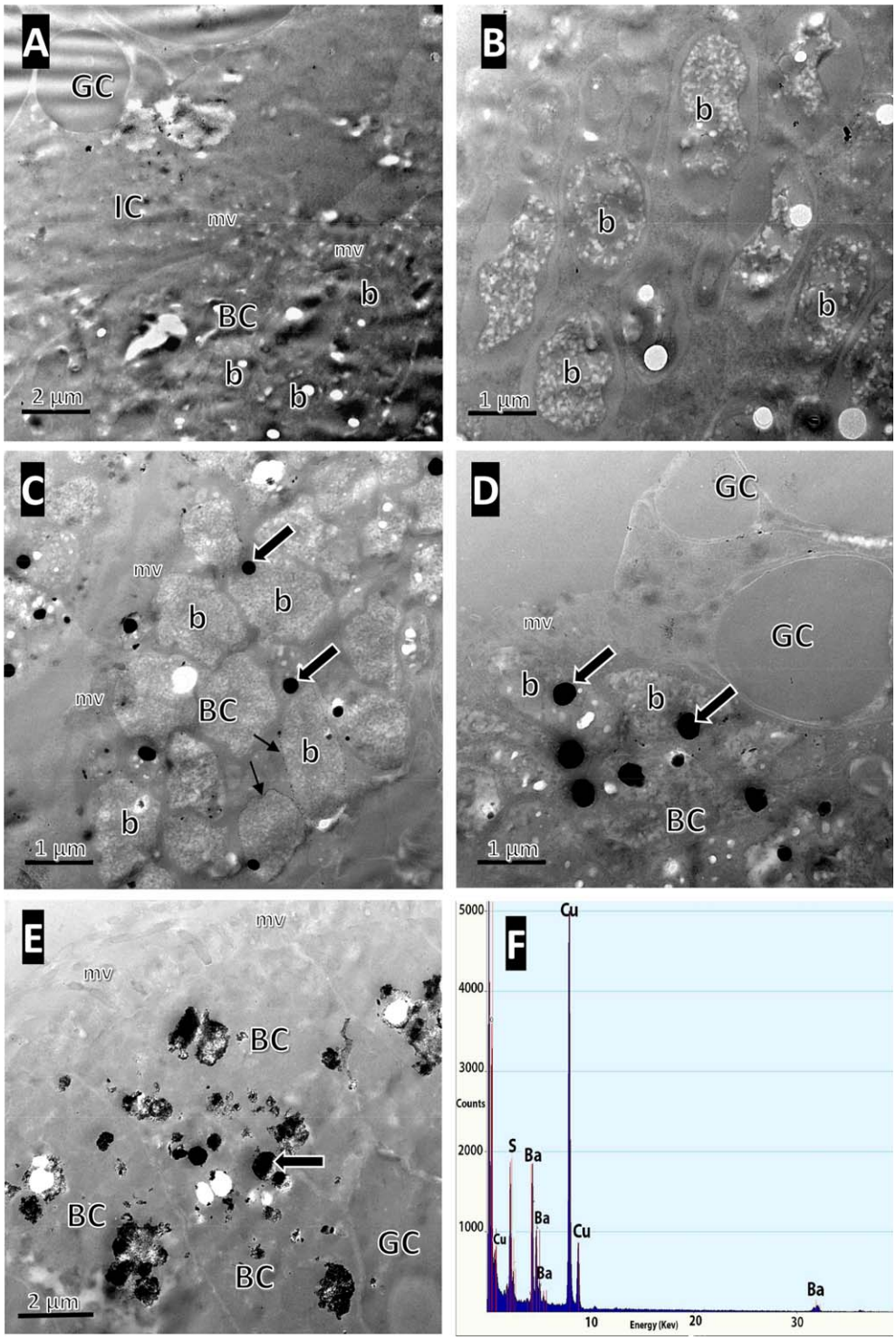

Fig. 2. Cytochemical detection of arylsulfatase and acid phosphatase in thin gill sections of *Codakia orbicularis*. **A,B:** Conversely to *C. orbiculata*, enzymatic activity in *C. orbicularis* was weak or absent in freshly collected individuals, and even after 3 weeks of starvation, no activity was detected either for arylsulfatase (A) or for acid phosphatase (B). **C,D:** Acid phosphatase activity in individuals starved for 6 weeks concern a large number of bacteria within most of the bacteriocytes composing each gill filament. In each bacteriocyte (BC), several bacteria (b) were degraded simultaneously by this enzyme. At the same time, granule cells (GC), which are devoid of bacterial endosymbionts, presented no enzymatic activity. **E:** In bivalves starved for 6 weeks, all the symbionts seemed to be affected by arylsulfatase degradation as shown by the numerous black areas observed through the cytoplasm of the bacteriocytes. **F:** EDX spectrum obtained from cytochemical detection of arylsulfatase activity by barium precipitation from black areas (black arrows) are shown in (C–E). Copper originated from the grid bars supporting the thin sections (mv: microvilli; N: nucleus). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

months and minimize the number of symbionts inside the gills. During our experiments, the appearance of the lucinid gills changed from thick and lightly beige in freshly collected bivalves (containing large symbiont populations with a large quantity of elemental sulfur) to thin and dark brown in starved bivalve individuals (containing fewer symbionts without elemental sulfur) (personal observations) (Caro et al., 2009; Lechaire et al., 2008; Pflugfelder et al., 2005). We suggest that the host can use this large amount of bacteria as a nutrient source through lysosomal degradation (Johnson and Fernandez, 2001). Although their digestive tract is reduced (Reid, 1990) particle feeding is still part of the nutrient strategy of symbiont-bearing lucinids (Duplessis et al., 2004). During starvation, enzyme activities were observed inside bacteriocytes close to, and within, the bacteria. We suggest that the host digests its bacterial endosymbionts during starvation to use the bacteria themselves as nutrients. The trophic pathway has been described in other marine chemotrophic symbioses (Boetius and Felbeck, 1995; Fiala et al., 1994; Herry et al., 1989; Kádár et al., 2007). For example, the lucinid *Lucina pectinata* digests its symbionts in large secondary lysosomes (Liberge et al., 2001). The enzymatic degradation of the symbionts in *Codakia* seemed different from that observed in *L. pectinata*, as no large secondary lysosomes with positive activity for the two enzymes tested were observed. In *Codakia* individuals, the gill-endosymbionts seem to be individually digested rather than in high numbers within large lysosomes as in *L. pectinata*. It seems that *Codakia* is less adapted to symbiont digestion than *L. pectinata*.

Most of the enzyme activity was observed inside the symbionts, suggesting that the detected enzymes could be produced by symbionts themselves and be active even after their death. Symbionts may have experienced sulfide and/or nutrient limitation inside the bacteriocytes. Following the death and lysis of symbionts, degraded products from the symbiont could possibly become incorporated by host cells helping the hosts to survive starvation. After a return to normal conditions, hosts could acquire new symbiosis-competent bacteria from their environment, restoring a "normal" metabolic pathway. It remains uncertain whether lucinids actively lyse bacteria to obtain nutrients during periods of host starvation, or whether the symbionts, starved of their energy source, undergo autolysis (with possible nutritional benefits to the host). Further studies are necessary to investigate how bacterial populations are controlled by lucinid hosts under natural conditions using metabolomics or stable isotope analyses.

ACKNOWLEDGMENTS

We need to thanks the Symbiomics program: "Sten König was funded through a Ph.D. grant from the EU Marie Curie Initial Training Network (ITN) "Symbiomics". The authors thank the three anonymous reviewers whose suggestions helped them to improve their manuscript.

REFERENCES

Barka T, Anderson PJ. 1962. Histochemical methods for acid phosphatase using hexazonium pararosanalin as coupler. J Histochem Cytochem 10:741–753.

Boetius A, Felbeck H. 1995. Digestive enzymes in marine invertebrates from hydrothermal vents and other reducing environments. Mar Biol 122:105–113.

Brissac T, Gros O, Mercot H. 2009. Lack of endosymbiont release by two Lucinidae (Bivalvia) of the genus *Codakia*: Consequences for symbiotic relationships. FEMS Microbiol Ecol 67:261–267.

Caro A, Gros O, Got P, Wit RD, Troussellier M. 2007. Characterization of the population of the sulfur-oxidizing symbiont of *Codakia orbicularis* (Bivalvia, Lucinidae) by single-cell analyses. Appl Environ Microbiol 73:2101–2109.

Caro A, Got P, Bouvy M, Troussellier M, Gros O. 2009. Effects of long-term starvation on a host bivalve *Codakia orbicularis* (Lucinidae) and its symbiont population. Appl Environ Microbiol 75:3304–3313.

Cavanaugh CM, Gardiner SL, Jones ML, Jannasch HW, Waterbury JB. 1981. Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila* Jones: Possible chemoautotrophic symbionts. Science 213:340–342.

Chandramohan D, Devendran K, Natarajan R. 1974. Arylsulfatase activity in marine sediments. Mar Biol 27:89–92.

Dorman DC, Moulin FJ-M, McManus BE, Mahle KC, James RA, Struve MF. 2002. Cytochrome oxidase inhibition induced by acute hydrogen sulfide inhalation: Correlation with tissue sulfide concentrations in the rat brain, liver, lung, and nasal epithelium. Toxicol Sci 65:18–25.

Duarte CM, Chiscano CL. 1999. Seagrass biomass and production: A reassessment. Aquat Bot 65:159–174.

Duplessis MR, Dufour SC, Blankenship LE, Felbeck H, Yayanos AA. 2004. Anatomical and experimental evidence for particulate feeding in *Lucinoma aequizonata* and *Parvilucina tenuisculpta* (Bivalvia: Lucinidae) from the Santa Barbara Basin. Mar Biol 145:551–561.

Durand PP, Gros O, Frenkiel L, Prieur D. 1996. Phylogenetic characterization of sulfur-oxidizing bacterial endosymbionts in three tropical Lucinidae by 16S rDNA sequence analysis. Mol Mar Biol Biotechnol 5:37–42.

Elisabeth NH, Gustave SDD, Gros O. 2012. Cell proliferation and apoptosis in gill filaments of the lucinid *Codakia orbiculata* (Montagu, 1808) (Mollusca: Bivalvia) during bacterial decolonization and recolonization. Microsc Res Tech 75:1136–1146.

Felbeck H. 1981. Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia pachyptila* Jones (Vestimentifera). Science 213:336–338.

Felbeck H. 1983. Sulfide oxidation and carbon fixation by the gutless clam *Solemya reidi*: An animal-bacteria symbiosis. J Comp Physiol 152:3–11.

Fiala MA, Michalski JC, Jolles J, Alonso C, Montreuil J. 1994. Lysosomic and lysozyme activities in the gill of bivalves from deep hydrothermal vents. C R Acad Sci III 317:239–244.

Fiala-Medioni A, Felbeck H, Childress JJ, Fisher CR, Vetter RD. 1990. Lysosomic resorption of bacterial symbionts in deep-sea bivalves. In: Nardon P, Glaninazzi-Pearson V, Grenier AM, Margulis L, Smith DC, editors. Endocytobiology IV: Fourth international colloquium on endocytobiology and symbiosis, INSA-Villeurbanne (France), July 4–8, 1989. Paris: Institut national de la recherche agronomique. pp. 335–338.

Frenkiel L, Mouëza M. 1995. Gill ultrastructure and symbiotic bacteria in *Codakia orbicularis* (Bivalvia, Lucinidae). Zoomorphology 115:51–61.

Glauert AM. 1991. Epoxy resins: An update on their selection and use. Eur Microsc Anal 5:15–20.

Gros O, Darrasse A, Durand P, Frenkiel L, Moueza M. 1996. Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve *Codakia orbicularis*. Appl Environ Microbiol 62:2324–2330.

Gros O, Frenkiel L, Mouëza M. 1998. Gill filament differentiation and experimental colonization by symbiotic bacteria in aposymbiotic juveniles of *Codakia orbicularis* (Bivalvia: Lucinidae). Invertebr Reprod Dev 34:219–231.

Gros O, Liberge M, Heddi A, Khatchadourian C, Felbeck H. 2003. Detection of the free-living forms of sulfide-oxidizing gill endosymbionts in the lucinid habitat (*Thalassia testudinum* environment). Appl Environ Microbiol 69:6264–6267.

Gros O, Elisabeth NH, Gustave SDD, Caro A, Dubilier N. 2012. Plasticity of symbiont acquisition throughout the life cycle of the shallow-water tropical lucinid *Codakia orbiculata* (Mollusca: Bivalvia): Symbiont acquisition in lucinid clams. Environ Microbiol 14: 1584–1595.

Herry A, Diouris M, Le Pennec M. 1989. Chemoautotrophic symbionts and translocation of fixed carbon from bacteria to host

tissues in the littoral bivalve *Loripes lucinalis* (Lucinidae). Mar Biol 101:305–312.
Johnson MA, Fernandez C. 2001. Bacterial symbiosis in *Loripes lucinalis* (Mollusca: Bivalvia) with comments on reproductive strategy. J Mar Biol Assoc UK 81:251–257.
Jørgensen BB. 1982. Mineralization of organic matter in the sea bed—The role of sulphate reduction. Nature 296:643–645.
Kádár E, Davis SA, Lobo-da-Cunha A. 2007. Cytoenzymatic investigation of intracellular digestion in the symbiont-bearing hydrothermal bivalve *Bathymodiolus azoricus*. Mar Biol 153:995–1004.
Lechaire J-P, Frébourg G, Gaill F, Gros O. 2008. In situ characterization of sulphur in gill-endosymbionts of the shallow water lucinid *Codakia orbicularis* (Linné, 1758) by high-pressure cryofixation and EFTEM microanalysis. Mar Biol 154:693–700.
Lewis PR, Knight DP. 1992. Cytochemical staining methods for electron microscopy. In: Glauert AM, editor. Practical methods in electron microscopy, Vol. 14. Amsterdam: Elsevier. 321 pp.
Liberge M, Gros O, Frenkiel L. 2001. Lysosomes and sulfide-oxidizing bodies in the bacteriocytes of *Lucina pectinata*, a cytochemical and microanalysis approach. Mar Biol 139:401–409.
Löffler B-M, Hesse B, Kunze H. 1984. A combined assay of three lysosomal marker enzymes: Acid phosphatase, β-D-glucuronidase and β-*N*-acetyl-D-hexosaminidase. Anal Biochem 142:312–316.
Ott J, Rieger G, Rieger R, Enderes F. 1982. New mouthless interstitial worms from the sulfide system: Symbiosis with prokaryotes. Mar Ecol 3:313–333.
Patel S, Patel B. 1985. Effect of environmental parameters on lysosomal marker enzymes in the tropical blood clam *Anadara granosa*. Mar Biol 85:245–252.
Pflugfelder B, Fisher CR, Bright M. 2005. The color of the trophosome: Elemental sulfur distribution in the endosymbionts of *Riftia pachyptila* (Vestimentifera; Siboglinidae). Mar Biol 146:895–901.
Pipe RK, Moore MN. 1985. The ultrastructural localization of lysosomal acid hydrolases in developing oocytes of the common marine mussel *Mytilus edulis*. Histochem J 17:939–949.
Reid R. 1990. Evolutionary implications of sulphide oxidizing symbioses in bivalves. In: Morton B, editor. The Bivalvia. Hong Kong: Hong Kong University Press. pp. 127–140.
Reynolds LK, Berg P, Zieman JC. 2007. Lucinid clam influence on the biogeochemistry of the seagrass *Thalassia testudinum* sediments. Estuar Coast 30:482–490.
Rogers D. 1960. Acid phosphatases of *E. coli*. Arch Biochem Biophys 89:97–104.
Schweimanns M, Felbeck H. 1985. Significance of the occurrence of chemoautotrophic bacterial endosymbionts in lucinid clams from Bermuda. Mar Ecol Prog Ser 24:113–120.
Stewart FJ, Cavanaugh CM. 2006. Symbiosis of thioautotrophic bacteria with *Riftia pachyptila*. Prog Mol Subcell Biol 41:197–225.
Streams ME, Fisher CR, Fiala-Medioni A. 1997. Methanotrophic symbiont location and fate of carbon incorporated from methane in a hydrocarbon seep mussel. Mar Biol 129:465–476.
Taylor JD, Glover EA. 1997. The lucinid bivalve genus *Cardiolucina* (Mollusca, Bivalvia, Lucinidae): Systematics, anatomy and relationships. Bull Nat Hist Mus Zool Ser 63:93–122.
Van Dover CL. 2000. The ecology of deep-sea hydrothermal vents. Princeton, NJ: Princeton University Press.