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

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Thioautotrophic bacterial endosymbionts are degraded by lysosomalhost digestion during starvation: case study of two lucinids -C*odakia orbicularis* and *Codakia orbiculata.*

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Abstract

The Caribbean bivalves*Codakia orbicularis*(Linné, 1758)and *C. orbiculata*(Montagu, 1808) live inseagrassbeds of*Thalassiatestudinum*and harborintracellular sulfur-oxidizing Gamma-proteobacteria. Thesebacterial symbionts fix CO_2 via the Calvin Benson Cycle and provide organic compounds to the bivalve. In case of starvation, no reduced sulfur compoundsand no organic particle foodareavailable; the symbionts could be considered asthe sole nutrient source by the host. In previous studies it was shown that the intracellular bacterial population decreased considerably during starvation and that the bacteriawere not released by the bivalves(Brissac et al., 2009).In the present study, the activity oftwolysosomal marker enzymes (acid phosphatase and arylsulfatase) was detected usingcytochemical experimentscoupled with EDX-TEM (Energy Dispersive X-ray- Transmission Electron Microscopy) during sulfide and organic particle starvation. Thedegradation of bacterial endosymbionts began after two weeks of starvation in *C. orbiculata* and after three weeks in*C. orbicularis*.Such degradation process seems continuousthrough months and could be responsible for the disappearance of the bacterial endosymbionts within the gills during starvation.These data suggest that the host usesymbionts as a nutrient source to survive a hunger crisis. The carbon transfer from the symbionts to the hostcould be flexibleand could consist in transfer of organic matter, "milking", under normal feeding conditions and digestion of the symbionts,"farming",under starved conditions.

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 Lucinidaeharbors chemoautotrophic Gamma-proteobacteria in specialized gill cells called bacteriocytes(Taylor et al., 1997).Manylucinid bivalves inhabit seagrass beds, where they dominate the sedimentary infauna(Reynolds et al., 2007). The seagrassecosystem is a very productive system and is enriched in organic matter(Duarte et al., 1999). The anaerobic degradation of organic matter in seagrass bed sediments by sulfate reducers produces reduced sulfur compounds such ashydrogen sulfide(Jørgensen, 1982). Reduced sulfur compounds (herein called sulfides) are toxic to most plants and animals (Dorman et al., 2002).Lucinidbivalvesliveat the interface ofoxic 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 oxidizesulfides, produce organic matterthrough 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 translocate 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 to other bivalves without endosymbionts. Lucinids provide their endosymbionts withsulfide and oxygen through permanently water pumping,oxygen act as terminal electron acceptor.

Codakiaorbicularis(Linné, 1758) and *C. orbiculata*(Montagu, 1802) inhabit the shallow water seagrass bed of *Thalassiumtestudinum*in the Caribbean Sea. Both lucinidshave been studied with regard to the interaction between host and symbionts in the last years, these systems represent an attractive model to investigate symbiosis. According to 16S rRNA sequence analysis, both *Codakia*speciesharbor the same and only one 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 symbiontswere 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 theirwhole life byphagocytosis at the apical pole of the bacteriocytes(Elisabeth et al., 2012).The symbionts of both lucinids are not released by the host, so the intracellular life of the symbiontsseems 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 areup to seven sub-populations of thissymbiont phylotype within a single bivalve. These bacterial sub-populationsdiffer 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 et al., 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 starvationthe organization of the gills in *C. orbicularis* changes and the bacterial population decreasesby aboutone third per month(Caro et al., 2009). After six months of starvation, no symbiont is detectable in the gills, the main part of which is then occupied by granule cells. The reduction of bacteriocyte'svolumeoccurs parallel to the reduction of the number of bacterial symbionts(Caro et al., 2009). Due to the fact that no bacteria were detectable in theseawater 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 to host-driven lysis, as a mean of obtaining nutrients from these bacteria. Indeed, bivalves with chemoautotrophic symbionts are known to digest and use symbionts as a nutrient source(Herry et al., 1989; Boetius and Felbeck, 1995; Fiala et al., 1994; 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 *Bathymodiolusazoricus*(Kádár et al., 2007). In this case the authorshypothesized that the autolysis of the bacteria is part of the cell cycle and that a bacterial acid phosphatase is involved in the processof the autolysis. Acid phosphatase and arylsulfatase are common enzymes in gram-negative bacteria(Rogers, 1960; Chandramohan et al., 1974). In eukaryotes, acid phosphatase and arylsulfatase are lysosomal markers that have been detected the Mollusca (Patel and Patel, 1985; Patel and Patel, 1985; Pipe and Moore, 1985; Liberge et al., 2001). Lysosomes are eukaryotic

membrane bound cell organelles with hydrolytic enzymes to chemically deteriorate polymers and bacteria. Acid phosphatase and arylsulfatase are 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 nutrient source through lysosomal degrdadtion.

Material and Methods

Sampling of bivalves

Adultindividualsof*Codakia orbicularis* and *C.orbiculata*, were manually collected in *Thalassiumtestudinum*seagrass beds on "îletcochon" (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 50L plastic tanks with 0.22 µm filtered seawater at 26°C up to six 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 seven 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 two weeks through the experiment (i.e 5 months) while for *C. orbicularis*, sampling took place three weeks after initial field collection then every two weeks.

Cytochemestry

The gills of the bivalves were dissected and fixed (2% glutaraldehyde, 1% paraformaldehyde in0.1 M cacodylateadjusted to 900 mOsm,) for 2h at 4°C. The fixed gills were washed overnight at 4°C in 0.2M sodium cacodylatebuffer with an adjusted osmolarity at 1800 mOsmwith NaCl and Ca_2Cl. The gills were sliced in sodium cacodylate buffer into 100 µm sections using a Vibratome OCT Slicer at 4°C. Only one enzyme activity 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 Hopsu-Havu et al.(1967 cited in, Narbaitz, 1995). Briefly, the 100µm sections weretransferred directly to the enzyme reaction solution (60 mM sodium acetate, 15 mMacetic acid, 8 mg/ml sulfate p-nitrocatechol, 60 mM bariumchloride) and incubated for 45 min at 37°C. After the incubation,the samples were washed in cacodylate buffer in several changes of sodium cacodylatebuffer overnight.

Acid phosphatasewas detected bya method from Barka and Anderson(Barka et al., 1962). The sections were pre-incubated for 10 to 20 min at 37°C in 50 mM acetate buffer with 15 mMacetic acid. The enzymatic reaction took place in the pre-incubation buffer containingan additional 0.1 M sodium ß-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 was formed as a result of enzyme activity during the incubation stage.

For both enzymes a negative control was prepared therefore the 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 aceton series and embedded in Epon-araldite resin according to Glauert (1991). Ultra-thin (100 nm) sections were made using an ultracut E microtome andtwo grids containing 4-5 sections were observed with a Tecnai G2 TEM at 200 kv. The barium or lead precipitates were detected using a 30mm^2 EDX detector. The EDXS analysiscorresponds 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 bivalveschanged visibly from thick andbeige to thin and dark brown. The mortality of the bivalves in the sterile sea water tanks was low: around one to five percent per week. Although the lethality was higher during the first three weekscompared to the later five 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 weak positive reaction: only few black areas were visible in a couple of bacteriocytes(fig 1 A/B) while most of the bacteriocytes throughout the gill filaments are free of lysosomal enzyme activity (data not shown). This data suggests that adult individuals in their natural habitat can digest symbionts, probably in the frame of resorption of dead bacteria.After two weeks of starvation, the acid phosphatase activitywas detected close to symbionts, while other gill cells were free of enzyme activity (fig

1 C/D). Likewise, the activity of the arylsulfatase increased during starvation time, after two months most of the symbionts inside the bacteriocytes are affected by an arylsulfatase activity (fig 1 E). Afterthe beginning of bacterial degradation in the gills, the lysosomal 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 period of starvation (5 months and more), the lysosomal activity globally decreases as less bacteriocytes are present through the lateral zone which now contains mostly granule cells. However, in the few remaining bacteriocytes which contain only few bacterial symbionts, there is still a lysosomal digestion occurring. No black areas were observed in granule cells and intercalary cells.

For *C. orbicularis* the story looked similar. After three weeks of starvation,no positive signal was detected for arylsulfatase and acid phosphatase in the gills of *C. orbicularis*, indicating very weak to no lysosomal activity(fig 2 A/B). In *C. orbicularis,*bothenzyme activities were detected aftersix weeks of starvation (fig 2 C/D/E). 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(fig. 1F/2F).In the negative controls, no barium or lead was detectable (not shown).

Discussion and Conclusion

The present study uses cytochemical methods to explain how lucinidbivalves 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 decreasesby one third per month (Caro et al., 2009) while no released bacteria aredetectable using CARD-FISH(Brissac et al.,2009). Lysosomes were rarely observed in freshly collected gills of *C. orbicularis*(Frenkiel et al., 1995). In our study in freshly collected *Codakia* bivalves, symbiont digestion were rarely observed.These data suggest that adult individuals in their natural habitat can digest symbionts, probably in the frame of resorption of dead bacteria and symbiont population controlling. In deep sea bivalves it was suggested that lysosomal

symbiont digestion is a way of controlling symbiont population (Fiala-Medioni et al., 1990). During starvation, lysosomes in the gills in both bivalvesbecomelarger and more abundant(Caro et al., 2009). Moreover, Elisabeth et al. (2012) describeda massive decrease of 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 signalling processes(Stewart et al., 2006).In the laboratory,it is possible to maintain adult bivalvesunder starvation conditionsfor up to six 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 population with a big amount of elemental sulfur) to thin and dark brown in starved bivalve individuals (containing less symbionts without elemental sulfur) (personal observations)(Johnson et al., 2001; Lechaire et al., 2008; Caro et al., 2009). We suggest that the host can use this large amount of bacteria as nutrient source through lysosomal degradation. Although their digestive tract is reduced (Reid, 1990)particle feeding is still part of the nutrient strategy of symbionts-bearing lucinids(Duplessis et al., 2004).During starvation, lysosomal enzyme activities were observed inside bacteriocytes close to,and within,the bacteria. We suggest that the host digestsits bacterial endosymbiontsduring starvation to use the bacteria themselves as nutrients (i.e. farming). The trophic pathway has been described in other marine chemotrophic symbioses(Herry et al., 1989; Fiala et al., 1994; Boetius and Felbeck, 1995; Kádár et al., 2007). For example, the lucinid*Lucina pectinata*, digest its symbionts in large secondary lysosomes(Liberge et al., 2001). The enzymatic degradationof the symbionts in *Codakia* appeared different from thatobserved in *L. pectinata*as no large secondary lysosomes with a positive activity of the two lysosomal enzymes tested were observed during this study in *C. orbicularis* bacteriocytes. In *Codakia*individuals, the gill-endosymbionts seem to be individually digested compared to multiple bacteria digestionwith large lysosomes 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 symbiontsindicating that the detected enzymes could be produced by symbionts themselves and be active even after their death.Symbionts could not find sulfides and/or enough nutrients inside the bacteriocytes due to the fact that stressed hosts preferred to sustain their own metabolism at the expense of its symbiotic population. So, once died and autolysed, the degraded products from the

symbionts could possibly become incorporated by host cells helping the hosts to survive starved conditions. Then, in better conditions, hosts will acquire new symbiosis-competent bacteria from its 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 studiesare necessary to investigate how bacterial populations are controlled by lucinid hosts under natural conditions using metabolomics or stable isotope analyses.

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Figure 1: Cytochemical detection of lysosomal enzymes arylsulfatase and acid phosphatase in thin gill sections of*Codakia orbiculata.*

A-B: Very few bacteriocytes from freshly collected individuals presented a couple of bacterial endosymbionts (*) partially degraded due to the activity of arylsulfatase (A) and to acid phosphatase (B). **C-D:** In individuals starved for two weeks, the number of bacteria degraded by lysosomal enzymes like acid phosphatase increases with numerous bacteriocytes affected. In each bacteriocyte (BC), several bacteria (b) are degraded by this enzyme suggesting a high lysosomal activity from host. In the same time, other gill cells (intercalary cell (IC) and granule cell (GC)) devoid of bacterial symbionts present no lysosomal activity. **E:** In bivalves starved for two month, most of the bacteria inside the bacteriocytes appear 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 indicated by black arrows in pictures C and D. Copper originates from the grid bars supporting the thin section.

(BC: bacteriocytes; GC: granule cells; IC: intercalary cells; mv: microvilli; N: nucleus)

Figure 2: Cytochemical detection of lysosomal enzymes arylsulfatase and acid phosphatase in thin gill sections of*Codakia orbicularis.*

A-B: Conversely to *C. orbiculata*, lysosomal activity in *C. orbicularis* was weak or absent in freshly collected individuals and even after three weeks of starvation, no activity was detected either for arylsulfatase (A) or for acid phosphatase (B). **C-D:** Acid phosphatase activity from six weeks starved individuals concerns a large number of bacteria within most of the bacteriocytes composing each gill filament. In each bacteriocyte (BC), several bacteria (b) are degraded simultaneously by this enzyme confirming a strong lysosomal activity from the host cell. In the same time, granule cell (GC), which are normally devoid of bacterial endosymbionts, present no lysosomal activity. **E:** In bivalves starved for six weeks, all the symbionts appear 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) in pictures C-E. Copper originates from the grid bars supporting the thin section.

(BC: bacteriocytes; GC: granule cells; IC: intercalary cells; mv: microvilli; N: nucleus)