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RESEARCH ARTICLE

In situ characterization of sulphur in gill-endosymbionts of the shallow water lucinid *Codakia orbicularis* (Linné, 1758) by high-pressure cryofixation and EFTEM microanalysis

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Abstract In this study, *Codakia orbicularis* gill-tissues were cryo-fixed by using high-pressure freezing, a freeze substitution process and finally by cryo-embedding in Lowicryl. Ultrathin sections were then used for an EFTEM microanalysis. Results show that intracellular bacterial symbionts contain elemental sulphur in periplasmic vesicles as indicated by conventional TEM. When sulphur is temporarily depleted in the environment, such structures may act as energy sources for bacterial metabolism. Moreover, sulphate was detected in the cytoplasm of the bacterial symbionts, suggesting the oxidation of elemental sulphur, located in periplasmic granules, to sulphate (the final step in sulphur oxidation) by these chemoautotrophic bacteria. To assess the effects of host starvation on the bacterial sulphur content, adult individuals of *C. orbicularis* were maintained in starvation for 6 weeks in sterile artificial seawater depleted in sulphur. During starvation, both (1) the number of bacteria inside the bacteriocytes and (2) the number of periplasmic granules per prokaryotic cell decreased. The content of the remaining periplasmic granules had been modified to sulphate. This observation suggests that bacterial gill-endosymbionts used the elemental sulphur in their periplasmic granules as stored substrate for oxidation in order to produce energy in case of sulphur depletion.

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Introduction

Codakia orbicularis (Linné, 1758) is known to harbour endosymbiotic sulphide-oxidizing bacteria in specialized cells of the gill filament named bacteriocytes (Frenkiel and Mouëza 1995). This tropical, shallow-water bivalve establishes symbiotic relationships with γ-Proteobacteria (Durand and Gros 1996) similar to those described in hydrothermal vent invertebrates (Cavanaugh et al. 1981; Felbeck et al. 1981). Such bacterial interactions have been intensively studied regarding the physiological activity of the symbionts compared to host activity. Only few of these studies focused on the respiratory metabolism of the symbionts (Arndt et al. 2001; Duplessis et al. 2004).

In thioautotrophic symbiotic models, conventional TEM (cTEM) provides excellent documentation at the ultrastructural level but gives no information concerning the chemical composition of the granule structures observed in the bacterial symbionts. The main problem in chemical investigations from thin-sections is that sulphur not bound in proteins is quickly lost during the fixation, dehydration, and embedding processes. Thus, symbiont and/or organelle fractions must be purified from tissues without chemical fixation before energy dispersive X-ray (EDX) microanalysis (Liberge et al. 2001) or Raman spectroscopic analysis (Pasteris et al. 2001). Even with these precautions, the

localization and characterization of the intracellular sulphur is difficult and in situ information in the host tissue is not available.

Some authors have shown that energy-filtered transmission electron microscopy (EFTEM) microanalysis (electron spectroscopic imaging: ESI and parallel electron energy-loss spectroscopy: PEELS) is a method well suited for detection of iron polyphosphates (Lechaire et al. 2002) in bacteria granules from the *Riftia pachyptila* tube and sulphur compounds in endosymbiotic bacteria (Lechaire et al. 2000; Krieger et al. 2000) from the trophosome of *R. pachyptila* (Vestimentifera) and *Inanidrilus leukodermatus* (Oligochaeta), respectively. More recently, investigations have focused on host organelles from the bacteriocytes of the bivalve *Lucina pectinata* (Lechaire et al. 2006). However, the sulphur content of the bacterial gill-endosymbiont colonizing lucinid bivalves was not addressed.

The main focus of the present study was to determine the location and the chemical form under which putative sulphur components were stored and mobilized in the bacterial endosymbionts of the model for thioautotrophy, the bivalve *C. orbicularis*.

Materials and methods

Sample collection

Adult *C. orbicularis* specimens were hand-collected in *Thalassia testudinum* seagrass beds on the island of Guadeloupe in the French West Indies. Bivalves were kept alive in vivo and transported within 2 days of collection to the lab in Paris where cryofixation was performed.

As a control, 12 specimens were kept in sterile artificial seawater (reconstituted with sterile ultrapure-water) without added sulphide. The starvation experiment was conducted at room temperature, ranging from 25 to 28°C. The water was highly oxygenated using an aquarium pump and renewed twice a week to avoid potential toxic effect due to increasing concentrations of ammonia, the nitrogen waste product of the clams. Six individuals were randomly collected after 6 weeks and cryofixed as controls.

Fixation and tissue processing

Small pieces of tissues were dissected from freshly delaminated gills and immediately placed in platelets specific for high-pressure freezing according to Lechaire et al. (2006).

HP freezing was followed by automatic freeze substitution (AFS, Leica) in acetone (instead of alcohol, in order to minimize loss of sulphur) from −90 to −20°C. The sample was then infused with Lowicryl K4 M resin (Polysciences, Inc.) at −20°C as previously described in Lechaire et al. (2006). Ultrathin sections were collected onto carbon-coated 700-mesh copper grids before EFTEM analysis. For morphological analyses, sections were contrasted by 2% aqueous uranyl acetate for 7 min followed by a lead citrate coloration.

Gill tissue samples were also chemically fixed as previously described (Frenkiel and Mouëza 1995) for morphological controls before embedding in epoxy resin.

EFTEM analysis: PEELS and ESI procedures

The EFTEM observations were performed using a LEO 912 Omega transmission electron microscope (LEO Electron Optics GmbH, Oberkochen, Germany) at 120 kV. Acquisition was accomplished with the ESIvision program (versions 3. 0 Soft-Imaging Software, SIS, GmbH, 48153 Münster). To improve the accuracy of the analysis, spectra were acquired from different sulphur references (elemental sulphur and sulphur compounds) as described recently (Lechaire et al. 2006). For PEELS acquisition, the primary magnification was set to select only one granule in the area delimited by the entrance aperture of the spectrometer. For ESI acquisition and to minimize the radiation damage we used the three-window method (Jeanguillaume et al. 1978; Reimer et al. 1992).

Results

Bacteriocyte and symbiotic ultrastructure

In *C. orbicularis*, the bacteriocytes represent one of the major cell types of the gill filament (Fig. 1) according to cTEM observations. The cytoplasm of the bacteriocytes, mostly located in the one-third of the lateral zone, are filled with intracellular bacteria that are characterized by numerous periplasmic empty vesicles (Figs. 1, 2). This type of host cell is characterized by a rounded apical pole with short microvilli that is in contact with the circulating seawater, a basal nucleus, and few mitochondria (Fig. 2). Bacteria are usually enclosed individually and present the double membrane typical for Gram negative bacteria (Fig. 3). The bacterial cytoplasm usually essentially contains DNA, ribosomes, and empty clear vesicles located in the periplasmic space (Fig. 3).

Cryotechniques coupled with the EFTEM observations made at zero-loss energy (Figs. 4, 5, 6) permitted the visualization of the bacterial symbionts. Because the samples could not be oriented during the cryo-embedding process, it is difficult to obtain similar section planes for an easy comparative study with cTEM. However, the bacterial symbionts could be observed inside the gill-tissue. Bacteriocytes from individuals freshly collected from the field harbour

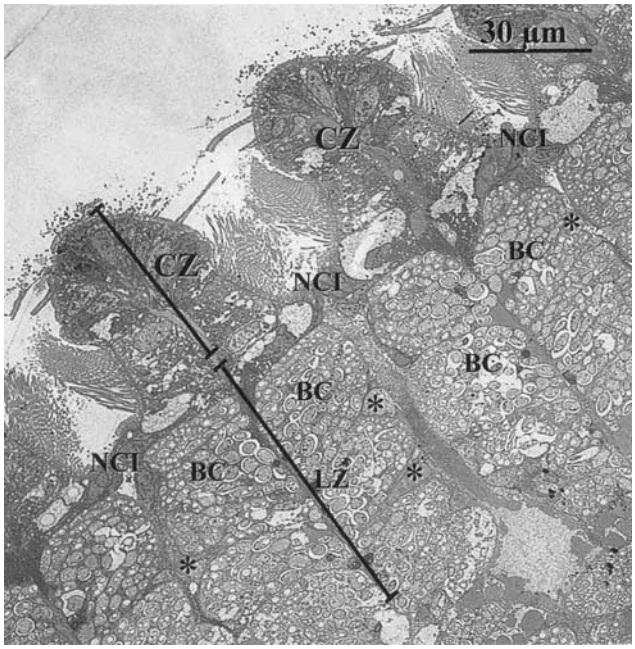

Fig. 1 Low magnification image of gill filaments from adult individuals dissected immediately upon recovery. Each gill filament is characterized by a ciliated zone (*CZ*) separated from the lateral zone (*LZ*) by several non-ciliated intermediary cells (*NCI*). Bacteriocytes (*BC*) filled with chemoautotrophic-symbionts are part of the lateral zone with numerous intercalary cells characterized by their nucleus in an apical position (*asterisks*)

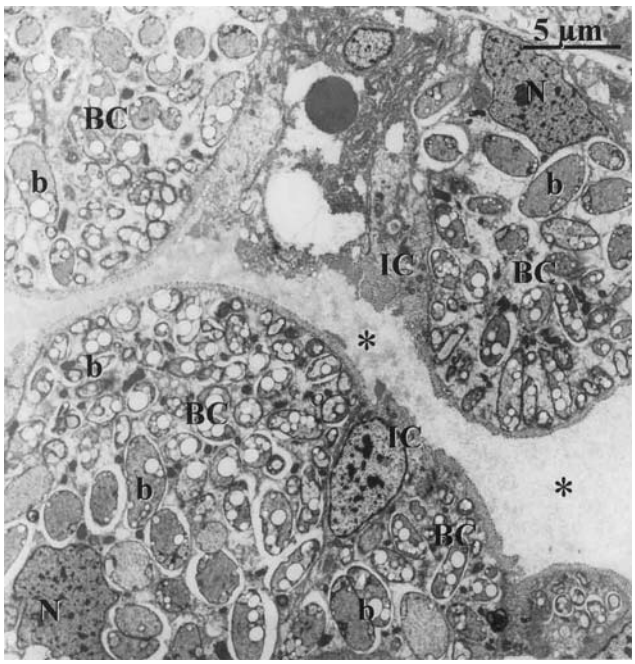

Fig. 2 cTEM view of the lateral zone of a gill filament from a freshly collected clam. Bacteriocytes (*BC*) have a basal nucleus (*N*) and a rounded apical pole developing a broad contact with pallial sea-water (*asterisk*). The cytoplasm is crowded by bacteria (*b*) that are usually individually enclosed inside bacteriocyte vacuoles. Intercalary cells (*IC*) that are regularly interspersed between bacteriocytes are devoid of bacterial symbionts

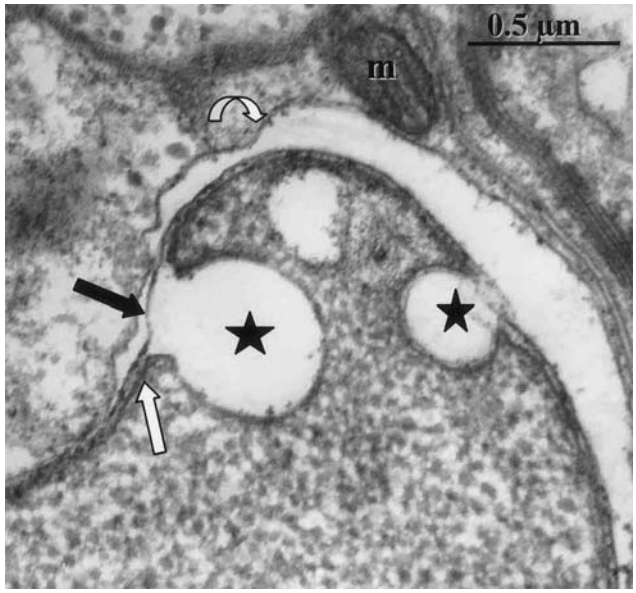

Fig. 3 High magnification image of an envacuolated thioautotrophic bacterium focusing on sulphur granules. Such granules, which appear as empty vesicles (*stars*) with cTEM, are located in the periplasmic space between the inner membrane (*white arrow*) and the outer membrane (*black arrow*) of this Gram negative bacteria. *Curved arrow* shows the eukaryotic host membrane of the vacuole containing the symbiont, *m* mitochondria

numerous bacterial symbionts per host cell containing periplasmic spaces with dense material (Figs. 5, 6) opposite to results obtained by cTEM (Figs. 1, 2, 3). In starved individuals, it was more difficult to find bacteria from the bacteriocyte sectioned indicating that the number of bacterial symbionts has decreased within such host cells during the starvation period. Such data were recently confirmed using molecular technique as CARD-FISH from histological sections (Caro and Gros, unpublished data).

Chemical composition of periplasmic granules

The ESI method was performed in order to image the location of the sulphur in the periplasmic granules. Figure 7a illustrate the presence of sulphur (S-distribution) in such prokaryotic granules from the bacteria observed in Fig. 6. When the S-distribution image was superimposed onto the high-contrast image (HCI) recorded at 250 eV (Mixmap image, Fig. 7c), sulphur was observed localized inside the periplasmic vesicle. The sulphur is distributed homogenously within the granule.

The PEELS method allowed us to confirm the presence of sulphur in the dense vesicle (Fig. 7e) with a specific sulphur spectrum (edge closed to 160 eV) obtained after background subtraction. The presence of phosphorus in the same area analysed was tested by the PEELS method: no significant signal was obtained (data not shown). Only the

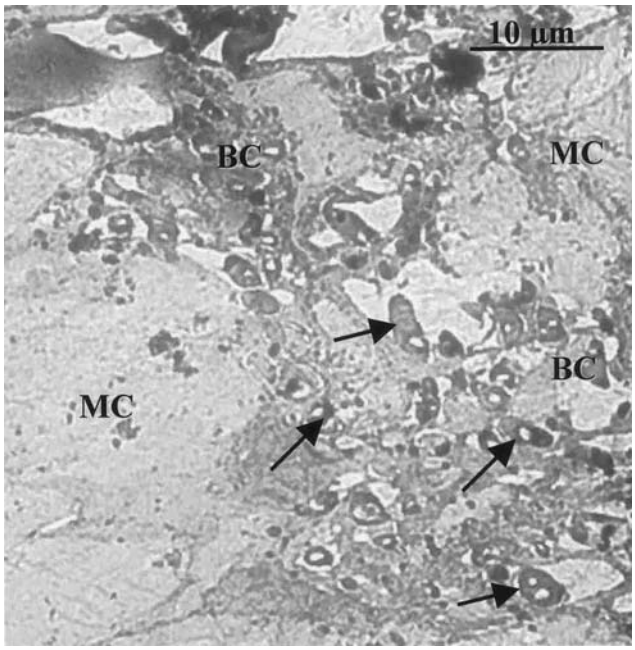

Fig. 4 Low magnification image of an EFTEM observation of the lateral zone of the *C. orbicularis*-gill of a freshly collected specimen. Zero-loss filtered image of an unstained ultrathin section showing various mucocytes (*MC*) and few bacteriocytes (*BC*) harbouring numerous bacteria (*arrows*)

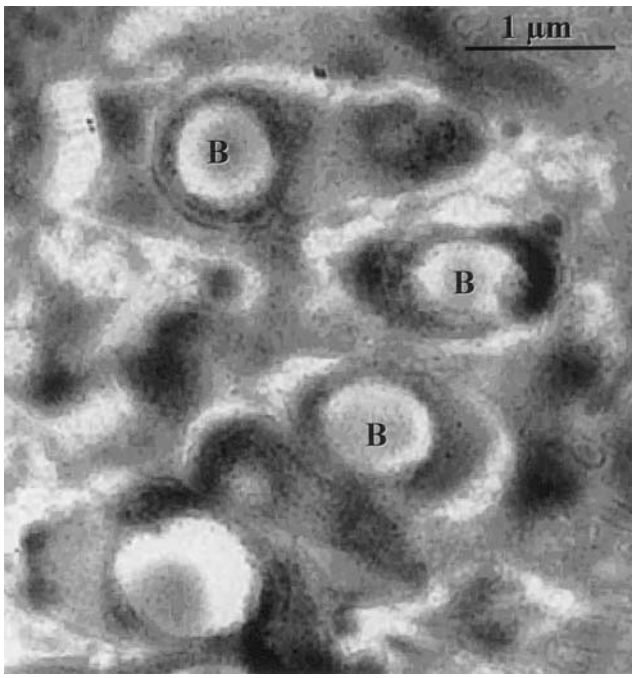

Fig. 5 EFTEM observation of the gill-endosymbiont from a freshly collected clam. Zero-loss filtered image of an unstained ultrathin section showing bacteria containing electron luscent granules (*B*)

profile of the spectrum obtained with the reference S° (Lechaire et al. 2006) is similar to the profile of the spectrum obtained with the sulphur contained in the periplasmic

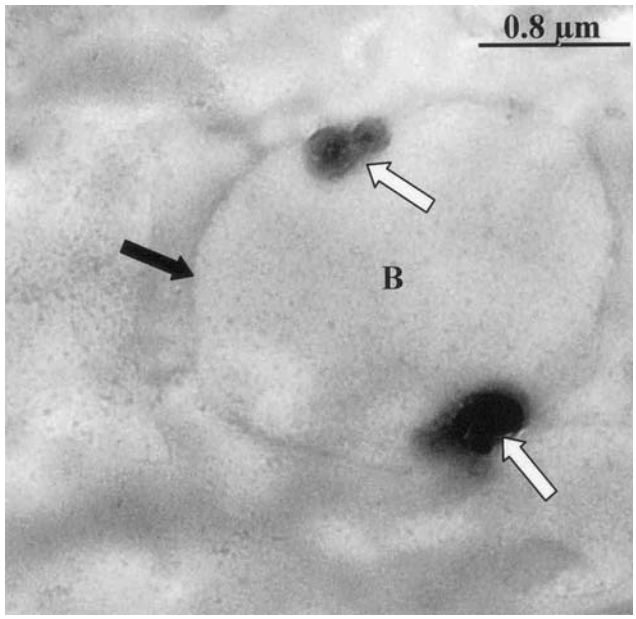

Fig. 6 EFTEM observation of one gill-endosymbiont from a freshly collected clam. High contrast image of an unstained ultrathin section showing one bacterium (*B*) containing electron dense granules (*white arrows*). The bacterial symbiont is enclosed in a host vesicle which is indicated by a *black arrow*

granule (Fig. 7e). The other spectra profiles obtained from different redox states of sulphur (Lechaire et al. 2006) do not resemble the S° spectrum of the periplasmic granule.

Using the same kind of analysis, some bacteria showed sulphur distributed throughout the cytoplasm (Fig. 7b, d). The PEELS method confirms the presence of a mixture of S° and sulphate in the whole cytoplasm of some bacterial symbionts (Fig. 7f) rather than S° as observed in the periplasmic granules (Fig. 7e).

Evolution of the sulphur granules during starvation experiment

After 6 weeks of starvation in artificial sulphide free seawater (Fig. 8), both the number of bacteria inside the gills (this study) and the number of granules per prokaryotic cell decreased (Caro, personal communication). Sulphur compounds were detected in symbionts as shown in the S-distribution image (Fig. 8a, b) according to the mix-map images (Fig. 8c, d). The PEELS method allowed us to confirm the presence of sulphate (SO_4^{2-}) in the dense granules (Fig. 8e) located in the periplasmic space of some endosymbionts observed from 6 week-starved individuals. Elemental sulphur was not observed contrary to the individuals freshly collected in the field. Thus, the sulphur content had changed after 6 weeks of starvation indicating that the elemental sulphur located in the periplasmic vesicles was used as storage for the symbionts in case of depletion of sulphur

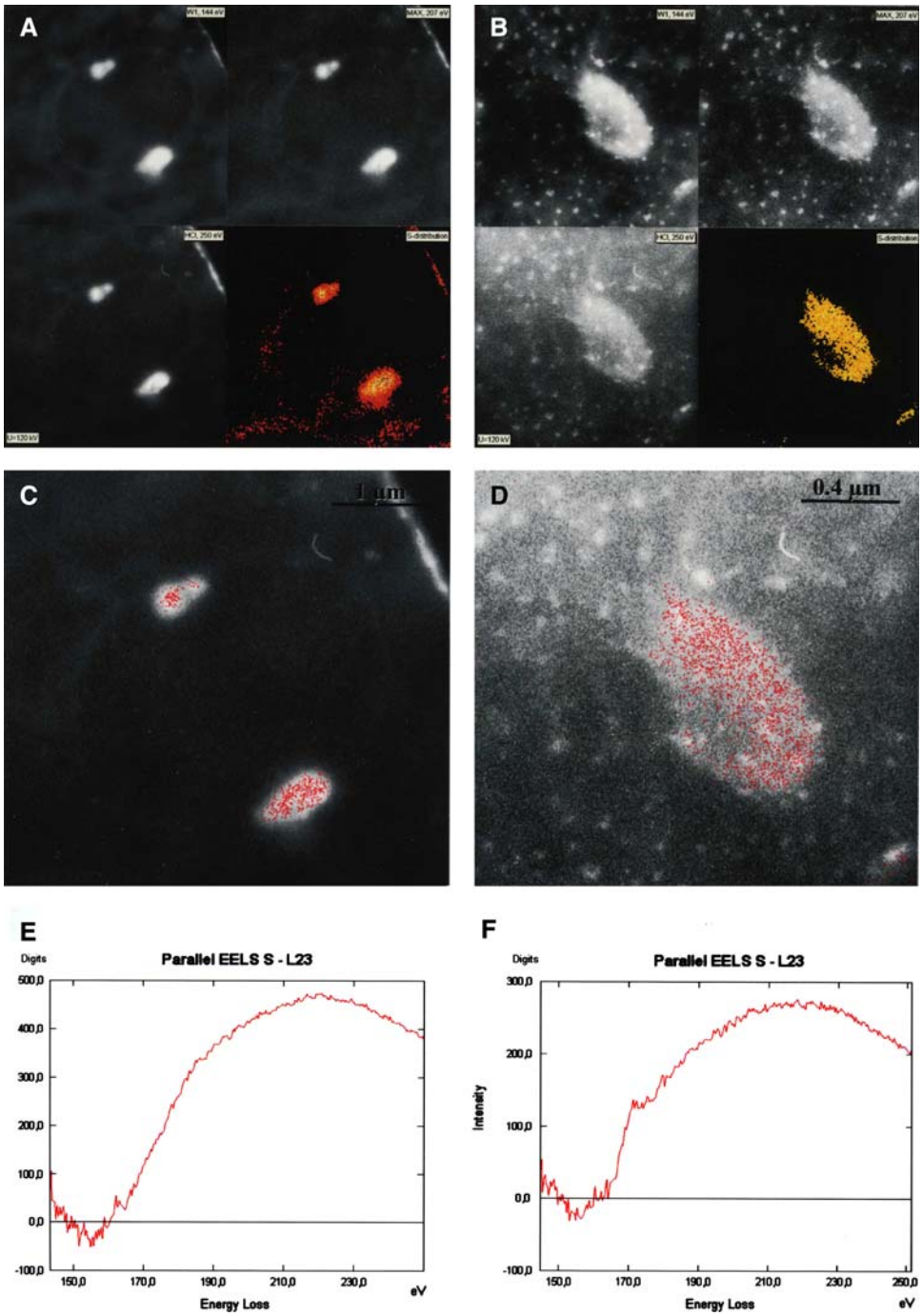

Fig. 7 **a–f** EFTEM observations of the gill-endosymbiont from a clam freshly collected in the field. ESI method. For the 3 window acquisition method only 2 images are shown: one image at the maximum energy of sulphur (Max 207 eV) and one image before (W1 144 eV) the sulphur edge (160 eV). A topographic reference image is recorded at 250 eV (high contrast image HCI 250 eV). At this energy, the contrast is inversed: the dense granules in the ultrathin section appears in *white*. The result is shown in the S-distribution images inside the dense granules located in the bacterial periplasm (**a**) or in the bacterial cytoplasm (**b**). Mixmap images: the net sulphur map is superimposed on the high contrast image. The sulphur distribution is homogenous in the granule (**c**) or in the cytoplasm (**d**). PEELS method. The spectrum obtained after back-ground subtraction corresponds to the elemental sulphur S° (**e**) in the granule whereas in **f** the spectrum corresponds to a mixture of S° and sulphate compounds (SO_4^{2-}) characterized by a small peak at 170 eV in the cytoplasm. The S-$L_{2,3}$ edge is closed to 160 eV

in the environment. The presence of a mixture of S° and sulphate was also detected in the cytoplasm of some bacteria (Fig. 8d, f) as for individuals freshly collected from the field (Fig. 7f).

Discussion

Because sulphur-oxidizing gill-endosymbionts are not cultivable yet, very few data are available on the sulphur metabolism of these bacteria (Arndt et al. 2001; Duplessis et al. 2004) or on the sulphur content of the bacterial symbionts in the host tissue (Vetter 1985; Pasteris et al. 2001). Recently, Pflugfelder et al. (2005) have suggested based on EELS analysis that the bacterial symbionts of *R. pachyptila* contain S° sulphur but were unable to determine the precise location inside the bacteria. Nothing is known about the symbionts colonizing Bivalvia even though a recent study was done on the sulphur content of a eukaryotic vesicle produce by the host in *L. pectinata* (Lechaire et al. 2006). Hofer and Golob

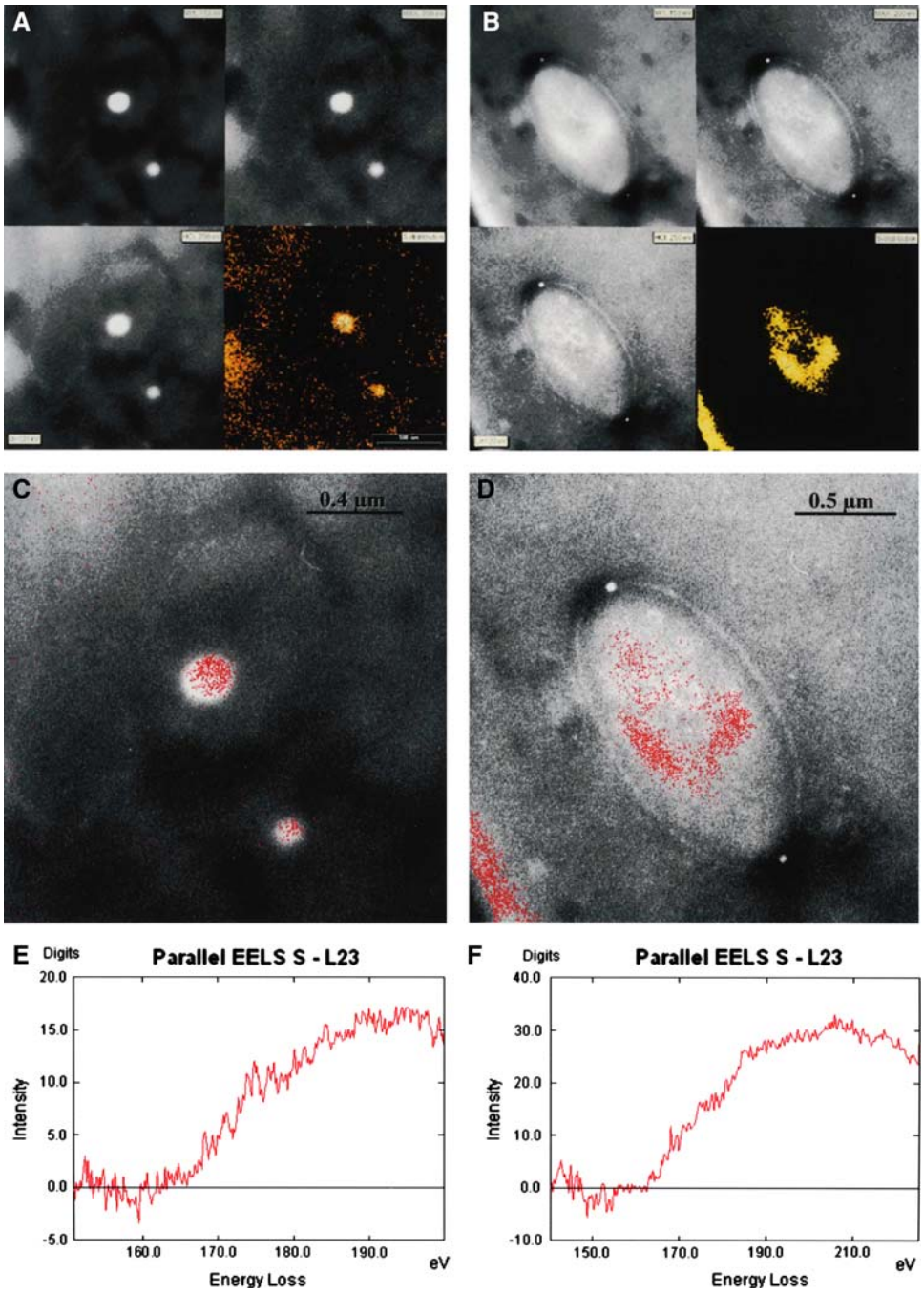

Fig. 8 **a**, **f** EFTEM observations of the gill-endosymbiont from an individual kept 6 weeks in starvation in artificial sterile seawater. ESI method with similar conditions as described for the Fig. 7. The result is shown in the S-distribution images: the bacterial symbionts still harbour sulphur compounds either in granules (**a**) or in the cytoplasm (**b**). Mixmap images: the sulphur map is superimposed on the high contrast image. The sulphur distribution is restricted to granules (**c**) or homogenous inside the bacterial cell (**d**). PEELS method. The spectra obtained after back-ground subtraction correspond to sulphate compounds (**e**) characterized by the small peaks at 175 and 185 eV in the granule or to a mixture of S° and sulphate compounds with a small peak at 170 eV (**f**) in the bacterial cytoplasm. The S-$L_{2,3}$ edge is at 160 eV

(1987) have shown that near-edge fine structures of EELS edges can be very useful as a fingerprint for rapid identification of chemical compounds such as sulphates in the EM. In marine invertebrates harbouring sulphide-oxidizing symbionts, EELS analysis has been done in the gutless oligochaete *Inanidrilus leukodermatus* (Krieger et al. 2000). The authors have detected sulphur in the larger symbionts within intracellular granules and to a lesser degree in the cytoplasm (Krieger et al. 2000). So, by this way they were able to confirm phylogenetic data indicating that the larger symbiont was a sulphur-oxidizing bacterium. However, this paper did not indicate the oxydation level of the sulphur detected and, according to the fact that the fixation process used was designed to preserve antigenecity, the authors were unfortunately unable to identify which of the membrane-bound or non-membrane-bound globules were concerned. In our study, we try to improve the quality of the data obtained with EELS by using cryofixation under high-pressure freezing followed by freeze substitution and cryo-embedding processes in order to preserve the nature of chemical compounds.

The cryotechniques we used, i.e. HP freezing followed by freeze-substitution and cryo-embedding, allowed us to

observe and characterize the in situ content of the periplasmic vesicles of gill-endosymbionts colonizing *C. orbicularis*. This technique was successfully used by Lechaire et al. (2006) with another tropical lucinid, *L. pectinata,* to characterize sulphur vesicles produced by the host cells but did not attempt to show their presence in the symbionts. Periplasmic granules that are normally described as electron lucent using conventional transmission electron microscopic methods (fixation, embedding, and observation) can be observed in *C. orbicularis* (Frenkiel and Mouëza 1995).

The absence of solid sulphur crystals in the granules analyzed in this study suggest that these granules are actually elemental sulphur in the form of liquid-crystalline sulphur (Vetter 1985). Such elemental sulphur could serve as a store for energy production that is directly available for these prokaryotic cells or indirectly as described for the small host vesicles produced by the bacteriocytes of *L. pectinata* (Liberge et al. 2001; Lechaire et al. 2006).

In a previous study, Duplessis et al. (2004) demonstrated that the gill endosymbionts of *C. orbicularis* were able to use oxygen as the primary electron acceptor with a rapid oxygen consumption. The symbionts were able to produce hydrogen sulphide under anoxic conditions, supposedly from elemental sulphur stored in symbionts cells. Nitrate respiration was not detected in these sulphide-oxidizing bacteria (Duplessis et al. 2004). Therefore, the sulphur granules described in the periplasmic space of the symbionts in this study may serve as sulphur storage for the symbionts in case of temporary depletion of sulphur in the host environment.

To verify this hypothesis, we maintained clams in artificial sterile seawater without any sulphide and food (to avoid sulphide production by algae cultures) added. After 6 weeks of starvation, the remaining bacterial symbionts located in the bacteriocytes lost their elemental sulphur in their periplasmic granules. Bacterial gill-endosymbionts usually have fewer granules per cell indicating that they have used them as storage substrate during the time in the sulphide free artificial seawater used in this study. Caro et al. (2007) have shown that in freshly collected individuals, the symbiont population exhibited a great genomic heterogeneity with up to seven sub-populations of bacterial gill-endosymbionts that all depend on their sulphur content. The most common one is characterized by large cells with numerous sulphur granules while the others are characterized by smaller cells with only rare sulphur granules. After 2 months of starvation, the bacterial population consisted mostly of small bacteria without or with only rare granules (Caro and Gros, unpublished data).

In lucinids the sulphur metabolism appears to be different depending on the species considered. In *L. pectinata*, the host appears to store elemental sulphur in small vesicles that are produced by the host itself and are located at the basal pole of the bacteriocytes (Frenkiel et al. 1996; Liberge et al. 2001; Lechaire et al. 2006). Such sulphur storage may be usable for the bacterial symbionts (Frenkiel et al. 1996) when sulphur in the environment is depleted. On the other hand, in *C. orbicularis*, no sulphur granules can be observed by cTEM or be detected in the bacteriocyte's cytoplasm of the bivalve host (Frenkiel and Mouëza 1995; this study). The prokaryotic symbionts produce their own storage sulphur granules in periplasmic granules. Such storage of elemental sulphur can be used by the bacteria in oxidative metabolic pathways (Nelson and Fisher 1995) when sulphide is depleted in their environment.

Most of the tropical lucinids may have the same method of sulphur storage as that of *C. orbicularis* because based on 16S rDNA analyses 6 lucinid species have been described as harbouring the same gill-endosymbiont (Durand and Gros 1996; Gros et al. 2000, 2003a). Moreover, the gill-endosymbiotic bacteria are environmentally transmitted to the new host generation via a free-living symbiont (Gros et al. 1996, 2003b). No data are available about the sulphur content of such a free-living symbiont form. Whether they have similar sulphur storage granules in their periplasmic space is currently impossible to answer. However, newly endocytozed bacteria usually have very few or no periplasmic granules based on TEM analyse (Gros et al. 1996, 1998). These bacteria can be observed during the first steps of colonization of gill-cells in aposymbiotic juveniles located at the periphery of the bacteriocytes. Further investigations focusing on the appearance of such storage granules during the establishment of symbiosis will be necessary to complete our knowledge of sulphur metabolism in lucinid symbionts.

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References

Arndt C, Gaill F, Felbeck H (2001) Anaerobic sulphur metabolism in thioautotrophic symbioses. J Exp Biol 204:741–750

Caro A, Gros O, Got P, DeWit R, Trousselier M (2007) Assessment of the nucleic acid content, cell size, sulphur content, and respiratory activity of the sulphur-oxidizing symbiont of *Codakia orbicularis* (Bivalvia, Lucinidae). Appl Environ Microbiol 73:2101–2109

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

Duplessis MR, Gros O, Ziebis W, Robidart J, Felbeck H (2004) Respiration strategies utilized by the gill endosymbiont from the host lucinid *Codakia orbicularis* (Bivalvia: Lucinidae). Appl Environ Microbiol 70:4144–4150

Durand P, Gros O (1996) Bacterial host specificity of Lucinacea endosymbionts-interspecific variation in 16S rRNA sequences. FEMS Microbiol Lett 140:193–202

Felbeck H, Childress JJ, Somero GN (1981) Calvin–Benson cycle and sulphide oxidation enzymes in animals from sulphide-rich habitats. Nature 293:291–293

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

Frenkiel L, Gros O, Mouëza M (1996) Gill ultrastructure in *Lucina pectinata* (Bivalvia: Lucinidae) with reference to hemoglobin in bivalves with symbiotic sulphur-oxidizing bacteria. Mar Biol 125:511–524

Gros O, Darrasse A, Durand P, Frenkiel L, Mouëza M (1996) Environmental transmission of a sulphur-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). Invert Reprod Dev 34:219–231

Gros O, Frenkiel L, Felbeck H (2000) Sulphur-oxidizing endosymbiosis in *Divaricella quadrisulcata* (Bivalvia: Lucinidae): morphological, ultrastructural, and phylogenetic analysis. Symbiosis 29:293–317

Gros O, Liberge M, Felbeck H (2003a) Interspecific infection of aposymbiotic juveniles of *Codakia orbicularis* by various tropical lucinid gill-endosymbionts. Mar Biol 142:57–66

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

Hofer F, Golob P (1987) New examples for near-edge fine structures in electron energy loss spectroscopy. Ultramicroscopy 21:379–384

Jeanguillaume C, Tribbia P, Colliex C (1978) About the use of electron energy-loss spectroscopy for chemical mapping of thin foils with high spatial resolution. Ultramicroscopy 3:237–242

Krieger J, Giere O, Dubilier N (2000) Localization of RubisCO and sulphur in endosymbiotic bacteria of the gutless marine oligochaete *Inanidrilus leukodermatus* (Annelida). Mar Biol 137:239–244

Lechaire J-P, Frébourg G, Lopez-Garcia C, Gaill F (2000) Sulphur localization in *Riftia* endosymbionts. Biol Cell 92:24

Lechaire J-P, Shillito B, Frébourg G, Gaill F (2002) Elemental characterization of microorganism granules by EFTEM in the tube wall of a deep-sea vent invertebrate. Biol Cell 94:243–249

Lechaire J-P, Frébourg G, Gaill F, Gros O (2006) In situ localization of sulphur in the thioautotrophic symbiotic model *Lucina pectinata* by cryo-EFTEM microanalysis. Biol Cell 98:163–170

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

Nelson DC, Fisher CR (1995) Chemautotrophic and methanotrophic endosymbiotic bacteria at deep-sea vents and seeps. In: Karl DM (ed) Deep sea hydrothermal vent. CRC Press, Boca Raton, pp 125–167

Pasteris JD, Freeman JJ, Goffredi SK, Buck KR (2001) Raman spectroscopic and laser scanning confocal microscopic analysis of sulphur in living sulphur-precipitating marine bacteria. Chem Geol 180:3–18

Pflugfelder B, Fisher CR, Bright M (2005) The color of the trophosome: Elemental sulphur distribution in the endosymbionts of *Riftia pachyptila* (Vestimentifera; Siboglinidae). Mar Biol 146:895–901

Reimer L, Zepke U, Moesch J, Schulze-Hillert S, Ross-Messmer M, Probst W, Weimer E (1992) EELSpectroscopy. Carl Zeiss, Oberkochen

Vetter RD (1985) Elemental sulphur in the gills of three species of clams containing chemoautotrophic symbiotic bacteria: a possible inorganic energy storage compound. Mar Biol 88:33–42