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Research article

In situ localization of sulphur in the thioautotrophic symbiotic model *Lucina pectinata* (Gmelin, 1791) by cryo-EFTEM microanalysis

Jean-Pierre Lechaire*†[1], Ghislaine Frébourg*, Françoise Gaill* and Olivier Gros‡

*UMR 7138 Systématique, Adaptation, Evolution, Université Pierre et Marie Curie, Bât A, 4ème étage, 7, Quai Saint Bernard 75005, Paris, France, †Service de Microscopie Électronique, IFR 83 Biologie Intégrative, Université Pierre et Marie Curie, Bât A, 4ème étage, 7, Quai Saint Bernard 75005, Paris, France, and ‡UMR 7138 SAE, Département de Biologie, Université des Antilles et de la Guyane B.P. 592, 97159 Pointe-à-Pitre Cedex, Guadeloupe, France

Background information. Lucina pectinata is a large tropical lucinid known to harbour sulphide-oxidizing bacteria in specialized gill cells. Conventional TEM (transmission electron microscopy) has shown that bacteriocytes also harbour visibly 'empty' vesicles whose chemical content remains, to date, only roughly determined.

Results. In the present study, *L. pectinata* gill tissues were cryo-fixed as fast as possible by performing high-pressure freezing before a freeze-substitution process and finally performing a cryo-embedding in Lowicryl. Ultrathin sections were then used for a cryo-EFTEM (where EFTEM stands for energy-filtered TEM) microanalysis. Results show that bacteriocytes within the gill tissues contain elemental sulphur in small vesicles produced by the host itself. In instances of sporadic depletion of sulphur in the environment, such structures may act as energy sources for bacterial endosymbionts.

Conclusions. The cryo-EFTEM techniques represent (i) the only method used to date to locate and preserve sulphur at the cellular level and (ii) a powerful tool for sulphur metabolism analysis in thioautotrophic symbiont relationships.

Introduction

Lucina pectinata (Gmelin, 1791), like all lucinids described to date (Reid, 1990; Gros et al., 2000, 2003a), harbours endosymbiotic, sulphide-oxidizing bacteria located in specialized gill cells: the bacteriocytes (Frenkiel et al., 1996). This tropical, shallow-water bivalve (Warmke and Abbott, 1962; Abbott, 1974) establishes symbiotic relationships with Proteobacteria similar to those described in hydrothermal vent invertebrates (Cavanaugh et al., 1981; Felbeck et al., 1981). Although sulphide-oxidizing symbiotic relationships were initially reported 20 years ago, attempts to cultivate symbiotic bacteria remain unsuccessful. As a result, little information exists about the sulphur contents of bacterial granules (Vetter, 1985; Pasteris et al., 2001) and/or other sulphur storage in the bacteriocytes (Liberge et al., 2001). The initial metabolism of sulphide in the *L. pectinata* symbiont-containing gills appears to occur not in the bacterial symbionts but rather in the bacteriocytes (Liberge et al., 2001).

Ultrastructure studies have shown that the *L. pectinata* bacteriocytes contain three kinds of cytoplasmic inclusions (Frenkiel et al., 1996): (i) bacteria that are individually enclosed in host cell vesicles, (ii) lysosome-like bodies and (iii) electron-lucent vesicles located in the basal pole near the nucleus. cTEM (conventional transmission electron microscopy) provides

[1]To whom correspondence should be addressed (email jean-pierre.lechaire@snv.jussieu.fr).
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Abbreviations used: TEM, transmission electron microscopy; cTEM, conventional TEM; EDX, energy-dispersive X-ray; EFTEM, energy-filtered TEM; ESI, electron spectroscopic imaging; HCI, high-contrast image; PEELS, parallel electron energy-loss spectroscopy.

excellent documentation at the structural and ultrastructural levels, but give no information concerning the chemical composition of such structures *in situ* (Liberge et al., 2001). Symbiont and/or organelle fractions must be purified from tissues without chemical fixation before EDX (energy-dispersive X-ray) microanalysis (Liberge et al., 2001) or Raman spectroscopic analysis (Pasteris et al., 2001). Liberge et al. (2001) showed high levels of sulphur in a partly purified fraction containing small vesicles from *L. pectinata* bacteriocytes. Under these conditions, however, EDX techniques did not allow for the localization and characterization of the intracellular sulphur, restricting the possibility of chemical modification or loss of such compounds.

In previous studies, some authors have shown that EFTEM (energy-filtered TEM) microanalysis [ESI (electron spectroscopic imaging) and PEELS (parallel electron energy-loss spectroscopy)] is a well-suited method for detection of iron polyphosphates (Lechaire et al., 2002) in bacteria granules from the *Riftia pachyptila* tube and sulphur in endosymbiotic bacteria (Krieger et al., 2000; Lechaire et al., 2000) respectively from the trophosome of *R. pachyptila* (Vestimentifera) and *Inanidrilus leukodermatus* (Annelida).

Elemental sulphur is known to dissolve in solvents like those commonly used in cTEM after chemical fixation and during alcohol dehydration and epoxy resin embedding of biological samples (Vetter, 1985; Truchet et al., 1998; Pasteris et al., 2001). Consequently, the small electron-lucent vesicles produced by the *L. pectinata* bacteriocytes at their basal pole appear empty in ultrathin sections.

Since the use of dehydration solvents and embedding resins results in extraction of elemental sulphur in cTEM methods, Vetter (1985) suggested the use of cryotechniques. This author proposed, after glutaraldehyde fixation, a quick-freezing of the specimen, cryoprotected in 30% (v/v) glycerol, in liquid Freon-22 followed by freeze-etching. Impressive improvements in the preservation of very labile structures can be obtained by carrying out freeze-substitution, and embedding and polymerization exclusively at low temperatures. Biological materials prepared under these optimum conditions can be used for both morphological and microanalytical investigations (Edelman, 1990). To date, high-pressure freezing is the only method capable of instantly immobilizing *in situ* diffusible components in thick biological specimens (200 μm) without any cryoprotection (Shimoni and Müller, 1997). The biological specimens are physically fixed in a vitrified state. The components of such a vitrified tissue remain embedded in their native milieu.

As the success of microanalytical experiments strongly depends on sample preparation (Leapman and Andrews, 1991), we have performed all cryotechniques including high-pressure freezing, followed by a freeze-substitution and cryo-embedding in Lowicryl K4M at –20°C before sectioning to preserve the sulphur in the cells before cryo-EFTEM microanalysis.

The purpose of the present study was to determine the location and chemical form under which these putative sulphur reserves were stored and mobilized. As demonstrated in this study, use of all cryotechniques combined with cryo-EFTEM microanalysis represent a powerful tool for the investigation of sulphur localization and microbial metabolic interactions in thioautotrophic symbiotic relationships.

Results

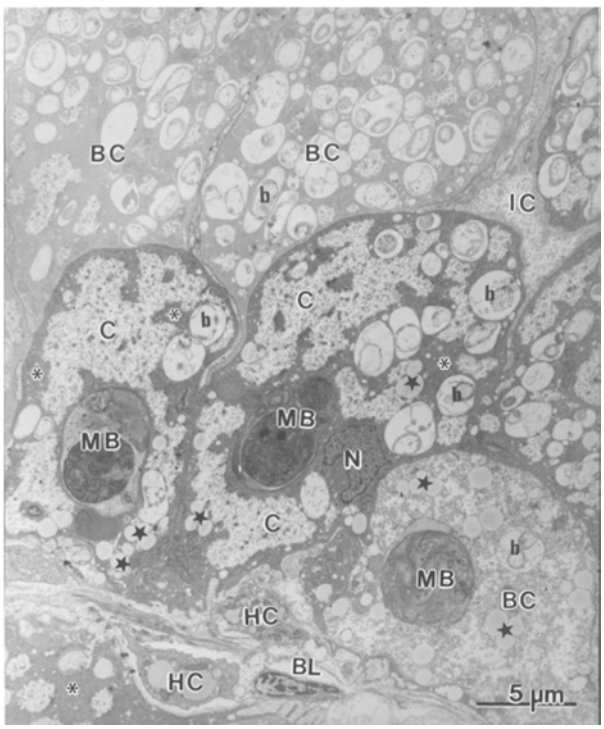
Bacteriocyte ultrastructure by cTEM

As described previously (Frenkiel et al., 1996), bacteriocytes represent the major cell type of the lateral zone of the gill filament. Bacteriocytes are characterized by a patchy cytoplasm with electron-dense haemoglobin-containing zones and electron-lucent areas (Figure 1). Various inclusions can be observed in bacteriocytes with a typical pattern from apical to basal pole. Envacuolated bacterial symbionts occupy most of the apical pole, while in the basal part they become scarce. The cytosol contains almost lysosome-like bodies characterized by membrane whorls and empty small vesicles usually located near the nucleus (Figure 1).

Morphological aspects after the use of cryotechniques

Cryotechniques coupled with the EFTEM observation (Figure 2A) permitted the visualization of the bacteriocyte's basal pole. In this case, the observation is made at zero-loss energy, enhancing the contrast of the unstained ultrathin section. Numerous bacteria in enlarged periplasmic spaces are visible. Two dense vesicles were observed: a round one (1.4 μm in diameter) and an elongated one (1.4 × 0.3 μm). The last one corresponded to a deformation of the resin under the electron beam due to the vicinity of a hole.

Figure 1 | Ultrastructure of *L. pectinata* bacteriocyte
The cytosol of the bacteriocytes usually presents a patchy aspect with dark haemoglobin-containing areas (asterisks) and clear granular areas (C). The envacuolated bacterial symbionts (b), appearing small and badly preserved by conventional fixation process, are almost located in the upper part of the bacteriocyte while the basal part contains a nucleus (N), large lysosome-like microbodies (MB) and small electron-lucent vesicles (stars). BC, bacteriocytes; IC, intercalary cells; HC, haemocytes; BL, blood lacuna.



Chemical composition of vesicles

Elemental mapping with ESI uses energy-filtered images at an element-specific energy loss from which a background image is subtracted (three-window method, Figure 2B). Thus the ESI method was performed in order to image the location of the sulphur in the bacteriocyte's basal pole. Figure 2(B) illustrates the presence of sulphur (S-distribution) in the electron-dense vesicles from the basal part of the bacteriocytes. When the S-distribution image was superimposed on to the HCI (high-contrast image) recorded at 250 eV (Mixmap image, Figure 2C), we observed that sulphur was localized inside the vesicle. At this energy loss, the contrast is inversed: a hole in the resin appeared black instead of white (compare with Figure 2A). The S localization is not distributed homogenously in the vesicle.

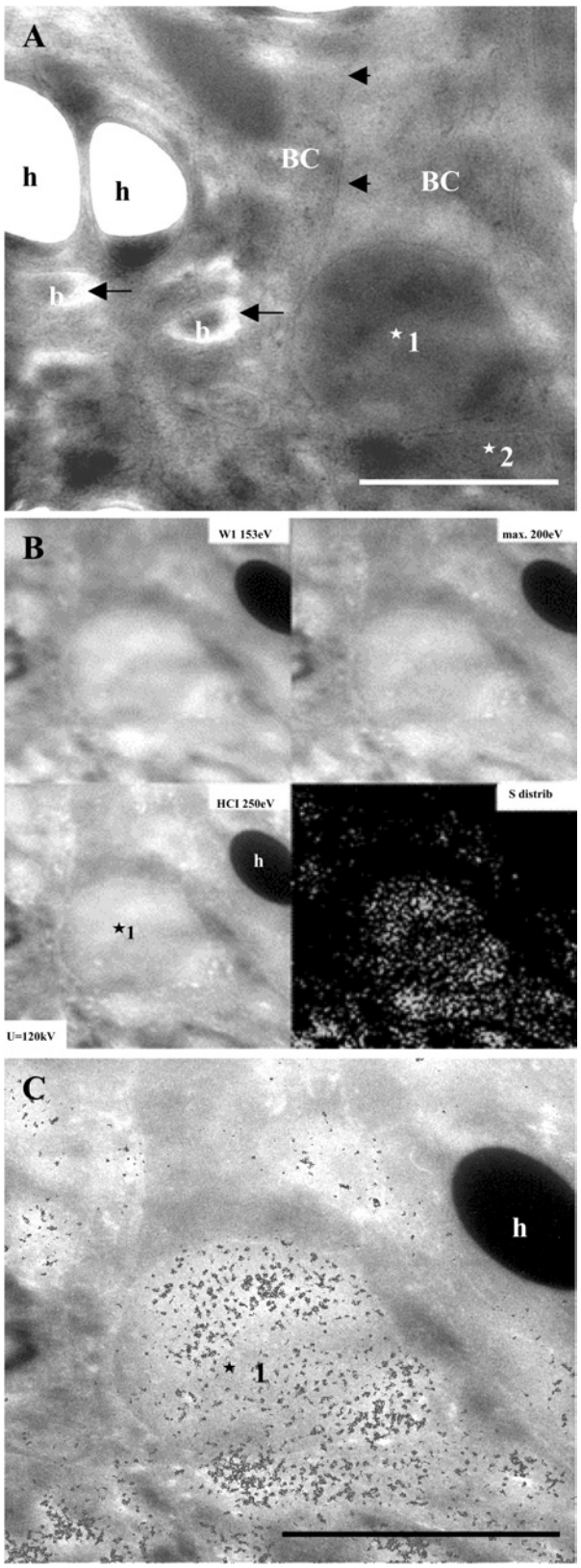
PEELS records the intensity as a function of energy loss from selected regions. The PEELS method allowed us to confirm the presence of sulphur in the dense vesicle (Figure 3C) with a specific sulphur spectrum (edge = 160 eV) obtained after background subtraction. The reference spectrum shown in Figure 3(A) corresponded to a reference specimen of elemental sulphur S. Only the profile of the spectrum obtained with the reference S is similar to the profile of the spectrum obtained with the sulphur contained in the dense vesicle. The other spectral profiles (Figure 3B) obtained from different redox states of sulphur (sulphate, sulphite) do not fit with the S spectrum of the dense vesicle.

Discussion

Most research on thioautotrophic symbioses has been conducted on hydrothermal vent systems, where these associations were first described in the early 1980s (Cavanaugh et al., 1981; Felbeck et al., 1981). Physiological studies, which represent an important tool but are difficult to perform due to these species pressure requirements, are generally limited to carbon transfer using carbon labelled isotopes (Distel and Felbeck, 1988; Herry et al., 1989) or bacterial metabolism pathway from bacterial purified fractions (Hentschel and Felbeck, 1993; Hentschel et al., 1993, 1996). These methods yield no data on sulphur metabolism in animals. Recently, a couple of studies attempted to investigate sulphur metabolism in bivalves (Arndt et al., 2001; Duplessis et al., 2004).

The shallow water bivalve *L. pectinata* is known to possess small sulphur-containing vesicles near the basal pole of the bacteriocyte cytoplasm as well as sulphur periplasmic vesicles in the bacterial symbionts (Frenkiel et al., 1996). The cryotechniques we used, i.e. high-pressure freezing followed by freeze-substitution and cryo-embedding, allowed us to observe and characterize *in situ* the content of the dense vesicles observed at the basal pole of *L. pectinata* bacteriocytes. Furthermore, the patchy aspect of the cytoplasm obtained from chemically preserved samples (Frenkiel et al., 1996) is not observed with such cryotechniques (results not shown). Thus the patchy aspect, with clear and dark areas representing cytoplasmic localization of intracellular haemoglobin,

Figure 2 | EFTEM observations of the bacteriocytes



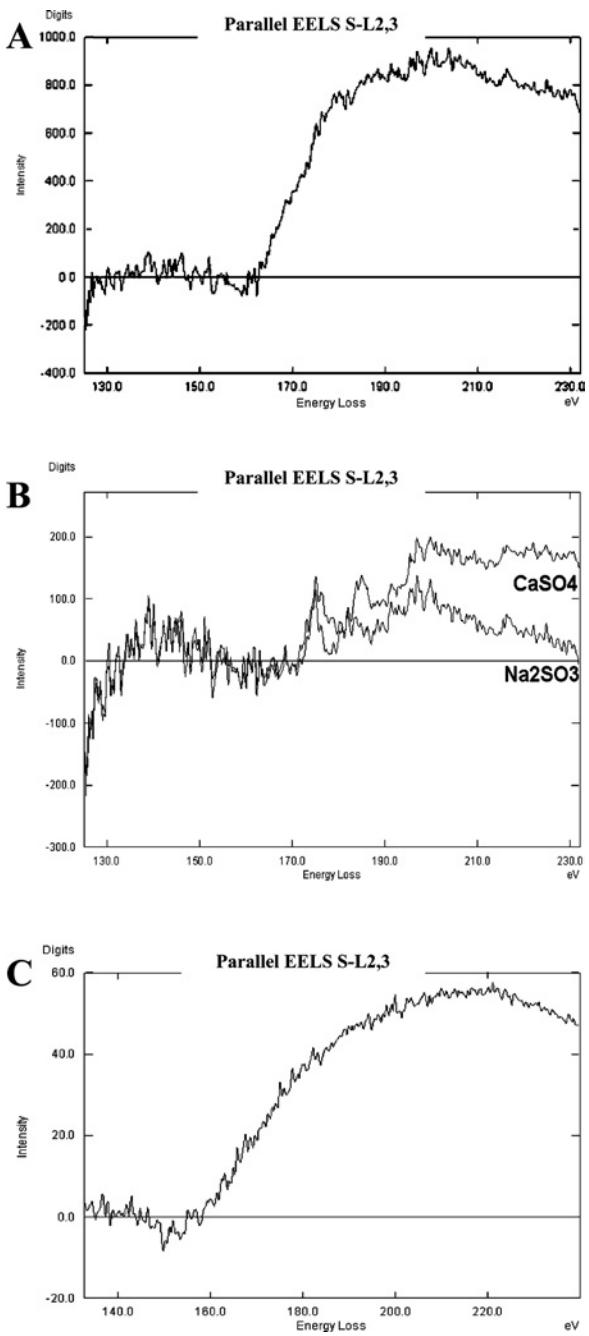
(**A**) Zero-loss filtered image of an unstained ultrathin section showing the basal poles of two bacteriocytes (BC) separated by a lateral membrane (arrowheads). The bacteriocyte on the left contains numerous bacteria (b) individually enclosed in host vacuoles (arrows). The bacteriocyte on the right shows two electron-dense vesicles (stars): a spherical one (star 1, 1.4 μm in diameter) and an elongated one (star 2, 1.4 μm × 0.3 μm). The form of vesicle 2 is due to the resin deformation at the vicinity of a hole. Two white holes (h) are visible on the left. Scale bar, 1 μm. (**B**) ESI method. For the three-window acquisition method, only two images are shown: one image at the maximum energy of sulphur (Max 200 eV) and one image before (W1 153 eV) the sulphur edge (160 eV). A topographic reference image is recorded at 250 eV (HCl 250 eV). At this energy, the contrast is inversed: a hole (h) in the ultrathin section appears in black. The result is shown in the S-distribution image at the basal pole of BC2. The sulphur map shows that the electron-dense vesicles contain sulphur (star). (**C**) Mixmap image: the net sulphur map is superimposed on the HCl. The sulphur distribution is not homogeneous in the vesicle. Scale bar, 1 μm.

would constitute an artifact created by the fixation process rather than a characteristic of this species. Further analysis will allow us to confirm this hypothesis.

These vesicles were normally described as electron lucent after cTEM treatment (Frenkiel et al., 1996; Liberge et al., 2001). Using purified fractions of such vesicles, Liberge et al. (2001) have shown that the latter could contain sulphur according to EDX analyses. Nevertheless, their results did not include any more information about the chemical speciation of such sulphur products. Vetter (1985) got similar results studying the sulphur content of three other clams (*Lucinoma annulata*, *Calyptogena elongata* and *Lucina floridana*). All of these previously published studies have used symbiont and/or organelle fractions purified from host tissues without chemical fixation before EDX analysis (Vetter, 1985; Liberge et al., 2001) or Raman spectroscopic analysis (Pasteris et al., 2001). Such techniques did not, however, permit the localization and characterization of the sulphur product *in situ*, restricting the possibility of chemical modification or loss of such compounds. This is the first study to permit *in situ* cellular observations of a thioautotrophic symbiosis model as well as one that allows the analysis of sulphur.

Figure 3 | The PEELS method

(**A**) Reference S spectrum obtained after background subtraction from a specimen test of elemental sulphur S. The edge is at 160 eV and the maximum at 200 eV. (**B**) Reference spectra obtained from sulphite (Na_2SO_3) and sulphate ($CaSO_4$) powders. Their profiles are different from the S profile and show two small peaks between 170 and 190 eV. (**C**) A specific sulphur spectrum (S-$L_{2,3}$ edge around 160 eV) is obtained from the spherical electron-dense vesicle. The profile of this spectrum is to compare with the reference S spectrum in (**A**).



Radiation damage is a major factor influencing the detection limit in many types of specimen (Egerton, 1986b). Even if the analysed element is not removed by the electron beam, mass loss of matrix elements may interfere with the measurement of elemental ratios. It is now well established that radiation damage effects are greatly reduced by TEM at low temperatures (Cosslett, 1978; Luther, 1992; Shillito et al., 1997). This is also the first time that studies of symbiotic associations, thin sections and liquid-nitrogen cooling are combined. The liquid-nitrogen-cooled sections were observed to be stable, with very few planar movements that occurred in ambient temperature sections viewed at very low dose rates. Hall and Gupta (1974) point out that cooling may greatly reduce the loss of mass ('chemical' damage) in sections of biological tissue. The observed effect must be due to secondary phenomena involving the local environment in the specimen. Among the factors that are mentioned as being of possible importance in reducing secondary reactions are the 'cage' effect in solids in which neighbouring molecules restrict the diffusion of reactive species and a decrease in the probability of occurrence of some of the secondary chemical reactions at low temperatures (Glaeser and Taylor, 1978). In conclusion, liquid nitrogen should be used in energy-loss microanalysis whenever it is necessary to minimize the loss of light elements (Egerton, 1986b).

Element microanalysis with EFTEM can be performed in different ways (Körtje, 1996): ESI and PEELS methods. The ESI method allowed us to image the location of sulphur in the dense vesicles at the bacteriocyte's basal pole. The PEELS method allowed us to discriminate the elemental sulphur type by comparison with different reference spectra (elemental sulphur S and other sulphur compounds) and to specify that these electron-dense vesicles contain elemental sulphur S. Elemental sulphur was already described in free-living sulphur-oxidizing bacteria as well as in marine invertebrate gill endosymbionts (Pasteris et al., 2001). These authors even suggested that the bacterial sulphur vesicles located in the periplasm could be dominated by microcrystalline solid elemental sulphur. A microcrystalline state of the sulphur could represent a stored sulphur compound with high solubility and reactivity that may be important to bacteria in cases of lack of sulphur in the environment. In our case, however, elemental sulphur is

described in the host cytoplasm. The absence of solid sulphur crystals in the vesicles suggested that these vesicles are actually elemental sulphur in the form of liquid-crystalline sulphur (Vetter, 1985) that could serve as an energy stock. Experiments are in progress to determine how such vesicles could evolve during a long starvation period under stress environment without any source of sulphide and/or food.

Cryotechniques such as those described in this paper could represent an important tool for further investigations in other chemosynthetic symbiosis models and especially those involving sulphide-oxidizing bacterial endosymbionts. Direct analyses or techniques developed specifically to preserve the original sulphur product are required to characterize bacterial or host vesicles in various thioautotrophic symbiosis models. Research is in progress in our laboratory to investigate the sulphur metabolism according to host physiology in another bivalve species model, *Codakia orbicularis*. The latter is the only marine invertebrate with chemoautotrophic bacterial endosymbionts for which the environmental transmission mode has unequivocally been demonstrated (Gros et al., 2003b).

Materials and methods

Sample collection

Adult *L. pectinata* specimens were hand-collected in mangrove swamp black mud on the island of Guadeloupe in the French West Indies (Jackson, 1973). Bivalves were kept alive in the laboratory in sterile seawater until cryofixation, which was performed within 3 days of the sample collection.

Fixation and tissue processing

Small pieces of tissues were dissected from freshly delaminated gills and immediately placed in platelets specific for high-pressure freezing (Müller and Moor, 1984). The sample was sandwiched between two aluminium platelets specially adapted for the following freeze-substitution procedure: for high-pressure freezing, the sample 'sandwich' was enclosed in a holder that clamped the two platelets firmly together and then placed in the pressure chamber of the high-pressure freezing machine (HPM 010, Bal-Tec, Balzers, Liechtenstein). In this pressure chamber, two jets of liquid nitrogen under a pressure of 2100 bar were directed from opposite sides on to the sandwiched specimen surface. Immediately following collapse of the pressure, the specimen holder was withdrawn and plunged into liquid nitrogen. The holder was opened and the sample 'sandwich' removed under liquid nitrogen. The frozen specimen was stored in liquid nitrogen for further treatment.

High-pressure freezing was followed by a freeze-substitution in an automat (AFS; Leica, Milton Keynes, U.K.) in acetone (instead of alcohol, in order to minimize loss of sulphur) from –90 to –20°C. The sample was then infiltrated with Lowicryl K4M resin (Polysciences, Biovalley, France) at –20°C. The resin was polymerized during a 48 h UV exposure. For EFTEM microanalysis, the samples were not osmicated in order to avoid scattering artifacts (Bordat et al., 1998). Osmium and/or heavy metals usually used for contrast in classical electron microscopy are not used in EFTEM microanalysis, in order to avoid the appearance of these elements in the element distribution image. Ultrathin sections (50 nm) were obtained with an ultramicrotome (Ultracut R, Leica) using a diamond knife (Diatome, Biel, Switzerland). Ultrathin sections were collected on to uncoated 700-mesh copper grids within 15 s of flotation to reduce contact with water, and were not contrasted for EFTEM.

Gill-tissue samples were chemically fixed as described previously (Frenkiel et al., 1996) for morphological controls before embedding in epoxy resin.

EFTEM analysis

The EFTEM observations were performed using a LEO 912 Omega transmission electron microscope (LEO Electron Optics, Oberkochen, Germany) equipped with a LaB_6 source, and operated at 120 kV. The LEO 912 features an in-column spectrometer (magnetic omega-type electron energy-loss filter) (Egerton, 1986a; Crozier, 1995). In order to reduce drift upon irradiation, the ultrathin sections (50 nm) were laid on an uncoated 700-mesh hexagonal copper grid with thin bars (Agar Scientific, Oxford Instruments, Orsay, France). Parallel electron energy-loss spectra were recorded with a cooled slow-scan CCD (charge-coupled-device) camera (Proscan, Penzing, Germany) equipped with a 1024×1024 pixel-sized chip and operating in a 14-bit mode. Acquisition was accomplished with the ESIvision program (version 3.0 Soft-Imaging software, SIS, Münster, Germany).

The PEELS procedure

To improve the accuracy of the analysis, spectra were acquired from different sulphur reference specimens (elemental sulphur S and sulphur compounds). For S, a fine powder obtained from S crystals (Prolabo, VWR International, France) was deposited on to a thin (5 nm) carbon film. The same procedure was used for the other sulphur compounds: sulphite and sulphate, which are known as transient sulphur intermediates in the energy-producing sulphide-oxidizing pathway in sulphide-oxidizing bacteria (Minic and Hervé, 2004).

In order to minimize beam-irradiation damage and to stabilize the thin film of S powder and the ultrathin section during the PEELS procedure, the recording was made at liquid nitrogen temperature under cryo-EFTEM conditions (–170°C). The specimens were transferred to the cryotransmission electron microscope LEO 912 Omega using the Gatan 626 DH cryotransfer specimen holder (Gatan, Warrendale, PA, U.S.A.) and maintained at –170°C. The Gatan cold stage controller (model 900) was used for temperature control.

The primary magnification was set to ×31 500 in order to select only one bacteriocyte vesicle or sulphur crystal in the area delimited by the entrance aperture of the spectrometer (set at 100 μm). The spectrum magnification was set at ×125. The acquisition time was 1 s and the integration maintained up to 30 s. For the sulphur spectra, a background subtraction was performed according to an exponential law model: $I = A \exp^{-rE}$

(where I is the intensity, E the energy loss and A and r variable parameters for the optimum adjustment; Quintana et al., 1998). At lower energy loss, the power-law formula is less appropriate (Quintana et al., 2001) and more complicated functions may be required (Egerton and Leapman, 1995). After background subtraction, a smoothing operation is made applying a filter-type Envelope to the standard spectra. This filter procedure is useful for spectra recorded with low intensity and serves to reduce the noise in the spectra.

ESI procedure

Due to the microscope's Koehler-type illumination, irradiation can be adjusted. Setting emission current to 6 μA and the condenser system to 0.63 mrad resulted in a dose rate of 2.5×10^3 electrons $\cdot$ nm$^{-2} \cdot$ s^{-1}. Exposure time was 1 s for each image. The entrance aperture of the spectrometer was set at 1.5 mm. The spectrometer slit width was set to 15 eV, and the primary magnification to ×20 000. All images were corrected for the camera offset and gain variations. After averaging 2×2 pixels, the effective pixel size on the resulting 512×512 images was 1.4 nm. For ESI acquisition and for minimizing the radiation damage, we used the three-window method (Jeanguillaume et al., 1978; Reimer et al., 1992).

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References

Abbott, R.T. (1974) American Seashells, Van Nostrand Reinhold, New York

Ardnt, C., Gaill, F. and Felbeck, H. (2001) Anaerobic sulfur metabolism in thioautotrophic symbioses. J. Exp. Biol. **204**, 741–750

Bordat, C., Bouet, O. and Cournot, G. (1998) Calcium distribution in high-pressure frozen bone cells by electron energy loss spectroscopy and electron spectroscopic imaging. Histochem. Cell Biol. **109**, 167–174

Cavanaugh, C.M., Gardiner, S.L., Jones, M.L., Jannasch, H.W. and Waterburry, J.B. (1981) Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila* Jones: possible chemoautotrophic symbionts. Science **213**, 340–342

Cosslett, V.E. (1978) Radiation damage in the high resolution electron microscopy of biological materials: a review. J. Microsc. **113**, 113–129

Crozier, P.A. (1995) Quantitative elemental mapping of materials by energy-filtered imaging. Ultramicroscopy **58**, 157–174

Distel, D.L. and Felbeck, H. (1988) Pathways of inorganic carbon fixation in the endosymbiont-bearing lucinid clam *Lucinoma aequizonata*. Part 2. Analysis of the individual contributions of host and symbiont cells to inorganic carbon assimilation. J. Exp. Zool. **247**, 11–22

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

Edelman, L. (1990) Freeze-substitution and low temperature embedding. In EMAG-MICRO 89, Vol. 2: Biological, Institute of Physics Conference Series (Elder, H.Y. and Goodhew, P.J., eds.), pp. 763–768, Institute of Physics Publishing, Bristol

Egerton, R.F. (1986a) Instrumentation for energy-loss spectroscopy. In Electron Energy-loss Spectroscopy in the Electron Microscope, pp. 27–128, Plenum Press, New York

Egerton, R.F. (1986b) Applications of energy-loss spectroscopy. In Electron Energy-loss Spectroscopy in the Electron Microscope, pp. 291–352, Plenum Press, New York

Egerton, R.F. and Leapman, R.D. (1995) Quantitative electron energy-loss spectroscopy. In Energy-filtering Transmission Electron Microscopy (Reimer, L., ed.), pp. 269–290, Springer-Verlag, Berlin

Felbeck, H., Childress, J.J. and Somero, G.N. (1981) Calvin-Benson cycle and sulphide oxidation enzymes in animals from sulphide-rich habitats. Nature (London) **293**, 291–293

Frenkiel, L., Gros, O. and 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

Glaeser, R.M. and Taylor, K.A. (1978) Radiation damage relative to transmission electron microscopy of biological specimens at low temperature: a review. J. Microsc. **112**, 127–138

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

Gros, O., Liberge, M. and 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. and 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

Hall, T.A. and Gupta, B.L. (1974) Beam-induced loss of organic mass under electron-microprobe conditions. J. Microsc. **100**, 177–188

Hentschel, U. and Felbeck, H. (1993) Nitrate respiration in the hydrothermal vent tubeworm *Riftia pachyptila*. Nature (London) **366**, 338–340

Hentschel, U., Cary, S.C. and Felbeck, H. (1993) Nitrate respiration in chemoautotrophic symbionts of the bivalve *Lucinoma aequizonata*. Mar. Ecol. Prog. Ser. **94**, 35–41

Hentschel, U., Hand, S.C. and Felbeck, H. (1996) The contribution of nitrate respiration to the energy budget of the symbiont-containing clam *Lucinoma aequizonata*: a calorimetric study. J. Exp. Zool. **199**, 427–433

Herry, A., Diouris, M. and 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

Jackson, J.B.C. (1973) The ecology of Molluscs of *Thalassia* communities, Jamaica, West Indies. I: distribution, environmental physiology, and ecology of common shallow-water species. Bull. Mar. Sci. **23**, 313–350

Jeanguillaume, C., Tribbia, P. and 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

Körtje, K.-H. (1996) ESI contrast analysis: a new approach for element analysis with energy-filtering transmission electron microscopy (EFTEM). J. Microsc. **184**, 175–184

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

Leapman, R.D. and Andrews, S.B. (1991) Biological electron energy loss spectroscopy: the present and the future. Microsc. Microanal. Microstruct. **2**, 387–394

Lechaire, J.-P., Frébourg, G., Lopez-Garcia, C. and Gaill, F. (2000) Sulphur localization in Riftia endosymbionts. 4th Congress of the French Society of Electron Microscopy, Toulouse, 4–8 September 2000. Biol. Cell. **92**, 383

Lechaire, J.-P., Shillito, B., Frébourg, G. and 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

Liberge, M., Gros, O. and Frenkiel, L. (2001) Lysosomes and sulfide-oxidizing bodies in the bacteriocytes of *Lucina pectinata*, a cytochemical and microanalysis approach. Mar. Biol. **139**, 401–409

Luther, P.K. (1992) Sample shrinkage and radiation damage. In Electron Tomography: Three-Dimensional Imaging with the Transmission Electron Microscope (Frank, J., ed.), pp. 39–60, Plenum Press, New York

Minic, Z. and Hervé, G. (2004) Biochemical and enzymological aspects of the symbiosis between the deep-sea tubeworm *Riftia pachyptila* and its bacterial endosymbiont. Eur. J. Biochem. **271**, 3093–3102

Müller, M. and Moor, H. (1984) Cryofixation of thick specimens by high pressure freezing. In the Science of Biological Specimen Preparation (Revel, J.P., Barnard, T. and Haggis, G.H., eds.), pp. 131–138, SEM Inc., AMF O'Hare, IL

Pasteris, J.D., Freeman, J.J., Goffredi, S.K. and Buck, K.R. (2001) Raman spectroscopic and laser scanning confocal microscopic analysis of sulfur in living sulfur-precipitating marine bacteria. Chem. Geol. **180**, 3–18

Quintana, C., Marco, S. and Bonnet, N. (1998) Optimization of phosphorus maps from EFTEM images by using different background models and Multivariate Statistical Analysis (MSA) in chromatin. Electron Microsc. **1**, 711–712

Quintana, C., Lechaire, J.-P., Bonnet, N., Risco, C. and Carrascosa, J.L. (2001) Elemental maps from EFTEM images using two different background subtraction models. Microsc. Res. Tech. **53**, 147–156

Reid, R.G.B. (1990) Evolutionary implications of sulphide-oxidizing symbioses in bivalves. In The Bivalvia – Proceedings of a Memorial Symposium in Honour of Sir C.M. Yonge, Edinburgh, 1986 (Morton, B., ed.), pp. 127–140, University Press, Hong-Kong

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

Shillito, B., Koster, A., Walz, J. and Baumeister, W. (1997) Electron tomographic reconstruction of plastic-embedded organelles involved in the chitin secretion process. Biol. Cell **88**, 5–13

Shimoni, E. and Müller, M. (1997) On optimizing high-pressure freezing: from heat transfer theory to a new microbiopsy device. J. Microsc. **192**, 236–247

Truchet, M., Ballan-Dufrançais, C., Jeantet, A.-Y., Lechaire, J.-P. and Gaill, F. (1998) Le trophosome de *Riftia pachyptila* et *Tevnia jerichonana* (Vestimentifera): bioaccumulations métalliques et métabolisme du soufre. Cah. Biol. Mar. **39**, 129–141

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

Warmke, G.L. and Abbott, R.T. (1962) Caribbean Seashells, Livingston Publishing, Narberth, PA

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