Biometals (2009) 22:723–732
DOI 10.1007/s10534-009-9216-5

Ultrastructural evidence for iron accumulation within the tube of Vestimentiferan *Ridgeia piscesae*

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Received: 12 October 2008 / Accepted: 26 January 2009 / Published online: 8 February 2009

Abstract This study reports on the accumulation of iron within the tube wall of the deep sea vent macro invertebrate Vestimentiferan *Ridgeia piscesae* collected from Juan de Fuca ridge. Combining an array of approaches including environmental scanning electron microscope (ESEM), electron probe microanalysis (EPMA), X-ray microanalysis (EDS) and transmission electron microscope (TEM), we provide evidences for the influence of prokaryotic organisms on the accumulation of metals on and within the tube wall. Two types of iron-rich minerals such as iron oxides and framboidal pyrites are identified within or on the tube wall. Our results reveal the presence of prokaryotic organism is apparently responsible for the early accumulation of iron-rich minerals in the tube wall. The implications of the biomineralisation of iron in tube wall at hydrothermal vents are discussed.

Keywords Hydrothermal vent · Ridgeia piscesae · Tube wall · Prokaryotic organism · Iron · Biomineralisation

Introduction

One of the most metal-rich biotopes known is hydrothermal vent environment where geothermally heated water reacts with its host rock forming fluids that nourish a diverse array of geothermally dependent microorganisms. Recently, several studies focused on the interactions between organisms and the solid components of rocks and minerals in hydrothermal environment (Maginn et al. 2002; Edwards et al. 2005; Suzuki et al. 2006). The main motivation for these studies derives from the possibility that organisms in hydrothermal vent environment might have a large but underappreciated role in biomineralization, rock alteration and biogeochemical cycles of C, Fe, S and other elements (Holden and Adams 2003; Emerson and Moyer 2002; Edwards et al. 2005; Reysenbach and Shock 2002; Kelley et al. 2002).

It has been demonstrated that vent macro biota can influence metal accumulation and mineralization at modern hydrothermal sites (Juniper and Tebo 1995; Cook and Stakes 1995; Maginn et al. 2002). The process of metal accumulation and mineralization has been inferred from observations of a range of replacement mineralogy and the role of microorganisms has been debated (Juniper and Tebo 1995; Maginn et al. 2002). However, early metal accumulation processes controlled or induced by vent macro biota are particularly poorly understood so far, and are of importance in understanding the interaction between organism and environment, as well as the preservation of fossils in ancient vent deposits (Zbinden et al. 2004; Kádár et al. 2005, 2006a, b, c, 2008; Juniper and Tebo 1995; Cook and Stakes 1995).

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Ridgeia piscesae is the only species of vestimentiferan tubeworm on the Juan de Fuca Ridge and can exhibit significant phenotypic plasticity ranging from long and skinny to short and fat morphotypes (Southward et al. 1995; Gaill and Hunt 1986). The overall length of tubes potentially ranges from c. 18 mm to c. 1.5 m and can be gold, brown, grey, translucent white, or black in color (Jones 1985; Southward et al. 1995). The known endosymbionts of *R. piscesae* and other vestimentiferans found at hydrothermal vents are within the subdivision *ε -Proteobacteria* (Lane et al. 1985; Feldman et al. 1997). These gutless worms rely on their endosymbionts located in the trophosome to get nutrition. The tubeworms supply hydrogen sulfide to the endosymbionts to produce energy and then the latter use the energy to convert carbon dioxide into organic carbon to feed the worms (Urcuyo et al. 2003). These tubeworms are covered with chitinous tube, which supports their bodies and protects them from the surrounding extreme environment (Cary et al. 1993). The chitinous tube is the only part of tubeworm which can be preserved as a geological fossil (Cook and Stakes 1995; Little et al. 2004). Although it is thought that microorganisms within the tubes might play a potential role in the mineralization of tube wall, there are few ultrastructural evidences to clarify it (Juniper and Tebo 1995; Maginn et al. 2002; Cook and Stakes 1995; Tunnicliffe and Fontaine 1987).

This study examines the possible reasons for Fe accumulation in the tube wall of Vestimentiferan *Ridgeia piscesae*. Applying ESEM, EPMA, TEM and EDX approach, evidences are provided to the presence of a large amount of prokaryotic organisms with potential role in Fe accumulation in the tube. This study enhances our understanding of the metal-microorganism-macro invertebrate interaction in hydrothermal vent environment, as well as formation mechanisms of vent fauna fossil throughout the geological record.

Materials and methods

Sampling

A joint China and US submersible dive expedition by R/V Atlantis and submersible Alvin was undertaken on the Juan de Fuca (JDF) ridge in the northeastern Pacific Ocean from 13 August to 3 September in 2005. Vestimentiferan *Ridgeia piscesae* samples were sampled from a diffuse flow environment at Main Endeavour hydrothermal field during Alvin dive 4135 (Fig. 1). Recovered *Ridgeia piscesae* samples were fixed with 4% glutaraldehyde and 8% formaldehyde, respectively and stored at 2°C. Transmission electron microscope, environmental scanning electron microscope, electron probe micro-analysis and backscattering micro-analysis were used to examine the samples.

Analysis methods

An FEI Quant 400 environmental scanning electron microscope was used to examine the samples under a low vacuum mode. The advantage of ESEM over traditional scanning and transmission electron microscopy (SEM and TEM) is that SEM and TEM require a substantial amount of sample preparation, potentially altering the architecture and composition of specimens, whereas ESEM does not. A genesis energy dispersive spectrometer (EDS) was used to assess the elemental composition of the precipitates. Analyses were performed at an accelerating voltage of 20 keV for 100 s, live time.

For transmission electron microscope (TEM) analysis, tube samples were sliced into small pieces, and fixed with 2% glutaraldehyde for 2 h, and then washed by PBS (DEFINE TERM PBS) three times, once every 10 min. The samples were then subsequently fixed

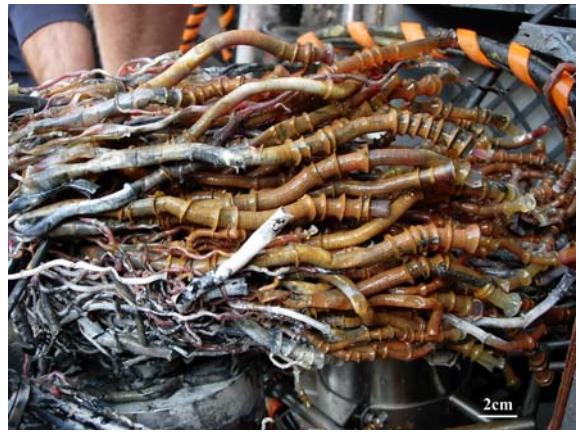

Fig. 1 Tubeworm samples (*Ridgeia piscesae*) collected in Main Endeavour hydrothermal field

with osmium tetroxide at 4°C for 2 h. A stepped dehydration process of increasing ethanol concentration treatments (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%, 10 min each) followed. After this procedure, the samples were transferred through epichlorohydrin and embedded in epoxy resin overnight. Ultrathin sections were prepared with a microtome equipped with a diamond knife and were placed on copper grids for TEM imaging. Finally, sections were stained with uranyl acetate and lead citrate to improve the contrast of cellular material. TEM was performed using an FEI Tecnai 12 transmission electron microscope and a JEM-2010HR transmission electron microscope at 100 kV of accelerating voltage. Energy dispersive X-ray spectroscopy (EDS) was employed at 100 kV on an Oxford INCA Energy TEM X-ray energy dispersive spectrometer.

JEOL JXA-8100 electron microprobe (EPMA) operating at 20 kV was used to determine the content of elements in the tubeworm wall and to get the back-scattered electron (BSR) image. The range of wavelengths used was 0.087–9.3 nm and the detection limit is estimated to be 100 ppm. Beam spot size and current are 1 μm and $2 \times 10^{-}8$ A, respectively.

Results

Occurrence and morphology of prokaryotic organisms

Prokaryotic organisms were observed on the inner tube wall surface, on the outer tube wall surface and within the tube wall. However, the number of microorganisms in the outer tube wall surface was notably less than that on the inner tube wall surface and within the tube wall. Microorganisms usually unevenly coated the inner tube wall surface or within the tube, and formed distinct layers within the tube wall itself. The morphology of these microorganisms was filamentous (Figs. 2, 3). These filamentous microorganisms, with a diameter of 0.1 μm or 0.3–0.5 μm, were apparently different in morphology from symbiotic microorganisms, which occur in the trophosome of the tubeworms (Cavanaugh et al. 1981). Most of microorganisms were enveloped by the metal precipitates, presumable Fe oxides. Some microorganisms were partly mineralized, while some microorganisms were completely mineralized. Numerous microorganisms were degraded on the inner tube wall surfaces and within

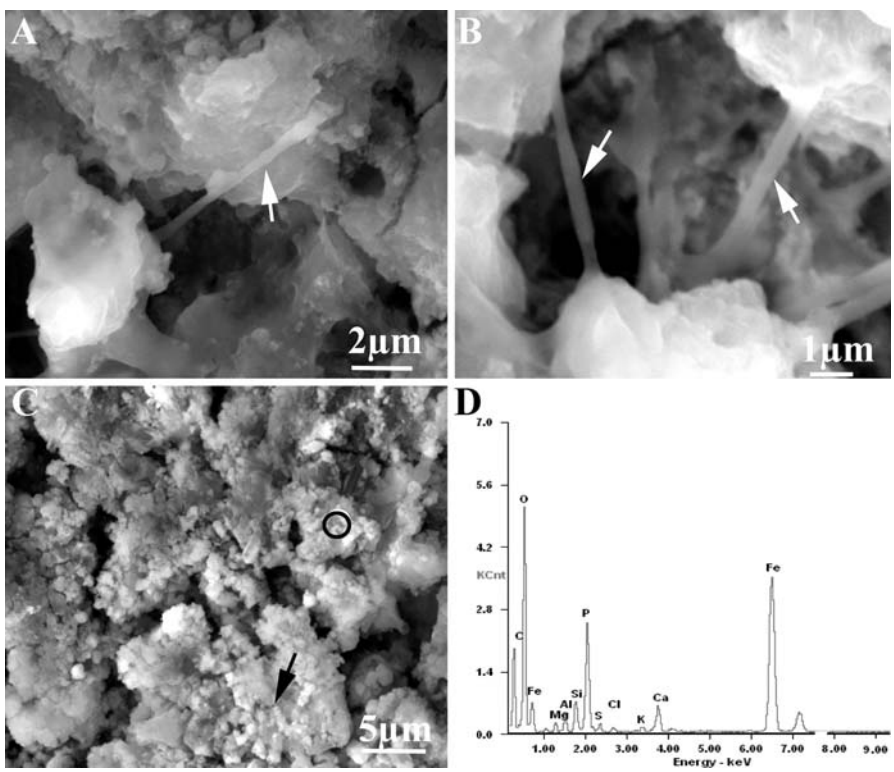

Fig. 2 **a**, **b** Environmental scanning electron microscope (ESEM) images of tubeworms show filamentous microorganisms (*white arrows*) occur in the interspace layer of the tube wall. **c** Numerous Fe oxides (*black arrow*) form on the outer tube wall surface. **d** The energy spectrum analysis of the outer tube wall surface (*black cycle*) in **c**. The contents of Fe, P, Ca and O are higher, indicating that Fe occurs as Fe oxides

the tube wall. Nanometer particles, probably products of microbial degradation, were observed on the inner tube wall and within the tube wall.

Fe-rich minerals

Two types of Fe-rich minerals, Fe oxides and Fe sulphides, were identified to be formed within the tube wall or on the tube wall. SEM results revealed a large amount of Fe oxide grains with the diameter of several micrometers to dozens of micrometers aggregated on the outer tube wall surface. EDS analysis showed these aggregates were rich in Fe and O as well as a minor amount of Ca, P, and Si, indicating that Fe possibly occurred as Fe oxide. EPMA results also revealed that Fe accumulating within the tube wall occurred as Fe oxide. In addition, Fe sulphide such as framboidal pyrite, which was raspberry-shaped spherical aggregates, 5–6 μm in diameter, composed of assemblages of tiny crystallites of 0.5–1 μm, was also occasionally observed on the inner tube wall surface (Fig. 4). No framboidal pyrite was identified within the tube wall or on the outer tube wall surface.

Intra-tube localization of Fe

In order to better understand the intra-tube localization of metallic elements and mechanism of their incorporation within the tube, semi-thin sections were prepared for electron microprobe and observed using back scattered imaging. Electron lucent areas indicate the heavy elements, which were identified to mainly

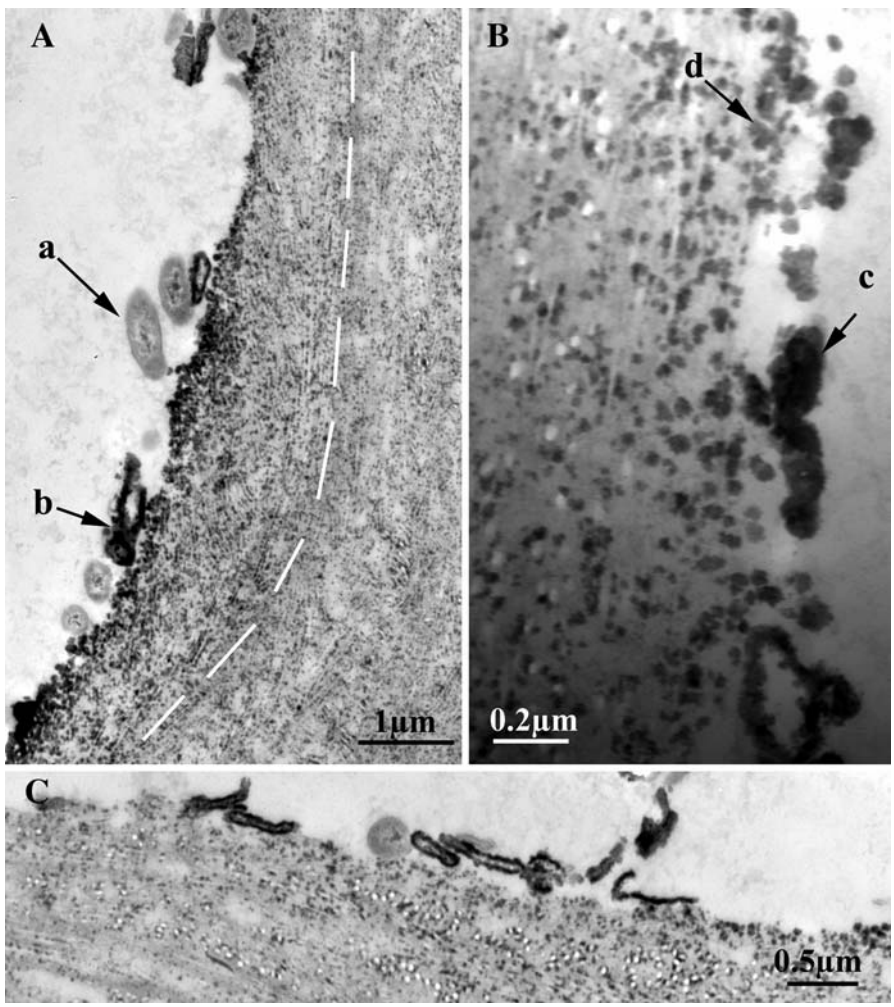

Fig. 3 **A–C** Transmission electron microscope (TEM) image of tube walls shows that microorganisms exist on the tube wall and form microbial layers parallel to the surface of tube wall. Some microorganisms do not undergo mineralization (**a**), while some are partly mineralized (**b**) and completely mineralized (**c**). Nanometer particles (**d**), probably products of microbial degradation, are observed on the inner tube wall and within the tube wall

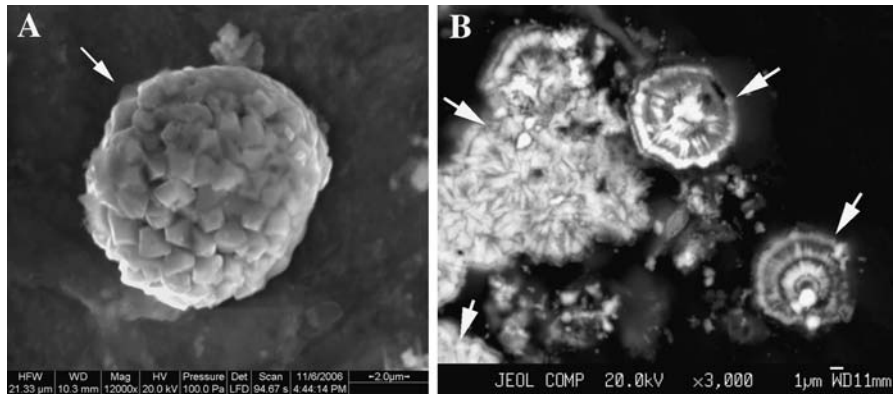

Fig. 4 Framboidal pyrites are observed on the surface of internal tube wall. **a** ESEM image. **b** BSE (back-scattered electron) image

consist of Fe through scanning for elemental composition. Two types of heavy metal distribution were showed in electron backscattering images and can be summarized as follows: (1) Electron lucent areas within the tube wall were parallel to the tube wall surface and exhibit a laminated structure. (2) Electron lucent degree was generally enhanced from inside to outside across the tube wall, which showed that the accumulation of Fe increased gradually as the same trend (Fig. 5). Black spots in Fig. 5 were inferred to be due to the presence of microbial cell moulds, which were formed by the bacterial degradation and mineralization (Verati et al. 1999; Maginn et al. 2002). The chemical element analysis from electronic probe showed Fe contents had a good positive correlation with Ca, P and Si, but had a negative correlation with S (Fig. 6).

Discussion

It has been shown that numerous microorganism-rich horizons are present on the surface and within the internal layer of the *Ridgeia piscesae* dwelling tubes in this study. TEM microphotographs with EDS revealed that the microorganisms in the tubes were partially or totally enveloped by Fe oxide, which indicating that Fe mineralization commonly happened on the cell of these microorganisms (Fig. 7). Major possible mechanisms for introducing Fe oxide

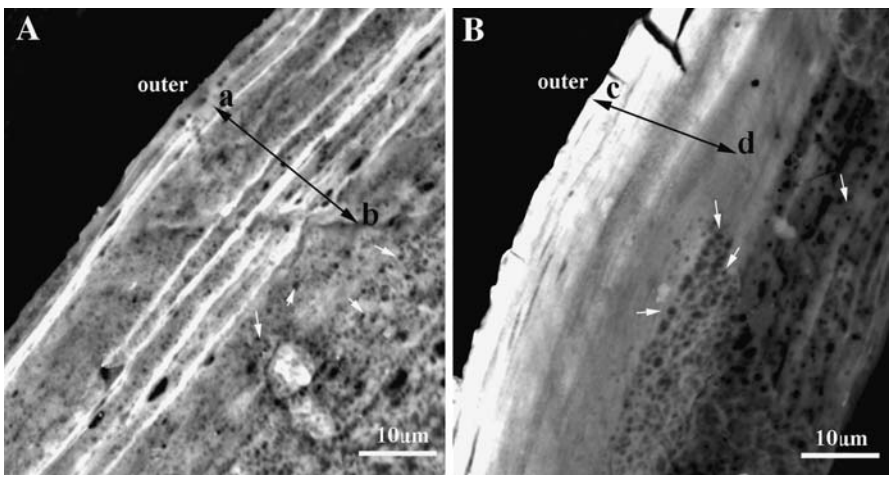

Fig. 5 BSE (back-scattered electron) images of tube walls. **a** Electron lucent areas within the tube wall are parallel to the tube wall surface and exhibit a laminated structure. **b** Electron lucent degree is generally enhanced from inside to outside across the tube wall. *Black spots* (*white arrows*) are inferred to be due to the presence of microbial cell moulds. Similar holes have been described from a thin oxidized veneer on the surface of smoker chimney from the Pito Seamount on the Easter Island microplateand and from the tube wall of *Alvinella pompejana*

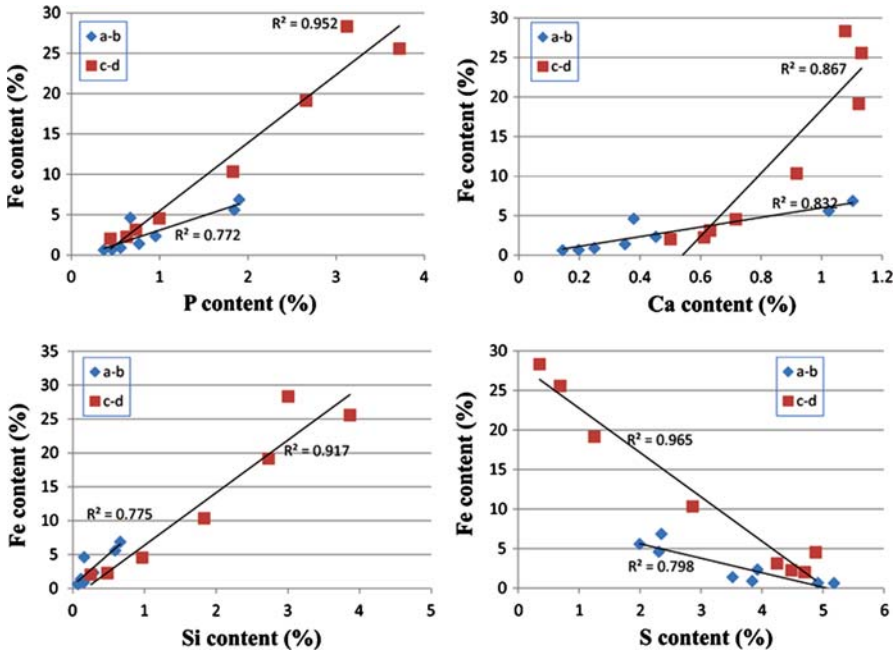

Fig. 6 Chemical element analysis of EPMA to line *ab* and *cd* in Fig. 5 shows Fe contents have a good positive correlation with Ca, P and Si, but have a negative correlation with S

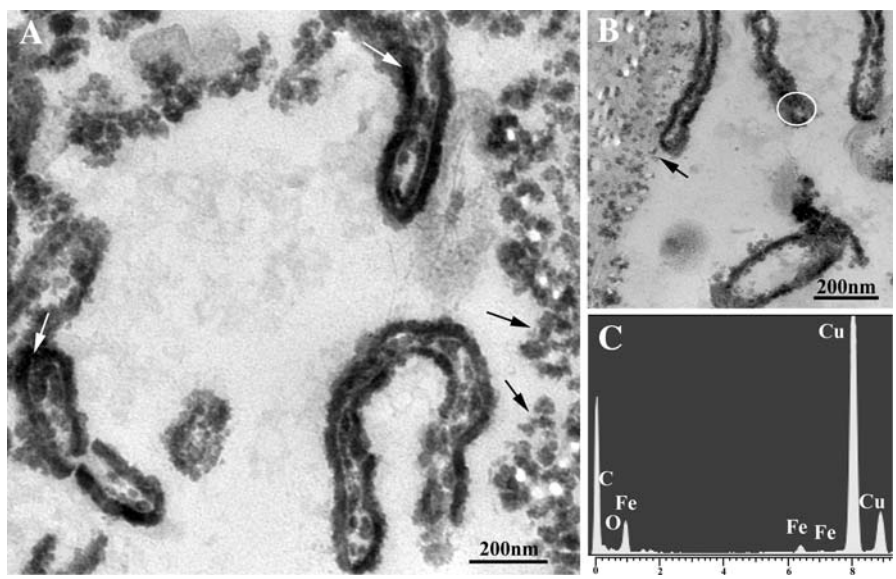

Fig. 7 TEM microphotographs with EDS reveal that the microorganisms in the tube wall are enveloped by Fe oxides. *White arrows* show the extracellular precipitates on the surface of cells. *Black arrows* point to nano particles possibly formed by the degradation of microorganisms. EDS analysis of extracellular precipitates (*white cycle*) in band show higher Fe and O are detected. Cu peak comes from Cu net

particles onto cellular surfaces of these microorganisms have been previously identified (Fortin and Langley 2005; Konhauser 1998). Biotic reactions responsible for the formation of biogenic Fe oxides include the microbial oxidation of Fe(II) to Fe(III) by a wide range of microorganisms under both acidic and neutral pH and oxic and anoxic conditions (Emerson and Weiss 2004; Hallberg and Ferris 2004; Emerson and Moyer 1997). In particular, aerobic and neutrophilic Fe-oxidizing bacteria such as *L. ochracea* and *G. ferruginea* in hydrothermal environment, are considered to lead to the precipitation of biogenic Fe oxides by oxidation of reduced Fe originating from deep-sea vents (Emerson and Moyer 2002; Edwards et al. 2003, 2004). In addition, extracellular Fe oxide formation also occurs as a result of passive reactions, whereby in situ chemical conditions favour the precipitation of the minerals on bacterial cell

walls and extracellular material (Beveridge and Fyfe 1985; Beveridge and Murray 1980; Ehrlich 2002). Microbial cell walls or capsule possess several types of surface reactive sites that can bind soluble Fe and other metal (Fein et al. 2002; Châtellier et al. 2001, 2004). The microorganisms will have metal ions bound to their cell membranes before they become trapped within the tube wall, due to their continual contact with the hot hydrothermal fluid that flows through the dwelling tube. Having metal ions in the cell membrane will induce further nucleation of ions from solution.

Moreover, once the individual microbial cells have become entombed and begin to degrade, the formation of degradation products (polysulphide ions, anionic organic functional groups, etc.) will also effectively enhance the biomineralization potential of the microbial laminae (Ferris et al. 1988). We observed that numerous nano particles were possibly formed by the degradation of microorganisms distributing on the surface and within the tube wall, where the degree of mineralization was obviously higher than that of ambient tube wall (Fig. 3). In some cases, the cells totally degraded and degradation products enveloped the residual holes (Fig. 8). Due to the horizontal distribution of the degradation products of microorganisms, Fe mineralization always occurred in these areas as distinct layers parallel to tube walls as showed in electron back-scattering images (Fig. 5a). This provides a possible explanation for the layered structure of fossils persevered in the geological record.

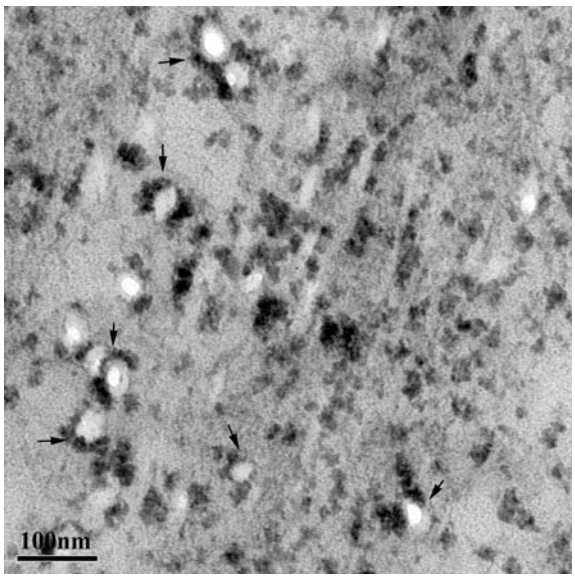

Fig. 8 TEM image shows degradation products of microorganisms distribute within the tube wall. *Black arrows* show that some cells totally degrade and degradation products envelope the residual holes

The degradation of organic matter in the tube wall is also likely to cause the Fe accumulation in the tube wall. With increasing thickness of the tube wall, the bioactivity of external wall organic matter decreases gradually. The organic compounds such as protein and chitosan would be degraded (Gaill and Hunt 1986, 1991). The products of degradation likely play an important role in adsorbing metal ions, which would effectively improve the mineralization potential and accelerate the process of Fe accumulation in the tube wall. The external surface of tube wall was the earliest exudates of internal organs and directly contacted with fluid, so its degree of Fe accumulation were the greatest in this study. The degree of Fe accumulation in the tube wall would show a decreasing trend from the outside to the inside of the tube due to the different degradation degree (Fig. 5b).

In addition to Fe oxides, a small number of framboidal pyrites were observed on the surface of internal tube wall as well (Fig. 4). Framboidal pyrite is very common in reducing sediments such as modern dark muds, or in ancient dark shales and limestones, and even among the anoxic pages of old books with ink containing sulfate. It has been recognized that they have a very close association with decaying flesh or organic matter, or bacterial origin passing through various Fe–S compounds has been commonly proposed (Love et al. 1984; Donald and Southam 1999; Posfai et al. 1998; Garcia-Guinea et al. 1997; Folk 2005). It is possible for hydrothermal solution containing Fe^{2+} and SO_4^{2-} to be fixed as pyrite by the action of sulphate-reducing bacteria (Bottrell and Morton 1992). Another possibility is that hydrothermal fluid directly provides H_2S for the precipitation of framboidal pyrite on the inner tube wall surface. Whatever the sulphide source, bacteria are implicated in the formation of some hydrothermal Fe sulphides by acting as sites for adsorption of Fe ions, by providing a locally reducing environment that favours precipitation of Fe, and by providing a source of elemental S for Fe sulphide precipitation (Jonasson and Walker 1987).

Chemical element analysis also provided information about the sources of elements in tube wall. As we know, P is an important element in organisms. The content of P in internal organs of *Ridgeia piscesae* ranged from 0.14 to 0.41% in this study, which was similar to that in other hydrothermal organisms (such as *Alvinellid polychaetes*). However, the content of P in tube walls was about more than 10 times that in tissues and also showed a decreased trend from outer wall to inner wall in most of samples, indicating that majority of P in tube walls was from ambient hydrothermal environment instead of from organisms. Moreover, the oxidation of H_2S by symbiotic bacteria in the trophosome has been considered to be very significant for the enrichment of S in tubeworms (Cavanaugh et al. 1981; Urcuyo et al. 2003). The content of S in inner wall (4.248–5.179%) was obvious higher than that in outer wall (0.353–2.862%) in this study, indicating that S in the tube wall might originate from the bio-oxidation of symbiotic bacteria in the trophosome, and then enter into inner wall as exudates of internal organs. Thus, the good positive correlation among Fe, P, Ca and Si in the tube wall indicates that these elements are likely accumulated from ambient hydrothermal environment (Fig. 6). However, the negative correlation between Fe and S showed that S had a different source from Fe and probably originated from the metabolism of symbiotic bacteria in the trophosome.

Although there remains a great deal of uncertainty regarding which microorganisms were actually present on or within the tube for the absence of molecular phylogenetic analysis, we indeed observed accumulation of Fe oxides on cellular surfaces of these microorganisms through ultrastructural analysis. Additionally, we suggest that the formation of framboidal pyrite on the tube might be related to microbial activities. The presence of microorganisms apparently is responsible for the early accumulation of Fe on or within the tube through biotic or abiotic passive reactions. The microbial layers effectively provide an initial template for further mineralization within tube wall. This biomineralization process that is active in hydrothermal tubeworms provides a viable mechanism for the preservation of the tube walls with fine-scaled organic structures in the fossil record.

Acknowledgments The authors are indebted to Dr. Maurice Tivey and Prof. Marvin Lilley for their kindly help during the sample collection. We thank the constructive remarks made by two anonymous reviewers. Special thanks also go to all the participants of the joint China and US submersible dive expedition cruise.

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