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Raman spectroscopic and laser scanning confocal microscopic analysis of sulfur in living sulfur-precipitating marine bacteria

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Abstract

Laser Raman microprobe spectroscopy and laser scanning confocal microscopy were used to determine the presence and speciation of sulfur in sulfur-oxidizing, marine bacteria from Monterey Bay, CA. The bacteria studied include: large, filamentous *Thioploca* and *Beggiatoa*, endosymbionts in the vesicomyid clam *Calyptogena kilmeri*, and a filamentous bacterium of undetermined species. All of these bacteria were shown spectroscopically to store elemental sulfur in submicrometer to several micrometer diameter vesicles. More detailed Raman spectroscopic study of the vesicles in *Thioploca* and *Beggiatoa* provided further chemical and structural characterization of the elemental sulfur. The sulfur is bonded in the common, stable S_8 ring configuration and is of an extremely fine-grained microcrystalline form. No additional (organo) sulfur compounds were detected spectroscopically in the vesicles under the low laser powers required to preserve the molecular structure of the sulfur. The present spectroscopic and optical data stand in contrast to reports and inferences of liquid-like elemental sulfur or homogeneous, complex sulfur compounds in other sulfur-oxidizing bacteria. The findings of this study are compatible with a model of sulfur vesicles as dominated by microcrystalline solid elemental sulfur, perhaps embedded in a matrix and/or confining membrane of organic material. The high reactivity and solubility observed in these vesicles is attributed to the extremely fine grain size of the solid elemental sulfur. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Bacteria; Sulfur; Vesicle; Raman spectroscopy; *Thioploca*; *Beggiatoa*

1. Introduction

Chemoautotrophic sulfur-oxidizing bacteria use the energy derived from the oxidation of hydrogen sulfide, bisulfite, or thiosulfate (using either oxygen or nitrate as the electron acceptor) to fix carbon (Schlegel and Bowien, 1989). Sulfur-oxidizing bacteria may exist as free-living large filaments or single cells (e.g., *Thioploca*, *Beggiatoa*, *Thiomargarita*, *Thiobacillus* spp., *Thiomicrospira* spp.) (e.g., Nelson and Hagen, 1995; Fossing et al., 1995; Schulz et al., 1999), endosymbionts in metazoans (e.g., Vetter, 1985; Nelson and Fisher, 1995), or epibionts on protists (Buck et al., 2000) or metazoans (Ott, 1996). All these bacteria share environments characterized by elevated levels of hydrogen sulfide produced by bacterial sulfate reduction in sediments, e.g., hydrothermal vents, cold seeps, or silled anoxic basins. Sulfur-oxidizing bacteria are thought to be important to global biogeochemical processes due to their

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widespread distribution, their (in some cases) high biomass and their coupled role in sulfur and nitrogen cycles (Fossing et al., 1995; Otte et al., 1999). An ultrastructural feature shared by all sulfur-oxidizing bacteria is the presence of membrane-bounded spherical to subspherical vesicles. These vesicles are thought to be sites of sulfur storage (Vetter, 1985; Steudel, 1989).

Numerous questions remain about the nature of the sulfur in these marine bacteria. The bacteria themselves can range down to micrometer scale, and the sulfur vesicles may be even smaller. The most basic question is whether the observed vesicles indeed contain sulfur. Due to the small volumes of material within putative sulfur vesicles, however, it is difficult to distinguish them from other vesicle-like storage structures, such as PHB (poly-β-hydroxybutyrate) bodies. Other properties of sulfur and its compounds, for instance, high vapor pressure and low melting point, hamper analysis. Sulfur is known for its instability under an electron beam, particularly, while under vacuum. These factors make it difficult to document the presence of sulfur in bacteria and, particularly, to identify exactly where the sulfur resides. In addition, questions remain concerning the chemical speciation of the sulfur, i.e., whether it occurs as elemental sulfur (in chains or rings) or as sulfur-bearing (organic) compounds, such as polythionates or organic polysulfides (Steudel, 1989; Prange et al., 1999). Although the diversity of sulfur-oxidizing bacteria is vast (Kelly, 1989; Nelson, 1989), the actual presence and nature of sulfur have been investigated in detail only for a few organisms, notably *Chromatium* (Hageage et al., 1970), *Beggiatoa* (Lawry et al., 1981), purple and green sulfur-oxidizing bacteria from the families Chromatiaceae, Ectothiorhodospiraceae and Chlorobiaceae (Prange et al., 1999), and endosymbionts of one lucinid and one vesicomyid clam species (Vetter, 1985). It currently is assumed that all other sulfur-oxidizing bacteria, both free-living and symbiotic, have similar mechanisms of processing the sulfur generated by chemosynthesis (with the apparent exception of sulfur-filamentous bacteria described by Taylor and Wirsen (1997) and Taylor et al. (1999)).

Techniques, such as transmission electron microscopy (TEM), X-ray diffractometry (XRD), electron microprobe analysis (EMPA) and X-ray absorption near-edge spectroscopy (XANES), have been applied to the sulfur vesicles in some bacteria (Hageage et al., 1970; Nicolson and Schmidt, 1971; Lawry et al., 1981; Vetter, 1985; Prange et al., 1999). TEM can provide excellent documentation of organic and inorganic morphology at the submicrometer level (Fig. 1). However, the technique calls for complex sample preparation in order to maintain the sample's morphology and to permit the necessary thinning of the material (Maier et al., 1990). Unfortunately, elemental sulfur readily dissolves in solvents used during the dehydration typical of tissue processing (e.g., Strohl et al., 1981; Vetter, 1985; this paper, Fig. 1(B), (D)). The sulfur that remains is subject to thermal degradation under the electron beam. TEM photomicrographs of known sulfur-precipitating bacteria, such as *Thioploca* and *Beggiatoa*, typically show minute transparent features that may be interpreted as "sulfur drop-out" regions and smaller electron-dense features that may be interpreted as residual sulfur (Maier et al., 1990; Vetter, 1985; this paper, Fig. 1). For samples in which the sulfur vesicles can be stabilized, selected-area electron diffraction techniques offer the ability to understand any crystalline structures of sulfur in individual vesicles. To our knowledge, no such analyses have been undertaken.

Many of the sample-handling and sample-stability problems of TEM also hinder EMPA of sulfur-bearing vesicles. EMPA has micrometer-scale spatial resolution and can provide qualitative or quantitative analysis of the elemental composition of the vesicles. However, both (1) detection of organic compounds (i.e., recognizing atomically light elements, such as C, H, N and O, in addition to "heavy" S), and (2) distinguishing the presence of organic compounds from the simple physical intergrowth of elemental sulfur with some organic material are very difficult with an electron microprobe. Thus, Lawry et al. (1981) and Vetter (1985) identified by EMPA the dominance of element sulfur in *B. alba* vesicles and in symbionts in the gills of *Lucinoma annulata*, respectively; however, they could not rule out the additional presence of organosulfur compounds.

In contrast to the *chemical* analytical capabilities of EMPA, X-ray diffraction provides an excellent means for documenting the specific crystalline *structure* of a material, thereby allowing for identifica-

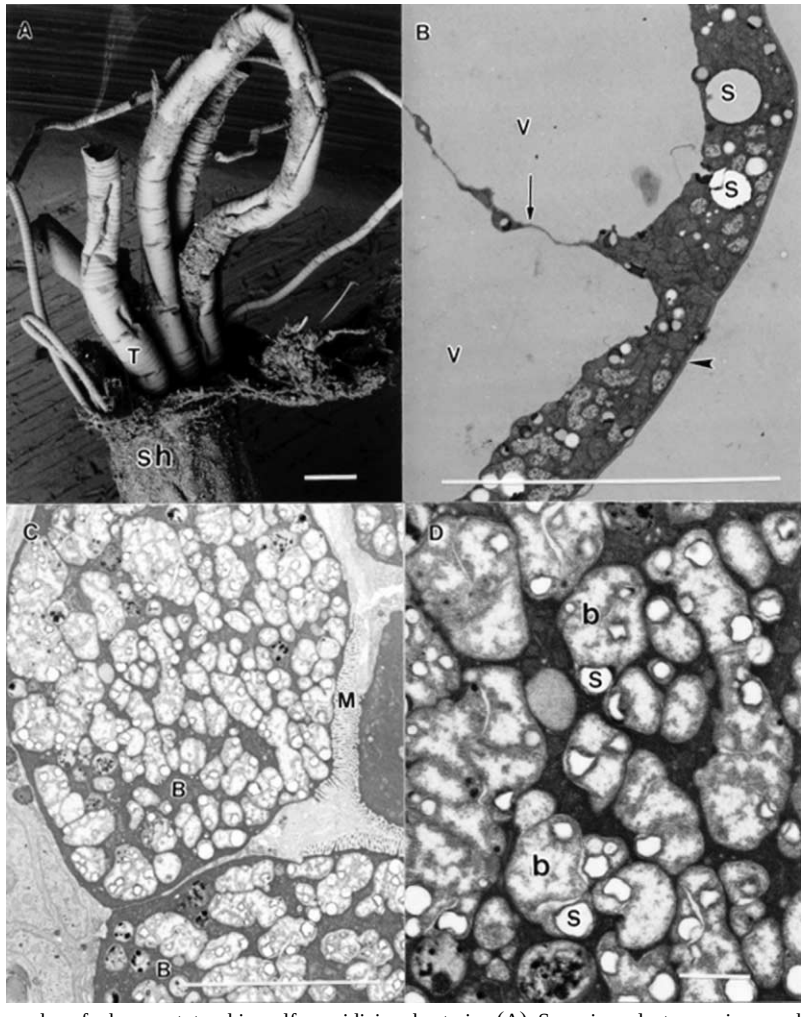

Fig. 1. Electron micrographs of chemoautotrophic sulfur-oxidizing bacteria. (A) Scanning electron micrograph of critical-point-dried *Thioploca* trichomes (T) inside a common polysaccharide sheath (sh). Scale bar = 100 μm. (B) Transmission electron micrograph of a single *Thioploca* trichome illustrating the cell wall (arrowhead), the septum (arrow) separating two adjoining cells, the large vacuoles (V) that comprise most of the cell volume, and the sulfur vesicles (S). Vesicles out of which the sulfur has been dissolved during the dehydration series appear bright white. Scale bar = 10 μm. (C) Transmission electron micrograph of the gill of *C. kilmeri* illustrating the bacteriocytes (B), one of which dominates the field of view, and the peripheral microvilli (M). Scale bar = 10 μm. (D) Transmission electron micrograph of the gill of *C. kilmeri* illustrating the individual bacteria (b) within a bacteriocyte (see (C)). The individual bacteria are complex, rounded, white and gray structures containing bright white empty sulfur vesicles (S). Scale bar = 1 μm.

tion. However, XRD is only sensitive to crystalline species, and the sample is subject to possible X-ray-induced degradation during analysis. In addition, sufficient sample material must be collected to enable detection. This type of "bulk" analysis does not yield direct information on the spatial distribution of sulfur within the bacteria. XANES, as implemented by Prange et al. (1999) through application of synchrotron radiation, not only can provide structural-chemical information on the absorbing atoms, but also can be applied to intact bacteria with their associated sulfur spherules. However, beam intensities on the spherules are very high; interpretation of the spectra in terms of actual sulfur species is complex and typically proceeds by comparison/analogy with spectra of known standards.

To avoid the limitations and difficulties of the above analytical techniques, we selected a combination of optical microscopy, particularly, laser scanning optical confocal microscopy, and laser Raman microprobe analysis to locate and determine the speciation of sulfur vesicles in four types of sulfur-oxidizing bacteria. There are several advantages to these two approaches. The optical confocal microscope, employing a combination of epireflection and autofluorescence imaging, can be used to determine the 3-D spatial distribution of highly refractile (i.e., showing high optical relief) sulfur precipitates in the intact organisms with a spatial resolution of ≤ 0.5 μm. Both techniques require minimal or no sample preparation, which ensures little artificial change in the sulfur compound. Provided that the intensity of the exciting laser beam is kept sufficiently low, these techniques do not alter the speciation or crystallinity of the sulfur-bearing entity.

The laser Raman microprobe can detect spectroscopically, with a spatial resolution of ~ 2 μm, the presence of "sulfur" in the bacteria. Moreover, a Raman spectrum also documents the speciation of the sulfur (i.e., elemental or specific compound), its state of crystallinity (coarsely crystalline, microcrystalline, amorphous/glass), and—if crystalline—its basic crystal structure (e.g., orthorhombic, monoclinic). As demonstrated in the present study, laser Raman microprobe analysis is a powerful technique for the investigation of microbial metabolic reactions and the pathways of element cycling in the crust and biosphere, including diagenetic processes.

2. Application of Raman spectroscopy to microbial sulfur speciation

Individual Raman spectral bands represent the energies of specific vibrational motions among bonded atoms within a compound. As such, the spectrum identifies the compound structurally (e.g., calcite or aragonite), thereby permitting its composition (e.g., $CaCO_3$) to be inferred. Of importance here is the fact that elemental sulfur can take on multiple structures, i.e., polymorphs, most of which are metastable at room temperature (Meyer, 1964). The Raman spectra differ for the individual crystal structures of elemental sulfur (Edwards et al., 1997), such as orthorhombic, which is the stable form at room temperature, and monoclinic, which is metastable at room temperature, but stable over a short temperature interval above about 96 °C (Meyer, 1964). The Raman spectra also reflect differences in the configuration among the atoms within a given molecular structure. As shown in Fig. 2, the Raman spectrum of the common S_8 ring configuration differs from that of the more rare S_6 arrangement. Laser Raman microprobe spectroscopy, therefore, is a very desirable technique to apply to the detection and characterization of sulfur vesicles in bacteria. It offers the high spatial resolution (on the order of micrometers)

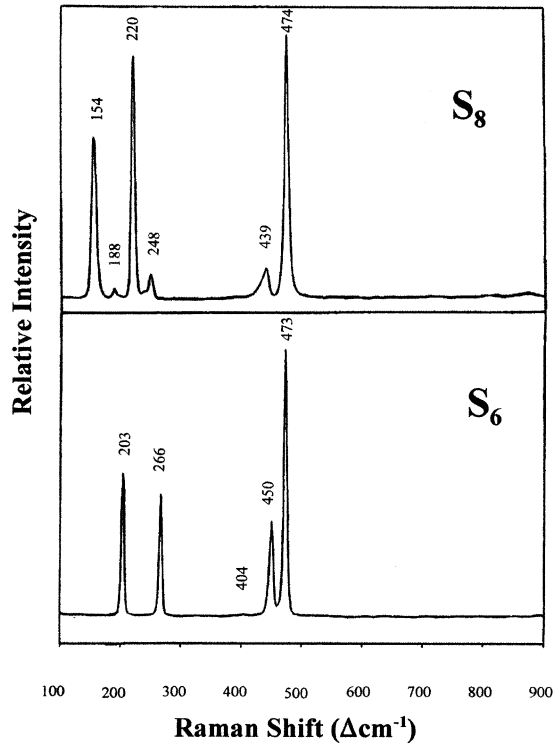

Fig. 2. Raman spectra of two different molecular configurations of elemental sulfur, S_8 and S_6. (Spectrum of S_6 is from unpublished data, courtesy of Dr. Mark E. Jason, Solutia Company, 1997). Raman spectra are plotted as intensity vs. a frequency term called wavenumber (cm^{-1}). It is not the absolute value of the frequency that is plotted, but rather the Raman shift, i.e., the shift of the detected frequency with respect to the frequency of the excitation laser. Those recorded shifts reflect the frequency of vibration of various motions of bonded atoms in a structure. A Raman spectrum, therefore, is a *molecular-structural fingerprint* of a compound or element.

needed for pinpoint analysis, little sample preparation, the ability to see exactly which feature is being analyzed, both chemical and structural information and rapid results (seconds to minutes, depending on the level of structural-chemical detail required).

3. Experimental procedures

3.1. Sampling

All samples for these analyses were collected in Monterey Bay, CA, from the sediment–water interface in water depths between 900 and 1000 m. The collections were carried out with the Monterey Bay Aquarium Research Institute remotely operated vehicle (ROV) *Ventana* supported by the research vessel *Point Lobos*. Two cold seep sites were sampled for the specimens analyzed: Clam Field (36°44.0′N, 122°2.0′W), where clams, *Thioploca* spp. and other bacterial mats were collected and Invert Cliff (36°46.4′N, 122°5.1′W), from which red and yellow *Beggiatoa* spp. were collected. Bacterial samples were collected through the use of corers 7 cm in diameter. Manipulator arms on the ROV inserted these corers into the sediment on the order of 15 cm deep, penetrating through bacterial mats on the sea floor. Clams (*Calyptogena kilmeri*) also were collected with the manipulator arm. At the end of the dive, sediment cores and clams were placed together with cold packs into thermally insulated containers for transport to the laboratory. Upon return to shore (<2 h after completion of the dive), the bacterial mats were separated (in a cold-room at 10 °C) from the sediment using cold, filtered seawater and gentle sieving. The mats were placed into 50-ml centrifuge tubes, filled completely (to exclude air as well as possible) with water that had overlain the sediment. Clams also were maintained in cold, running seawater (for 2 days) until prepared for shipping. Individual containers with bacterial mats and clams were placed into insulated packages together with cold packs and shipped by overnight delivery service to Washington University, where they were placed immediately into a refrigerator at 2–4 °C.

Live organisms were stored in cold sea water until they were removed for microscopic analyses. Free-living bacteria were transferred directly to a microscope slide, immersed in a few drops of sea water, and then covered with a microscope cover slip. The clams were still alive when they were opened to sample the endosymbionts. The gills were dissected and their contents smeared directly onto glass slides, after which they were covered with a few drops of sea water and then protected with a cover slip. The samples were viewed and analyzed within minutes or hours of the slide preparation.

3.2. Analytical techniques

3.2.1. Optical microscopy

The samples on glass slides were viewed in transmitted and reflected light with objectives of magnifications up to 80 × using an Olympus BH-2 polarizing microscope with a tungsten light source. The relatively high refractive index (2.04) of elemental sulfur makes it strongly refractile (high optical relief) and, thus, able to be imaged easily in transmitted light. The high refractive index also accounts for the relatively high reflectivity of sulfur with respect to other transparent materials. The extremely large birefringence of crystalline elemental sulfur (0.29) means that its birefringence can be detected under crossed polarizers even in crystals that are only 0.5 μm thick (Klein and Hurlbut, 1993).

3.2.2. Laser scanning confocal microscopy

Several individual trichomes (filaments) of *Thioploca* were imaged using a Zeiss LSM 410 laser scanning confocal microscope equipped with objectives up to 60 × and employing the 488-nm argon line for recording the epireflection image and the 568-nm argon line for generating autofluorescence images of fluorescence emission wavelengths greater than 590 nm. The two types of images (epireflection and autofluorescence) were recorded simultaneously (Fig. 3). Each dual image was taken in one *XY* plane and multiple *XY* sections were taken throughout the height of a trichome at 0.5-μm *Z* intervals. The individual *XY* images then were reassembled computationally to construct various three-dimensional views.

3.2.3. Laser Raman microprobe (LRM)

Two laser Raman microprobes were used for analyses. Both instruments consist of a spectrometer

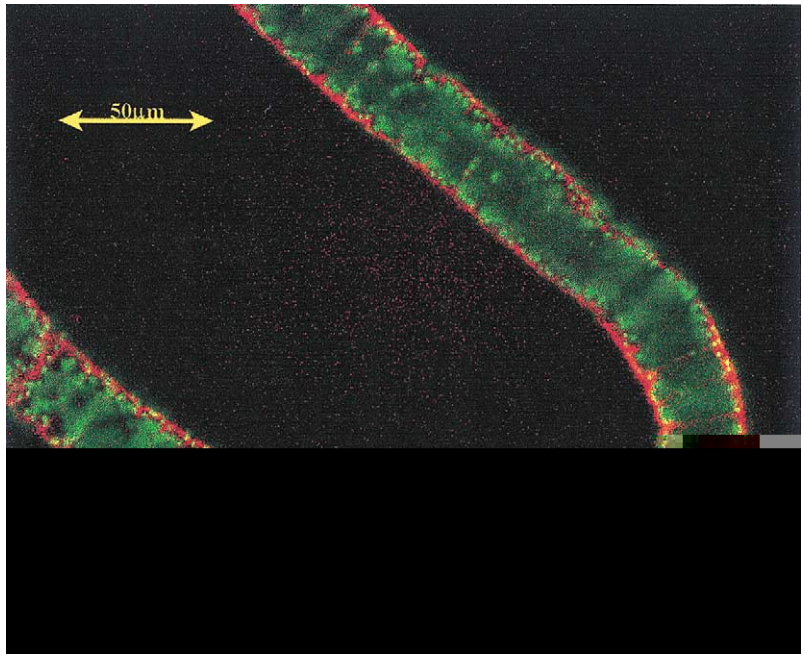

Fig. 3. Laser scanning confocal micrograph of a multicellular *Thioploca* trichome. Created by overlaying autofluorescence and epireflection confocal images. The fluorescence image, shown in red, is dominated by organic material of the thick cell walls and thin septa (finely dotted lines). The epireflection image, shown in green, is dominated by the relatively reflective elemental sulfur; where the sulfur (green) is fully enclosed in the cell wall (red), the sulfur appears yellow in this image. Micrograph shows that most of the cell is a fluid-filled vacuole and that sulfur vesicles are confined to cell walls and septa.

that is optically coupled to an Olympus research-grade microscope, which can be operated in transmission or reflection mode to view the sample and to focus the laser beam onto or into the sample. The laser beam spot is about 1 μm in diameter at its tightest focus, but broadens several fold as it penetrates the cover glass and enters the sample. Most analyses were done with a model S-3000 manufactured by Instruments SA. This is a multichannel Raman system with a triple spectrometer and employing an intensified photodiode array detector. The minimum recordable bandwidth (i.e., full width at half maximum) with our typical instrumental configuration was 11–12 cm^{-1} in the spectral region of interest. Analyses were made using the 514.5 nm green line of an argon-ion laser for excitation. An 80 × -ultra-long working-distance objective was used to deliver the laser beam to the sample. Spatial resolution was on the order of a few micrometers, and spectral resolution was approximately 7 cm^{-1}.

The other Raman microprobe that was used is a HoloLab 5000, manufactured by Kaiser Optical Systems. This is a spectrograph system employing a transmissive, volume holographic grating and a CCD detector with 1024 × 256 pixels. A 632.8-nm HeNe laser and a 50 × Olympus objective were used to deliver the laser to the sample. Spatial resolution was on the order of a few micrometers. In the region of interest, the spectral resolution was about 5 cm^{-1} and the minimum recordable bandwidth was 4–5 cm^{-1}.

The effect on the Raman spectra of the melting and quenching of sulfur was determined by varying the power of the laser beam in the Raman microprobe. For recording Raman spectra of sulfur in vesicles of 1–2 μm in diameter, laser power at the sample had to be maintained at or below 1.5 mW. For both instruments, laser power in excess of 3 mW at the sample led to immediate change (peak broadening) in the Raman spectra and in the morphology

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