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In Situ Analysis of Sulfur Species in Sulfur Globules Produced from Thiosulfate by *Thermoanaerobacter sulfurigignens* and *Thermoanaerobacterium thermosulfurigenes*▽†

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The *Firmicutes Thermoanaerobacter sulfurigignens* and *Thermoanaerobacterium thermosulfurigenes* convert thiosulfate, forming sulfur globules inside and outside cells. X-ray absorption near-edge structure analysis revealed that the sulfur consisted mainly of sulfur chains with organic end groups similar to sulfur formed in purple sulfur bacteria, suggesting the possibility that the process of sulfur globule formation by bacteria is an ancient feature.

The geochemical cycling of sulfur species plays an important role in energy generation and supports microbial communities in sulfur- and sulfide-rich environments (6, 25). Thiosulfate ($S_2O_3^{2-}$) is known to be one of the important products of biological oxidation or chemical oxidation of sulfides and plays a key role in the sulfur cycle in sediments (4, 11, 12). Thiosulfate can be oxidized to sulfate, disproportionated to sulfate and sulfide, reduced to sulfide under anaerobic conditions, and regarded as a widely used electron donor and acceptor for many microorganisms (1, 13). Recently, the thermophilic organism *Thermoanaerobacter sulfurigignens*, isolated from SO_2-emitting and sulfur-accumulating volcanic White Island (New Zealand), was described to convert up to 1 M thiosulfate to elemental sulfur and to tolerate sulfite up to 90 mM (15). The conversion of thiosulfate only to elemental sulfur instead to sulfide is no longer a taxonomic discriminating feature for distinguishing the *Firmicutes* genera *Thermoanaerobacterium* and *Thermoanaerobacter* (14, 18).

The formation of elemental sulfur (S^0) is ecologically important for several groups of microorganisms (7), and the chemical nature of the formed sulfur has been analyzed for a variety of bacteria (9, 10, 17, 19, 20, 21, 29, 30). However, most of the studies of the sulfur analysis focused on mesophilic phototrophic sulfur bacteria (5, 23). Thus, little information was available on the properties of sulfur globules produced in thermophilic chemoheterotrophic anaerobic *Firmicutes*.

Biologically produced sulfur can be stored inside and/or outside a cell, and the sulfur stored inside a cell exhibits properties different from that of sulfur stored outside a cell (15, 21). Previous works showed that the chemical natures of the sulfur and the surface properties of sulfur globules vary and differ in different groups of bacteria (20, 21). For example, sulfur in bacterial sulfur globules is liquid and rather amorphous compared to that in pure elemental sulfur (10) and shows low density and hydrophilicity (9, 17, 28, 29). So far, it has not been unequivocally demonstrated whether the sulfur produced by members of the thermophilic anaerobic *Firmicutes* is formed inside, outside, or both inside and outside cells and, subsequently, whether its location has an effect on the chemical structure and sulfur differentiation of the sulfur globules.

Both *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T and *Thermoanaerobacterium thermosulfurigenes* 4B^T were cultured heterotrophically in the presence of various concentrations of thiosulfate (between 10 and 500 mM) as a possible electron acceptor. The culture medium contained 0.5% (wt/vol) glucose as a carbon source supplemented with 0.1% (wt/vol) yeast extract, and the pH was adjusted to 6.5 (15). Thiosulfate solution was prepared anaerobically using the modified Hungate technique (16) and sterilized separately. The sulfur globules were produced as early as the mid-exponential growth phase but mainly at the end of the exponential growth phase and during the stationary phase. Since the appearance of the majority of extracellular sulfur globules was correlated with a decline in cell numbers, it was assumed that the extracellular sulfur globules were due mainly to cell lysis (Fig. 1A). However, scanning electron micrographs revealed that small sulfur globules were also produced outside the cells when they were grown with thiosulfate, which, however, were absent when the cells were grown without thiosulfate (Fig. 1B and C). The morphology of sulfur globules was identified by transmission electron microscopy by examining ultrathin sections (Fig. 2A to E). Energy-dispersive X-ray analysis confirmed that both types of globules contained sulfur (Fig. 2E). The intracellular

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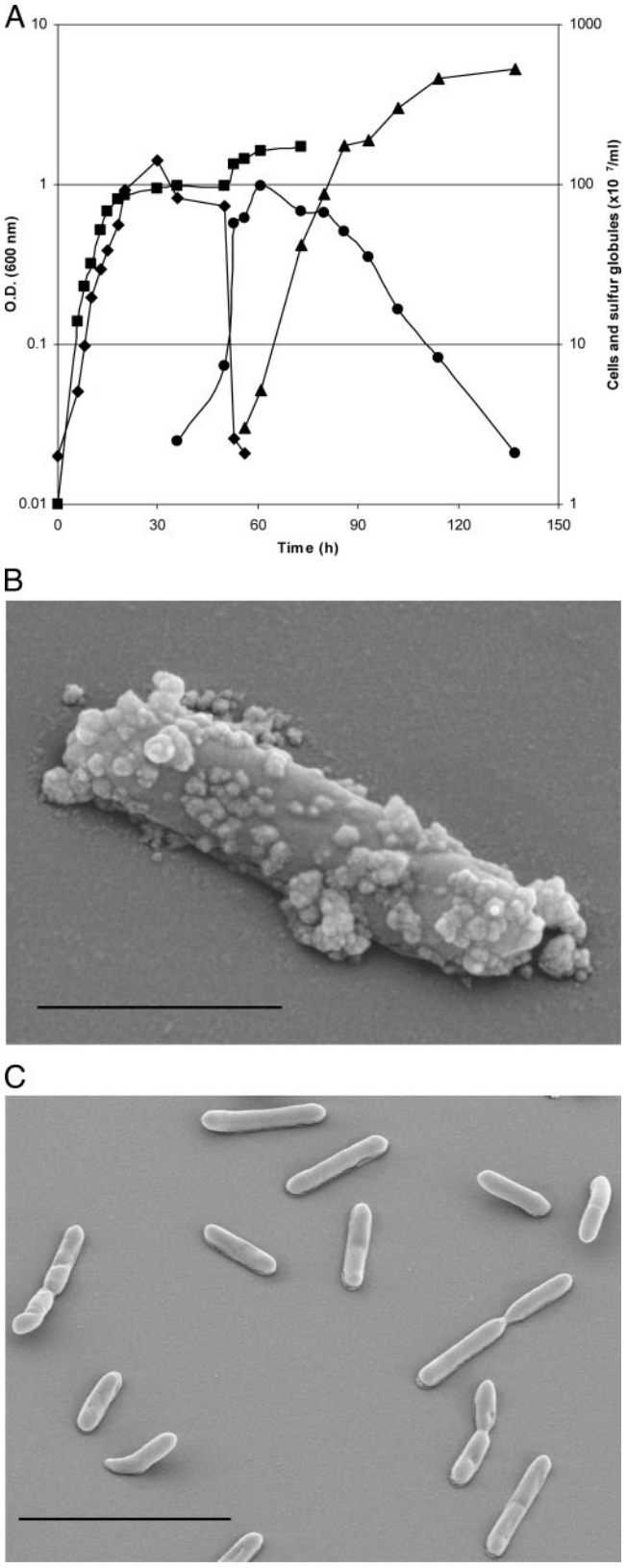

FIG. 1. Production of sulfur globules by *T. sulfurigignens* JW/SL-NZ826^T. (A) Measurement of the release of sulfur globules into the medium during a growth cycle. ■, optical density (O.D.) of the culture; ◆, cells without internal sulfur globules; ●, cells with internal sulfur globules; ▲, free sulfur globules in the medium. (B and C) Electron micrographs of a bacterium grown in the presence of 50 mM thiosulfate producing sulfur globules outside the cell (B) and of cells grown in the absence of thiosulfate (C). Bars, 1 μm (B) and 5 μm (C).

sulfur globules appeared to be enclosed by a membrane (not further characterized), as revealed by transmission electron microscopy (Fig. 2B). However, the observed structure could also be due to the mixtures of organic sulfanes with hydrophilic end groups (30).

X-ray absorption near-edge structure (XANES) spectroscopy, a powerful nondestructive tool for probing sulfur species in biological samples in situ (3, 20, 21, 23, 24), was used to analyze the sulfur species of globules formed in species of two genera from the phylum *Firmicutes*, *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T and *Thermoanaerobacterium thermosulfurigenes* 4B^T (14, 15, 27). XANES spectroscopy allowed us to use directly cultured bacteria in liquid media and to determine the valence of excited S atoms, the lengths of sulfur chains, and the type of the chemical bond in the second coordination shell of the excited sulfur atom (e.g., C-C single, double, or triple bonds) (24). Sulfur globules of *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T and *Thermoanaerobacterium thermosulfurigenes* 4B^T were prepared according to the procedure of Schmidt et al. (28) and Brune (2), with modifications. The cells were disintegrated by ultrasonication, the sulfur globules were separated by centrifugation, and the supernatant was removed with a pipette for analysis. Samples were prepared for XANES spectroscopy using the modified procedure of Prange et al. (21) (see the supplemental material for details). For the quantitative analysis of the spectra obtained from both bacteria, a wide variety of reference compounds of different sulfur species (representatives for a given class of an atomic environment) were measured and their respective relevances were tested (Fig. 3A). XANES spectra were recorded at the DCM beamline at the CAMD, Baton Rouge, LA, and analyzed quantitatively as described previously (8). The errors of the percentages of contribution of sulfur species (Table 1) were estimated to be less than ±10% (absolute value) (21, 22). The S K-edge XANES spectra with their accompanying WinXAS fits (26) for *Thermoanaerobacter sulfurigignens* (Fig. 3B, spectra a and b) and *Thermoanaerobacterium thermosulfurigenes* (Fig. 3B, spectra c and d) revealed that the sulfur in cells of these bacteria consisted mainly of sulfur chains (~80% had the structure R-S$_n$-R, and ~15 to 18% of minor substances had the sulfur structure C-S-H/C-S-S-C) (Table 1). Only small amounts of highly oxidized sulfur species such as sulfoxides and sulfonates were detected. The other tested reference species (S_8 rings, sulfate, thiosulfate), whose presence could be expected among the sulfur species since, e.g., S_8 ring sulfur is thermodynamically the most stable form of S^0 at ambient temperatures (20), were completely absent as determined with the fitting routine except in a residual thiosulfate-containing *T. thermosulfurigenes* culture sample taken 2 days after thiosulfate addition. The data presented here indicated that the sulfur species produced by *T. sulfurigignens* and *T. thermosulfurigenes* were comparable. The sulfur existed mainly as sulfur chains with, presumably, an additional

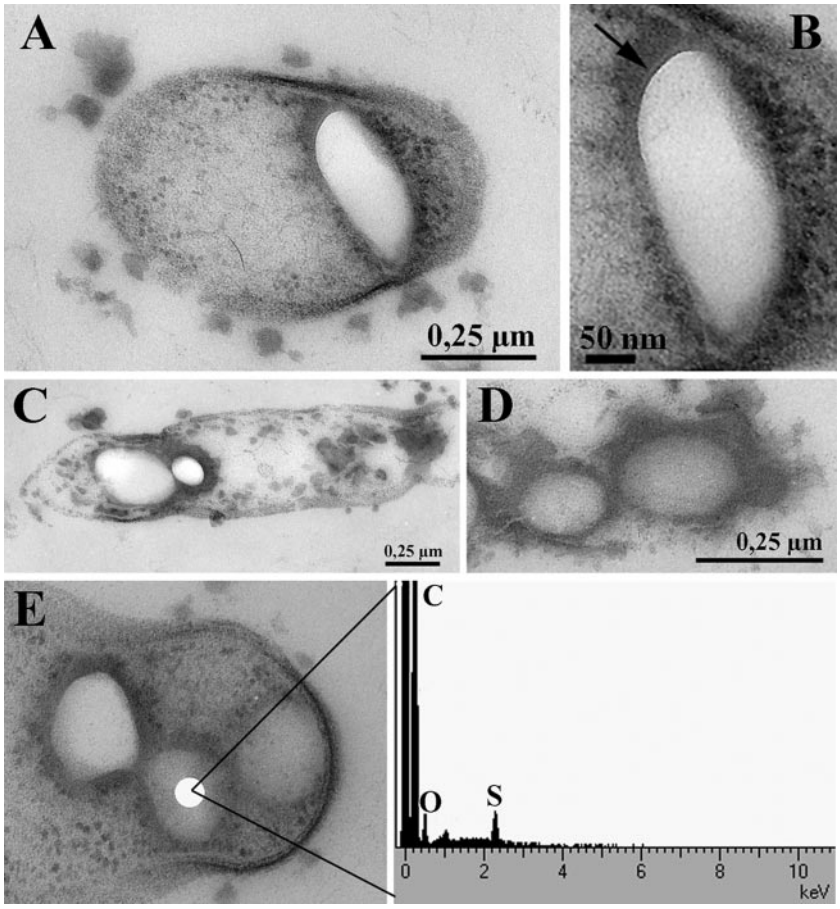

FIG. 2. Electron micrographs of *Thermoanaerobacter sulfurigignens* JW/SL-NZ826^T showing a sulfur globule inside the cell (A), the membrane around the sulfur globule (B), a cell containing sulfur globules in the process of lysis (C), sulfur globules in the culture from lysed cells (D), and the results of energy dispersive X-ray analysis indicating that the globules inside the cell contain sulfur (E).

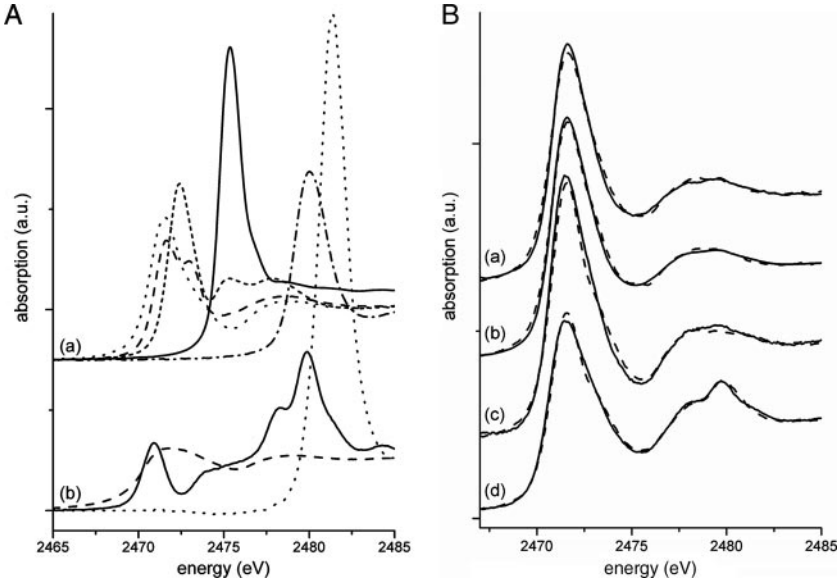

FIG. 3. Sulfur K-edge XANES spectra. (A) Spectra of the reference compounds which were used for fitting the bacterial spectrum. (a) Polymeric sulfur (dotted line) (cf. Table 1), oxidized glutathione (line of long dashes), reduced glutathione (line of short dashes), dimethyl sulfoxide (solid line), and cysteic acid (line of dots and dashes); (b) S_8 rings (line of long dashes), sodium thiosulfate (solid line), and zinc sulfate (dotted line). a.u., arbitrary units. (B) Spectra of *Thermoanaerobacter sulfurigignens* (solid lines) and accompanying WinXAS fits (dashed lines). (a) *T. sulfurigignens* fed with 50 mM thiosulfate; (b) *T. sulfurigignens* fed with 500 mM thiosulfate; (c) *Thermoanaerobacterium thermosulfurigenes* fed with thiosulfate (4 days after thiosulfate addition); (d) *T. thermosulfurigenes* fed with thiosulfate (2 days after thiosulfate addition).

TABLE 1. Results of fitting the sulfur K-edge XANES spectra of *Thermoanaerobacterium thermosulfurigenes* and *Thermoanaerobacter sulfurigignens* to the sum of the reference spectra

Sample	% Contributed by sulfur species[a]:					
	Thiosulfate	Glutathione (reduced)	Glutathione (oxidized)	Polymeric sulfur	Dimethyl sulfoxide	Cysteic acid
Thermoanaerobacter sulfurigignens fed 50 mM thiosulfate	—	10	5	83	—	1
Thermoanaerobacter sulfurigignens fed 500 mM thiosulfate	—	8	10	79	1.6	1.3
Thermoanaerobacterium thermosulfurigenes (2 days after thiosulfate addition)	10	13	15	50	—	—
Thermoanaerobacterium thermosulfurigenes (4 days after thiosulfate addition)	—	3	24	71	—	—

[a] With percentages of contribution by different sulfur species in the sulfur analysis, errors were less than ±10%. —, the contribution was <1%.

"organic compound" present in the form of mono- or bis-organyl sulfanes.

Based on previous results, it was assumed that different sulfur species in the sulfur globules reflect the different metabolic properties and ecological niches (21). Sulfur chains in anaerobically grown cells differed from those of aerobically grown cells (21). Interestingly, the sulfur species determined in this study, formed from thiosulfate reduction by anaerobic thermophilic *Firmicutes*, are very similar to those formed from the oxidation of sulfide by mesophilic phototrophic sulfur bacteria (21, 22). The phototrophic sulfur bacteria (phyla *Proteobacteria* and *Chlorobi*) are regarded as evolutionarily quite distant from the family *Thermoanaerobacteriaceae* of the phylum *Firmicutes*. The atmosphere of early Earth was sulfur rich, so anoxygenic photosynthesis using reduced sulfur compounds as electron donors prevailed among purple sulfur bacteria (e.g., *Chromatiaceae* and *Ectothiorhodospiraceae*) and green sulfur bacteria (*Chlorobiaceae*). In this context, the hypothesis by Urich et al. (31) should be noted; based on the crystal structure of the sulfur oxygenase reductase from the thermoacidophilic archaeon "*Aquifex aeolicus*" and theoretical considerations, only linear sulfur and not cyclic sulfur species can serve as a substrate for this enzyme. Furthermore, Franz et al. (8) found evidence that *Allochromatium vinosum* uses only the sulfur chain fraction of elemental sulfur and is unable to take up *cyclo*-octasulfur.

The results observed in this study imply that the formation of sulfur globules in the dissimilatory uses of various sulfur compounds may be more widespread among bacteria than previously thought and existed before the distant phyla (i.e., *Proteobacteria*, *Chlorobi*, and *Firmicutes*) diverged. At this time, the possibility of horizontal gene transfer cannot be excluded; thus, a thorough comparative analysis awaits the availability of the corresponding genome sequences.

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