Arch. Microbiol. 99, 323—329 (1974)

Scanning Electron Microscopy of Thiobacilli Grown on Colloïdal Sulfur

J. Baldensperger*, L. J. Guarraia**, and W. J. Humphreys***

Department of Microbiology, University of Georgia, Athens, Georgia

Received March 6, 1974

Abstract. Autotrophic sulfur oxidizers, *Thiobacillus thiooxidans* and *T. denitrificans*, grown on colloïdal sulfur, were examined by scanning electron microscopy.

Physical attachement of *Thiobacillus denitrificans* to colloïdal sulfur was evidenced, and a metabolite precipitated by lead acetate suggesting that sulfides are an intermediate in the oxidation of elemental sulfur by the organism.

Key words: Thiobacillus — Sulfur Oxidation — Scanning Electron Microscopy.

Schaeffer *et al.* (1963) using the transmission electron microscope (E.M.) and a carbon replica technique have shown the erosion of a sulfur crystal by the aerobic sulfur oxidizer *Thiobacillus thiooxidans*. Scanning electron microscopy (S.E.M.) has been used by Roth (1971) to study the colonial morphology of various bacteria grown on solid media. In the present study an attempt was made to visualize the relationship between some thiobacilli and colloïdal sulfur.

Materials and Methods

Autotrophic aerobic and anaerobic thiobacilli were isolated from the estuary surrounding Sapelo Island, Georgia, USA. Stock cultures of aerobic thiobacilli were maintained on Starkey's medium (Postgate, 1966), while anaerobic thiobacilli were cultivated in Baalsrud's medium (Baalsrud and Baalsrud, 1954).

For SEM examination, both aerobic and anaerobic thiobacilli were grown on membrane filters coated with colloïdal sulfur, prepared by acidification of a 10% (w/v) sodium thiosulfate solution.

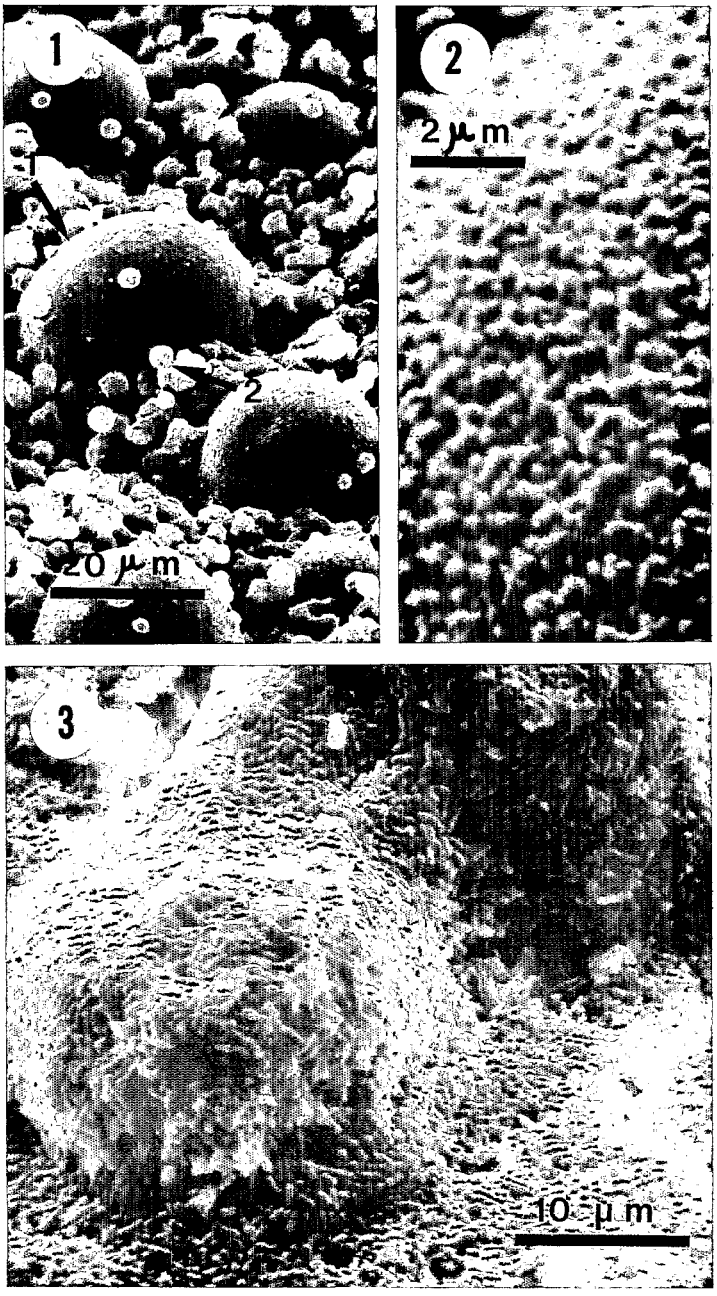
Light microscopy of the solution shows that sulfur granules are built by deposition of regular concentric layers, and the reaction is stopped by addition of water when the average size of the large granules is approximately 30 μm. The colloïdal suspension was passed onto membrane filters so that 30 mg of sulfur remained on the filter.

* Present address: Centre Orstom, B. P. 1386, Dakar, Senegal.

** Present address: Division of technical support, Environmental Protection Agency, Bldg. 2, Crystal Plaza, Arlington, Virginia, U.S.A.

Supported by Sea Grant D2263-123.

*** Department of Zoology, University of Georgia, Athens, Georgia, U.S.A.



Figs. 1—3

Appropriate dilutions of bacteria were passed through the sulfur filters and the membranes supporting *Thiobacillus thiooxidans* were placed on the surface of Petri dishes containing Starkey's medium devoided of sulfur and solidified by 1.5% noble agar. The filters supporting growth of *T. denitrificans* were placed in screw cap tubes containing Baalsrud's medium devoided of thiosulfate.

After 6 days of growth in the appropriate media, the filters were washed by filtration with distilled water, fixed for 2 hrs in a dilute solution containing: 4% paraformaldehyde, 10 ml; 2% glutaraldehyde, 10 ml; 0.1 M cacodylate buffer, 20 ml; filtrated sea water, 40 ml, and washed again by filtration with distilled water before air drying. A small piece of the filter was affixed to the specimen stub with silver conducting paint and coated with a layer of about 200 Å of Palladium-Gold in a vacuum evaporator. An uninoculated sulfur filter was prepared as a control by the same procedure.

For the detection of sulfides, filters supporting growth of *T. denitrificans* and control were placed overnight in 1 l of distilled water to wash out the salts of the medium, immersed for 30 min in a 2.5% (w/v) solution of lead acetate, washed by filtration and treated as described.

The specimen stubs were tilted to an angle of 45° and photographed with a Cambridge "Stereoscan" scanning electron microscope, using an acceleration voltage of 10 kiloVolts.

Results

Figs. 1 and 2 show two magnifications of the control sulfur filter. Large sulfur granules, average size 30 μm (arrow 1) are surrounded by smaller particles of sulfur, average size 5 μm (arrow 2). The surface appears to be covered by a layer of fine sulfur particles, average size 0.3 μm (Fig. 2).

Fig. 3 shows the sulfur granules covered by cells of the aerobic sulfur oxidizer *Thiobacillus thiooxidans*.

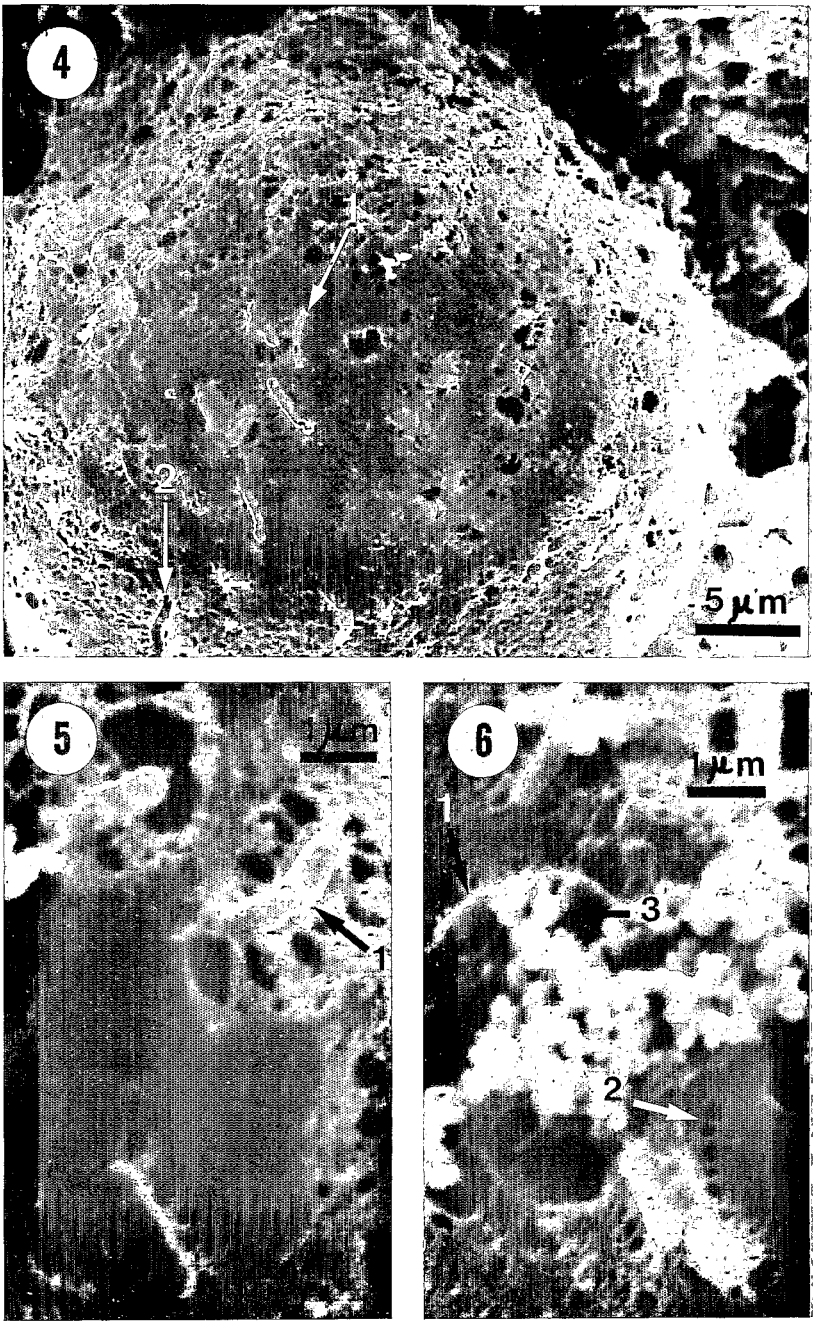
In Fig. 4 is shown a sulfur granule supporting growth of *Thiobacillus denitrificans*. Bacteria are seen on the surface (arrow 1). A furrow approximately corresponding to the size of a bacterium can be seen (arrow 2). The entire surface has been unevenly eroded.

A magnified sulfur granule is shown in Figs. 5 and 6. Arrow *1* in both figures points to a bacterial cell which is apparently eroding the sulfur granule. Bridges appear to exist between the bacterium and the sulfur granule, the bacterial cell resting on these bridges and dissolving away the sulfur, as evidenced by the holes on the surface on the granule. A possible cell print is seen in Fig. 6 (arrow 2). The surface of the sulfur granule after bacterial growth appears to be smooth as compared to the control (Fig. 2). The smaller particles of sulfur covering the surface before growth have been removed. However, arrow 3 in Fig. 6 points to particles of the same average size as seen on the control (Fig. 2), but covering the bacteria.

Fig. 1. Control sulfur granule, low magnification

Fig. 2. Surface of a control sulfur granule, high magnification

Fig. 3. Sulfur granules covered by cells of *Thiobacillus thiooxidans*



Figs. 4—6

A control sulfur granule treated with lead acetate is seen in Fig. 7. The sulfur filter supporting growth of *Thiobacillus denitrificans* and treated with lead acetate is shown in Fig. 8. The surface appears to be covered by a discontinuous precipitate.

Discussion

With the aerobic sulfur oxidizer *Thiobacillus thiooxidans* a continuous layer of cells is seen covering the sulfur (Fig. 3), and the bacteria could not be easily separated from the sulfur. We had previously observed by microscopic examinations that grinding in a mortar was necessary to detach *T. thiooxidans* from elemental sulfur after growth on this substrate.

On the contrary, examination of *Thiobacillus denitrificans* shows that most of the cells were separated from the sulfur granules. Furthermore, it was noticed that during growth many fragments of the film of colloïdal sulfur came apart from the membrane filters and sank down the bottom of the culture tubes. We suggest that the N_2 produced by denitrification during growth causes the cells to separate from the sulfur.

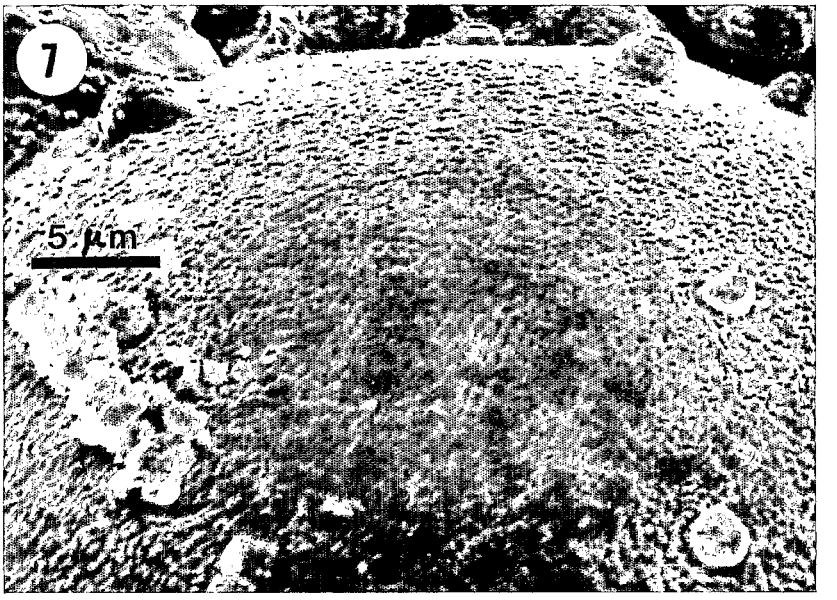
In Fig. 4, low magnification, some bacteria (arrow 1) and cell prints (arrow 2) can be observed. High resolution pictures (Figs. 5 and 6) show bacterial cell sunk in large cavities, and attached to the sulfur by bridges. This physical attachment was not seen by Schaeffer *et al.* (1963) using crystalized sulfur. The eroded surface and cell prints (Fig. 6, arrow 2) can be observed. The layer of smaller sulfur particles seen on the control (Fig. 2) was completely oxidized after 6 days by *Thiobacillus denitrificans*.

The physiological mechanism for the uptake of elemental sulfur by the thiobacilli has been suggested to be either by sulfides pathway in *T. neapolitanus* (Taylor, 1968) or by glutathion reduction in *T. thiooxidans* (Suzuki, 1965). In an attempt to determine if sulfides are an intermediate in the oxidation of elemental sulfur by *T. denitrificans*, lead acetate was used in the hope of precipitating lead sulfide on the surface of the cells. The salts of the medium were washed out, so that the precipitate observed on the sulfur granule supporting growth of *T. denitrificans* was due to a metabolite.

The metabolites which may precipitate with lead acetate are sulfate, thiosulfate or sulfide. Sulfate being very soluble would not accumulate

Fig. 4. Sulfur granule supporting growth of *Thiobacillus denitrificans*. Arrow *1*: bacterial cell; arrow *2*: cell print

Figs. 5 and 6. High magnification of *Thiobacillus denitrificans* grown on sulfur granule. Arrow *1*: bacterial cell; arrow *2*: cell print; arrow *3*: secondary deposit of sulfur



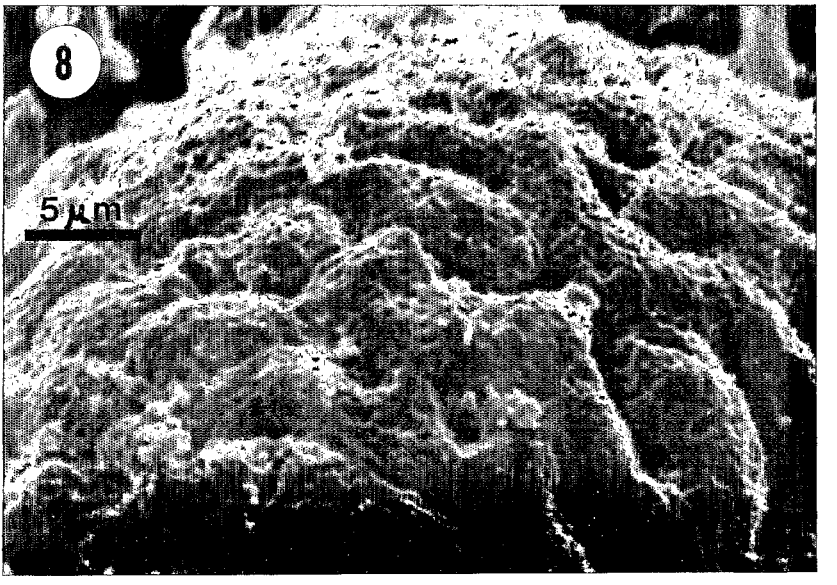


Fig. 7. Control sulfur granule treated with lead acetate

Fig. 8. Sulfur granule supporting growth of *Thiobacillus denitrificans* and treated with lead acetate

on the bacteria. It is unlikely that the precipitate seen in Fig. 8 is lead thiosulfate, since thiosulfate is actively metabolized by *Thiobacillus denitrificans* (Baalsrud and Baalsrud, 1954) and would not be excreted by the bacteria. It is suggested that the precipitate is lead sulfide since there appears to be a secondary deposit covering the bacteria on the untreated sulfur granules (Fig.6, arrow 3). According to the scheme of Vishniac and Santer (1957) elemental sulfur is formed during the sulfur oxidation pathway in thiobacilli. Our data suggest that elemental sulfur oxidation by *T. denitrificans* proceeds by the sulfide pathway, with a secondary formation of sulfur.

Acknowledgements. The authors wish to thank Dr. H. D. Peck, Jr., for helpful suggestions during the course of this work.

This work was supported by Grant USDC-Sea 1-36009.

References

Baalsrud, K., Baalsrud, K. S.: Studies on *Thiobacillus denitrificans*. Arch. Mikrobiol. **20**, 34—62 (1954)

Postgate, J. R.: Media for sulfur bacteria. Lab. Pract. **15**, 1239—1244 (1966)

Roth, I. L.: Scanning electron microscopy of bacterial colonies. In: Scanning electron microscopy. Proceeding of the fourth annual symposium. IIT Research Institute, Chicago, Ill. (1971)

Schaeffer, W. I., Holbert, P. E., Umbreit, W. W.: Attachment of *Thiobacillus thiooxidans* to sulfur crystals. J. Bact. **85**, 137—140 (1963)

Suzuki, I.: Oxidation of elemental sulfur by an enzyme system of *Thiobacillus thiooxidans*. Biochim. biophys. Acta (Amst.) **104**, 359—371 (1965)

Taylor, B. F.: Oxidation of elemental sulfur by an enzyme system from *Thiobacillus neapolitanus*. Biochim. biophys. Acta (Amst.) **170**, 112—122 (1968)

Vishniac, W., Santer, M.: The thiobacilli. Bact. Rev. **21**, 195—213 (1957)

Dr. J. Baldensperger
Centre Orstom
B.P. 1386
Dakar, Senegal