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SULFUR BACTERIA FROM A SOUTHWESTERN FLORIDA SINKHOLE: A PRELIMINARY REPORT

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INTRODUCTION

The recent discovery of a sulfide-containing sinkhole located near Venice, Florida has prompted interest among biologists, geologists, and archaeologists. The sink, about 220 feet deep (67 m), has been found to contain human bones, well preserved by hydrogen sulfide in the water which fills the hole. The saline water has a chlorinity of about 9.05 $\pm$ 2‰ (F. A. Kohout, U.S. Geological Survey). Warm water (ca. 38C) enters at the bottom of the sink at a rate of about 8.6×10^5 ft^3 per day. Considering the volume of the hole, the water has a residence time of about 20 days.

Our interest in the sinkhole stems from ongoing research concerning the microbial sulfur cycle in anoxic marine environments. In this report, we present some preliminary information on sulfur bacteria which we have cultured from a series of samples collected from the sink during the spring of 1975.

MATERIALS AND METHODS

Sampling

Samples were taken by SCUBA divers using sterile 50 ml plastic syringes. Immediately upon reaching the surface, the syringe contents were transferred into sterile, 35 ml glass bottles which had been pre-gassed with O_2-free N_2 gas and fitted with butyl rubber stoppers. The bottles were filled to overflowing with the water sample and immediately re-stoppered.

Enrichment

Media and culture conditions. Media for *Thiorhodaceae* were prepared according to Postgate (1967). Specific enrichment cultures were made for the following groups: *Chromatium* sp. and *Thiopedia; Chlorobium* sp.; and *Chlorobium thiosulfatophilum*. The culture medium with a salinity of 18% were added to sterile, gassed out 35 ml bottles and inoculated with 5 ml of sample. The bottles were incubated at 1 to 2 feet from a 40 watt incandescent light for a period of 3 months at 25 ± 3C.

Enrichment cultures for chemolithotrophic sulfur bacteria were made in the thiosulfate medium (TB) of Tuttle and Jannasch (1972) at 18% salinity and in sulfide gradient cultures containing 10 ml of a 4% NaCl, 4% agar solution overlayed after solidification with about 70 ml of the TB medium at pH 7. The TB medium cultures, contained in 16 x 120 mm test tubes, consisted of 5 ml of medium at pH 7 and a 1 ml inoculum; and the gradient cultures were prepared in 100 ml culture tubes with an inoculum of 5 ml. The cultures were incubated stationary at 25 ± 3C.

Isolation procedure. Isolations were made from the TB medium and sulfide gradient cultures by streaking onto TB agar plates as described previously (Tuttle and Jannasch, 1972). Isolated colonies were subcultured and maintained on TB agar slants. The isolates were compared on the basis of their colony morphology on TB agar and Nutrient Agar (Difco) containing 2% NaCl. Duplicate isolates were discarded.

Physiological testing. The representative isolates were tested for their ability to oxidize thiosulfate under aerobic conditions and to reduce

tetrathionate and thiosulfate under anaerobic conditions. Thiosulfate oxidation was determined in TB medium as previously described (Tuttle and Jannasch, 1972). Following incubation for 14 days at 22 ± 2C, the culture fluids were analyzed for pH, thiosulfate, tetrathionate, trithionate, and total cell protein. Uninoculated medium served as the control.

Determinations of anaerobic growth and tetrathionate or thiosulfate reduction were made using the pyruvate medium of Tuttle and Jannasch (1973). Following incubation at 22 ± 2C as noted in Tables 2 and 3, the cultures were examined for pH, thiosulfate, tetrathionate, trithionate, sulfide, and cell protein. Controls consisted of uninoculated medium with or without tetrathionate or thiosulfate.

Chemical determinations. Sulfide in the original samples was measured by the method of Packmeyer (quoted by Trüper and Schlegel,1974) and in the anaerobic growth experiments by the technique of Gilboa-Garber (1972). The latter method is not affected by the presence of thiosulfate at high concentration (Tuttle and Jannasch, 1973).

Thiosulfate, tetrathionate and trithionate were measured using the cyanolysis method of Kelly *et al.* (1969). Where necessary, sulfide interference was removed using the modification described by Tuttle and Jannasch (1973).

Total cell protein was determinated by the procedure of Lowry *et al.*; (1951) after the cell material had been worked with artificial seawater and digested with 10% (w/v) trichloracetic acid. The pH was measured with a Metrohm expanded scale pH meter (Brinkman Instruments).

RESULTS AND DISCUSSION

Sulfide production. Sulfide concentration versus depth is shown in Fig. 1. The erratic values cannot presently be explained, but could be caused by varying sulfide loss from the bottles during transit and storage before the sulfide determinations were made. The data show, however, that the entire water column contains sulfide. The absence of high sulfide values near the inflow (220 ft) suggest that the sulfide is formed *de novo* in the sink, and is not carried in with new water. If this is so, we can set 0.2 mmoles/m^3/day as the minimum rate of sulfide production assuming no sulfide oxidation occurs, the residence time of the water is 20 days, and the mean sulfide concentration in the sink is 4 µg at/l.

On the day of sampling, the divers observed a turbidity interface at about 190 ft depth. Water above was turbid while water below was clear. The visual observation taken with an experimental minimum and maximum in sulfide concentrations at 200 ft and 180 ft respectively (Fig. 1) indicate that the interface could represent a zone of sulfate reduction. The possibility needs to be further explored.

Photosynthetic bacteria. All our enrichments for photosynthetic sulfur-oxidizing bacteria (*Thiorhodoceae*) were negative. Whether this means that these organisms do not play a role in the potential sulfur transformations within the sink is not certain, because the bacteria may not have survived the abnormally long holding time between sampling and culturing. Nevertheless, we found no microscopic evidence that these organisms were present in the original samples at the time of sulfide determination.

Chemoautotrophic bacteria. The enrichment cultures with sulfide or thiosulfate as inorganic sources of energy and oxygen as the electron acceptor are specifically designed to culture chemoautotrophic sulfur bacteria (e.g., thiobacilli). The presence of these bacteria is evidenced by a marked decrease in the pH of the medium accompanied by the oxidation of a large portion of the inorganic sulfur substrate supplied. Even after prolonged incubation (3 months), we found no evidence for the presence of chemoautotrophs. In thiosulfate cultures, the pH increased rather than decreased, and the gradient cultures contained detectable sulfide after 3 months. Subsequent plating of samples from the enrichment cultures onto thiosulfate agar did not yield colonies of thiobacilli.

Heterotrophic thiosulfate-oxidizing bacteria. Based upon colony morphology, four distinct strains of bacteria were found after aliquots of TB medium enrichments were cultured onto thiosulfate agar. The distribution of these strains in the sink samples is shown in Fig. 1. Isolates B and F were generally present throughout the water column. Only strain a, found from the surface to 25 feet and then only at the inflow at 220 feet, appeared to have a well defined occurrence pattern. No statements concerning the quantitative abundance of these bacteria in the sink can be made on the basis of information presented here.

Under aerobic conditions (Table 1) the bacteria exhibited characteristics similar to heterotrophic thiosulfate-oxidizing bacteria previously found in a variety of marine environments (Tuttle and Jannasch, 1972, 1973a). The distinguishing features of growth in TB medium included a) the inability

to decrease the pH, b) the small amount of total thiosulfate oxidized and subsequent growth which occurred, and c) the formation of polythionates rather than sulfate as the major end product of thiosulfate oxidation.

Under anaerobic conditions (Tables 2 and 3), isolates B and C exhibited the physiological characteristics of some newly described facultatively anaerobic bacteria which reduce inorganic sulfur compounds other than sulfate at the expense of organic carbon as the oxidizable substrate (Tuttle and Jannasch, 1973). The increased growth in the presence of tetrathionate or thiosulfate as well as the accumulation of sulfide in thiosulfate containing cultures of both isolates to concentrations exceeding 1.7 mm indicate that the reductions were true dissimilations. The recovery of nearly all the added tetrathionate as thiosulfate (Table 2) and the decrease in pH indicates the following stoichiometry for tetrathionate reduction.

$$S_4O_6^{-2} + 2H^\circ \longrightarrow 2\ S_2O_3^{-2} + 2H^+$$

During thiosulfate reduction, the following reaction is indicated:

$$S_2O_3^{-2} + 2e^- \longrightarrow S^{-2} + SO_3^{-2}$$

where sulfite probably represents the missing sulfur which cannot be accounted for as sulfide, tetrathionate, or trithionate (Table 3). We have found this to be true for several other previously isolated strains (unpublished data). Isolates A and F exhibited little growth in the anaerobic cultures and did not reduce tetrathionate or thiosulfate.

SUMMARY

1. The sulfide profile suggests that sulfide is produced within the sink, probably by bacterial reduction of sulfate. In addition to sulfate reduction, sulfide may also be formed in part by the reduction of partially reduced inorganic sulfur intermediates such as thiosulfate and tetrathionate as evidenced by the anaerobic sulfur metabolism of two of the isolated bacterial species.

2. The potential for thiosulfate oxidation exhibited by the bacteria found indicates probable bacterial oxidation of sulfur compounds within the sink. The magnitude of this oxidation, however, would be dependent upon the availability of readily oxidizable organic energy sources since the microorganisms found cannot derive all the energy for growth from reduced sulfur compounds. Biological and chemical oxidation of sulfide or partially reduced inorganic sulfur intermediates would, of course, be dependent upon oxygen, or biologically alone, upon the presence of some alternate electron acceptor such as nitrate.

3. The presence of bacteria in the sink which metabolize sulfur only under aerobic conditions suggests that one cannot assume the absence of sulfide oxidation in the sink. Therefore, the estimate for sulfide production can only be considered a minimum value.

Future work

1. A sulfide profile, measured without retaining water samples, is needed to check the hypothesis of sulfate reduction accounting for sulfide in the sink.

2. An oxygen profile is needed to assess the possibility of chemical and biological sulfide oxidation.

3. We should look again for photosynthetic sulfur bacteria.

4. The occurrence of obligately anaerobic sulfate-reducing bacteria should be investigated, particularly with reference to possible interfaces and/or sulfide maxima.

5. The circulation pattern within the sink should be worked out.

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Table 1. Thiosulfate Oxidation by the Isolates in TB Medium.

Initial Thiosulfate Concentration = 40 mm, Initial pH = 7.0.

Cultures were shaken (100 rpm) for 14 days at 22 ± 2C.

Isolate	Final[b] pH	µmoles/ml $S_2O_3^{-2}$ Oxidized	µmoles/ml $S_4O_6^{-2}$ Formed	% Original $S_2O_3^{-2}$ Oxidized	% $S_2O_3^{-2}$ Oxidized to $S_4O_6^{-2}$	Growth mg Protein/l
A	7.61-7.78	2.497	0.944±0.124	6.2	75.6	0.05-0.15
B	7.46-7.48	1.926	0.646±0.050	4.8	67.1	0.06-0.08
C	7.54-7.55	2.846	0.808±0.038	7.1	56.8	0.04-0.06
F	6.80-6.81	0.510	0.137±0.013	1.3	53.7	0.00-0.02
Control[a]	6.77-6.81	0.000	0.000	-	-	0.00

[a] Uninoculated medium held at same conditions as cultures.

[b] All values represent duplicate cultures.

Table 3. Anaerobic Thiosulfate Reduction by the Isolates in the Medium of Tuttle and Jannasch (1973). The Cultures Were Incubated Stationary at 22 ± 2C for 10 days.

Isolate	Final pH	µmoles/ml $S_2O_3^{-2}$-S Lost	% $S_2O_3^{-2}$ Lost Recovered As $S_4O_6^{-2}$	$S_3O_6^{-2}$	S^{-2}	Growth,[c] mg Protein/l
A	8.22-8.25[b]	0.087[d]	131.0	0.0	0.0	0.20-0.22
B	7.29-7.33	1.323	10.0	4.1	65.5	6.54-7.36
C	7,33-7.35	1.335	10.8	2.5	65.5	8.14-9.70
F	8.24-8.26	0.075	65.0	35.7	0.0	0.02-0.04
Control[a]	8.22	0.132	100.0	0.0	0.0	0.0

[a] Uninoculated medium held at same conditions as cultures.

[b] All values represent duplicate cultures.

[c] Isolates B and C grew anaerobically in medium in which $S_2O_3^{-2}$ had been omitted. Protein concentrations were 3.48 and 3.14 for cultures B and C respectively. The corresponding pH values were 7.51 and 7.54. Control medium had a final pH of 8.22.

[d] Measured initial $S_2O_3^{-2}$ concentration = 4.759 µmoles/ml.