

Uptake Rates of Oxygen and Sulfide Measured with Individual *Thiomargarita namibiensis* Cells by Using Microelectrodes

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Gradients of oxygen and sulfide measured towards individual cells of the large nitrate-storing sulfur bacterium *Thiomargarita namibiensis* showed that in addition to nitrate oxygen is used for oxidation of sulfide. Stable gradients around the cells were found only if acetate was added to the medium at low concentrations.

The sulfur bacterium *Thiomargarita namibiensis* is a close relative of the filamentous sulfur bacteria of the genera *Beggiatoa* and *Thioploca*. It was only recently discovered off the Namibian coast in fluid sediments rich in organic matter and sulfide (15). The large, spherical cells of *Thiomargarita* (diameter, 100 to 300 μm) are held together in a chain by mucus that surrounds each cell (Fig. 1). Most of the cell volume is taken up by a central vacuole in which nitrate is stored at concentrations of up to 800 mM. The ability to accumulate nitrate is also found in larger, marine species of *Beggiatoa* (8) and *Thioploca* (3). The latter have been shown to use nitrate as an electron acceptor for the oxidation of sulfide to elemental sulfur and then to sulfate while they reduce nitrate to ammonia (13). Both *Beggiatoa* and *Thioploca* spp. show a phobic reaction towards oxygen even at low concentrations (5, 10). Higher oxygen concentrations in the bottom water (5 to 35 μM), when they occur off the Chilean coast during winter or El Niño events, dramatically reduce the *Thioploca* population (16). In contrast to this, *Thiomargarita* cells survive exposure to oxygen even at the concentrations of air saturation (15), although the bottom water overlying the sediments off Walvis Bay is usually anoxic.

Because *Thiomargarita* cells are not motile, the only way that the cells can come into contact with water containing nitrate is during intervals when the sediment gets resuspended in the water column. This can happen, for example, due to large sediment outgasings of methane, which occur regularly in the area inhabited by *Thiomargarita* (2, 17). During these events the highly fluidized sulfidic mud containing *Thiomargarita* cells may get mixed with oxygenated water containing nitrate. The purpose of this study was to investigate whether the cells merely survive such exposure to oxygen or whether *Thiomargarita* cells can use oxygen as an electron acceptor in addition to nitrate for oxidation of sulfide. Like the nitrate-storing species of *Beggiatoa* and *Thioploca*, *Thiomargarita* spp. have not been isolated yet in pure culture. Nevertheless, cells may be kept alive and growing in their native sediment for years. As *Thiomargarita* cells are not motile, it is not possible to draw

conclusions about their physiology by observing chemotactic behavior, as has been done successfully with *Beggiatoa* and *Thioploca* filaments (5, 10). However, because of the large size of *Thiomargarita* cells, they develop, around individual cells, measurable gradients of oxygen and sulfide that can be used for calculating uptake rates of oxygen and sulfide. Thus, the physiological reactions of individual cells to changes in oxygen and sulfide concentrations can be directly observed by observing changes in the uptake rates.

Experiments. The cells used for the experiments in this study were collected off Namibia during a cruise of the German RV *Poseidon* in May 1999 and were kept in their natural sediment at 5°C for more than 1 year. At approximately 3-month intervals the overlying water was removed and the sediment was resuspended several times in seawater enriched with nitrate (1 mM), which induced growth of *Thiomargarita* cells. During this treatment cells were exposed to oxygen. The experiments were conducted in a square polycarbonate chamber (7 by 7 by 7 cm) containing 250 ml of artificial seawater (36.4 g of NaCl per liter, 1 g of CaCl_2 per liter, 0.5 g of K_2HPO_4 per liter, 0.1 g of NaH_2PO_4 per liter; pH 7.3). The chamber was capped with a movable plate containing two holes for microelectrode access (Fig. 1). *Thiomargarita* cells were washed in medium and transferred to the chamber by sucking them up in the tip of a Pasteur pipette. After the cells were placed in the experimental chamber, acetate was added to a final concentration of 10 μM . The chamber was continuously flushed with either air or argon to control the oxygen concentration. Sulfide was added to the medium by passing the gas through a bottle containing 100 ml of carbonate buffer (pH 9.3) and sulfide at a high concentration (100-fold higher than the desired final concentration in the experimental chamber). Thus, the gas was supplemented with H_2S at the desired concentration before it was bubbled through the experimental chamber. After a stable concentration of sulfide was established, as measured with an H_2S microsensor, samples (1 ml) were taken, and the exact sulfide concentration was determined spectrophotometrically (1). The bubbling was adjusted so that the liquid in the chamber was well mixed and only a thin diffusive boundary layer (DBL) (approximately 140 μm) developed around the cells. The DBL around the cells (diameter, 220 μm) was found by moving the microsensor in 10- μm intervals away from the cell until no change in concentration was found. To measure fluxes of ox-

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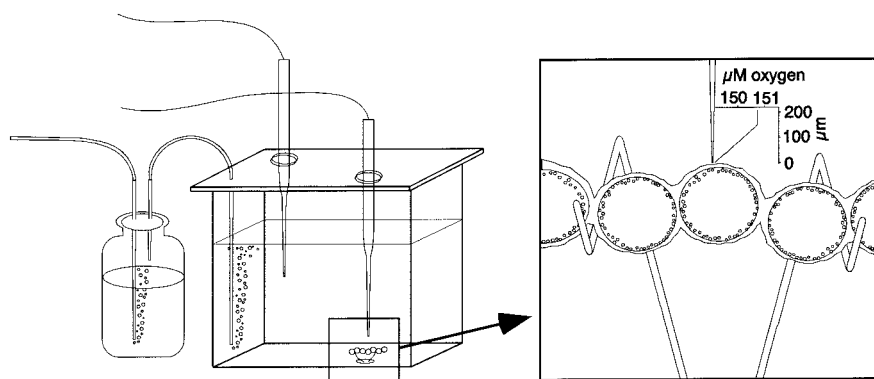


FIG. 1. Experimental setup. A chain of *T. namibiensis* cells was placed in a chamber with 250 ml of artificial seawater. The oxygen concentration in the medium was controlled by bubbling the medium with either air or argon. Sulfide was added by prior bubbling through a wash bottle containing a concentrated solution of sulfide. To ensure three-dimensional diffusion towards the cells, the chain of cells was elevated above the bottom of the chamber by clamping it between two platinum wires. One microelectrode (either oxygen or sulfide) was used to measure gradients towards the cells, while the other recorded the concentration of oxygen or sulfide in the bulk medium. The inset shows an example of oxygen flux without sulfide in the medium.

oxygen and sulfide towards a single cell (Fig. 2), a chain of *Thiomargarita* cells was fixed between two platinum wires at a distance of ca. 1 cm from the bottom of the chamber (Fig. 1). By using a preparation needle the two ends of the chain were placed into the V-shaped ends of the platinum wires. This design allowed three-dimensional diffusion toward the cell investigated. Vertical profiles of oxygen or sulfide were measured through the DBL of the cells at 10- μm intervals by using a Clark-type oxygen microelectrode with a guard cathode (14) or a sulfide microelectrode (6). The gradients through the DBL of the cells were found to be almost linear; therefore, the total flux towards the cell could be calculated by multiplying the linear flux ($J = D \times dC/dr$, where J is the flux, D is the diffusion coefficient, dC is the change in concentration between two points, and dr is the distance between these two points) by the surface area of the cell (diameter, 220 μm). To calculate the surface area involved in uptake, either the surface of a sphere ($4\pi r^2$) or the surface of the side of a cylinder ($2\pi rh$, where r is the radius and h is the height) could be assumed. In our case the height of the cylinder was twice the radius ($h = 2r$), so the two assumptions gave the same value. During the experiment whose results are shown in Fig. 3, the cells were lying on the bottom of the experimental chamber. The oxygen electrode was placed directly at the cell surface. Several times during the experiment, the electrode was moved 500 μm up and down to ensure that the bulk concentration of oxygen remained unchanged. In order to prove that oxygen and sulfide gradients resulted from physiological activity of the cells, selected active cells were exposed for 1 min to pure ethanol and returned to the chamber. These cells showed no gradients of oxygen or sulfide towards the cell surface. During this short exposure the cells were killed, but the internal sulfur inclusion did not dissolve. Also, addition of methanol to a final concentration of 1% in the medium led to immediate disappearance of sulfide and oxygen gradients around initially active cells. The abiotic oxidation of sulfide with oxygen is a slow process that occurs with half times between 0.4 and 65 h (9). As we flushed the medium continuously with both gases, this process should not have played a major role in these experiments.

Addition of acetate. In initial experiments freshly added cells showed pronounced gradients of oxygen and sulfide that slowly disappeared within 1 or 2 h. Only after sodium acetate was added to the medium at a final concentration of 10 μM did the oxygen gradients remain stable for at least 2 days, even when no sulfide was present in the medium. Further addition of 10 μM acetate did not enhance the oxygen flux towards the cells (data not shown). If the 10 μM acetate were consumed by the cell and used as an electron donor, this result would have corresponded to a maximum acetate flux of $6.5 \text{ pmol cm}^{-2} \text{ s}^{-1}$ and would have increased the oxygen flux by $13 \text{ pmol cm}^{-2} \text{ s}^{-1}$ ($\text{CH}_3\text{COOH} + 2 \text{ O}_2 \rightarrow 2 \text{ CO}_2 + 2 \text{ H}_2\text{O}$). As the oxygen flux was stabilized at only $1.1 \text{ pmol cm}^{-2} \text{ s}^{-1}$ and not increased even by the first addition of acetate, it is likely that the *Thiomargarita* cells in this experiment depended on acetate only as a carbon source and not as an electron donor. Acetate has also been shown to stimulate the sulfide uptake of *Thioploca* spp. (13) and the thiosulfate uptake of *Thiothrix* spp. (12) and can be used as a supplemental or sole carbon source by lithotrophic marine *Beggiatoa* strains (4). Nevertheless, the possibility that *T. namibiensis* grows autotrophically cannot be ruled out by the results of these experiments, because the special setup, which was designed to measure sulfide uptake in the presence of minimum oxygen concentrations and with a stable pH, did not allow bicarbonate-carbonate to be present in the medium. To maintain very low oxygen concentrations, it was necessary to bubble the medium constantly with argon, which would have stripped CO_2 from the medium.

Use of oxygen. The presence of sulfide in the medium clearly enhanced oxygen uptake (Fig. 2A), and likewise, sulfide uptake by the cells was enhanced by oxygen (Fig. 2B). These results suggest that *Thiomargarita* cells not only are able to survive exposure to oxygen but also may use oxygen as an electron acceptor. Addition of nitrate to the medium had no effect on the oxygen uptake. As judged by the sulfide uptake rates, the cells remained physiologically active even under oxygen concentrations close to saturation. This indicates that unlike *Beggiatoa* spp. (7, 10, 11), *T. namibiensis* is not obligately microaerophilic. The maximum sulfide flux was 7.5 pmol cm^{-2}

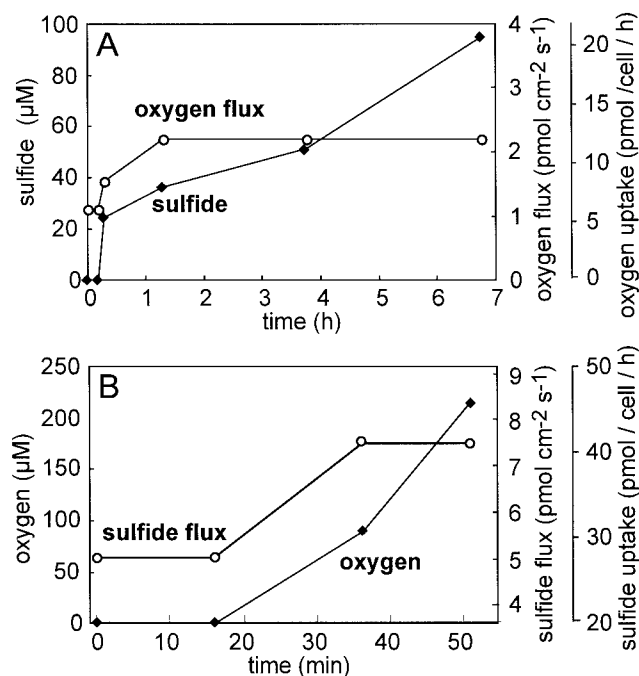


FIG. 2. Oxygen flux (A) and sulfide flux (B) towards an individual *Thiomargarita* cell with a diameter of 220 μm . The fluxes (\circ) were calculated from microprofiles measured through the 140- μm -thick DBL around the cell in the presence of a stable oxygen concentration of 150 μM (A) and a stable sulfide concentration of 320 μM (B). The changing bulk medium concentrations of sulfide (A) and oxygen (B) (\blacklozenge) were measured continuously with a separate electrode. The graph shows individual measurements. Therefore, the absolute numbers should be interpreted as examples of possible uptake rates found in *Thiomargarita* cells rather than as the typical rate for this genus. Numerous measurements showing the same type of response were obtained with cells lying on the bottom of the chamber, but quantification of fluxes from these profiles by using Fick's law may have led to overestimates and were therefore not included in the data set.

s^{-1} and could not be increased by increasing the oxygen concentration (Fig. 2B); likewise, the oxygen flux did not exceed $2.2 \text{ pmol cm}^{-2} \text{s}^{-1}$ after further addition of sulfide (Fig. 2A). It seems that with the rigorous stirring resulting from bubbling with gas, the maximum uptake rates occurred at sulfide and oxygen concentrations that are low (100 μM) compared to the concentrations that *T. namibiensis* can tolerate (air saturation). The observed sulfide flux under anoxic conditions (approximately $5 \text{ pmol cm}^{-2} \text{s}^{-1}$) (Fig. 2B) must have been supported by internally stored nitrate. Addition of oxygen increased the sulfide flux by ca. $2.5 \text{ pmol cm}^{-2} \text{s}^{-1}$. Thus, even though the sulfide flux could be significantly stimulated by oxygen, reduction of nitrate was still the more important process for sulfide uptake.

Cell response to sulfide under aerobic conditions. It was possible to monitor the reaction of an individual cell to pulses of sulfide in the medium by placing the oxygen electrode directly at the cell surface. A decline in the oxygen concentration represented higher uptake rates, and an increase indicated lower rates of oxygen uptake (Fig. 3). The response of the cell could be observed with very high temporal time resolution, but only relative changes in the uptake rate were visible. The fluxes

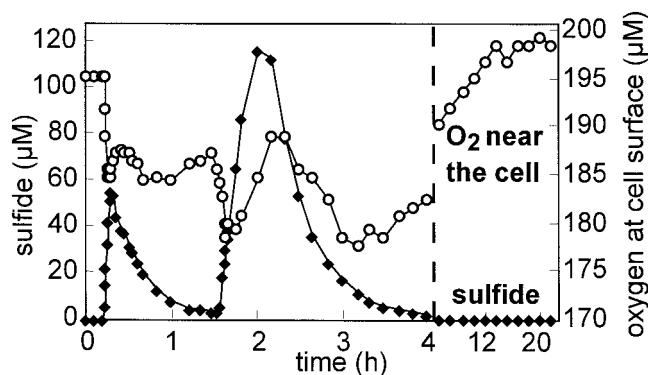


FIG. 3. Response of an individual *Thiomargarita* cell to changes in sulfide concentration (\blacklozenge) under oxic conditions. The sulfide sensor was placed in the bulk medium, and the oxygen electrode was placed directly at the cell surface (\circ). Thus, enhanced oxygen uptake rates are reflected by a decrease in oxygen concentration at the cell surface. The dashed line indicates a change in the x-axis scale.

could be obtained only from steady-state profiles. Two sequential pulses of sulfide (60 and 120 μM) both resulted in a sharp drop in the oxygen concentration, followed by an increase and then a decrease in the oxygen tension at the cell surface (Fig. 3). In both cases the minimum concentration at the cell surface was reached at sulfide concentrations of around 20 μM (24 and 18 μM , respectively). Together with the finding that oxygen uptake rates did not increase with sulfide concentrations above 37 μM , this suggests that under our experimental conditions *T. namibiensis* reached maximum sulfide fluxes at a sulfide concentration of around 20 μM even though it tolerates much higher concentrations. After both sulfide pulses the oxygen concentration at the cell began to rise again after the sulfide concentration dropped below approximately 9 μM , indicating that this sulfide concentration was too low to support maximal oxygen uptake rates. The oxygen concentration returned to the former value only 9 h after sulfide was completely removed from the medium (Fig. 3). Possibly, the accumulation of elemental sulfur under aerobic conditions with sulfide present led to the long-term effect of the sulfide pulses on the uptake rate of oxygen. A similar experiment in which oxygen was added under anoxic, sulfidic conditions resulted in a single drop and rise in the sulfide concentration at the cell surface (data not shown).

Implications. The results of our experiments show that even though *T. namibiensis* cells store high concentrations of nitrate, they can take up oxygen in the presence or absence of nitrate. As the oxygen uptake is greatly stimulated by the presence of sulfide and vice versa, oxygen seems to be used as an electron acceptor for the oxidation of sulfide, even though the sediments in which *T. namibiensis* is found are permanently anoxic. These observations suggest that even though most of the time *T. namibiensis* cells survive in sediments containing high sulfide concentrations with internally stored nitrate as their sole electron acceptor, they may be physiologically most active during times when the sediment is suspended. Suspension of sulfidic mud in oxic seawater should provide the cells with the opportunity to oxidize sulfide with oxygen. Even though such events may be relatively infrequent, the rapid response to oxygen and the relatively high uptake rates suggest that these short periods

might be the major times of energy gain, whereas nitrate might be used primarily to survive the time between sediment suspension events.

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REFERENCES

1. Cline, J. D. 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.* **14**:454–458.
2. Copenhagen, W. J. 1953. The periodic mortality of fish in the Walvis region; a phenomenon within the Benguela Current. *Investig. Rep. Div. Fish. S. Afr.* **14**:8–35.
3. Fossing, H., V. A. Gallardo, B. B. Jørgensen, M. Hüttel, L. P. Nielsen, H. Schulz, D. E. Canfield, S. Forster, R. N. Glud, J. K. Gundersen, J. Küver, N. B. Ramsing, A. Teske, B. Thamdrup, and O. Ulloa. 1995. Concentration and transport of nitrate by the mat-forming sulphur bacterium *Thioploca*. *Nature* **374**:713–715.
4. Hagen, K. D., and D. C. Nelson. 1996. Organic carbon utilization by obligately and facultatively autotrophic *Beggiatoa* strains in homogeneous and gradient cultures. *Appl. Environ. Microbiol.* **62**:947–953.
5. Huettel, M., S. Forster, S. Kloser, and H. Fossing. 1996. Vertical migration in the sediment-dwelling sulfur bacteria *Thioploca* spp. in overcoming diffusion limitations. *Appl. Environ. Microbiol.* **62**:1863–1872.
6. Jeroschewski, P., C. Steuckart, and M. Kühl. 1996. An amperometric microsensor for the determination of H_2S in aquatic environments. *Anal. Chem.* **68**:4351–4357.
7. Jørgensen, B. B., and N. P. Revsbech. 1983. Colorless sulfur bacteria, *Beggiatoa* spp. and *Thiovulum* spp., in O_2 and H_2S microgradients. *Appl. Environ. Microbiol.* **45**:1261–1270.
8. McHatton, S. C., J. P. Barry, H. W. Jannasch, and D. C. Nelson. 1996. High nitrate concentrations in vacuolate, autotrophic marine *Beggiatoa* spp. *Appl. Environ. Microbiol.* **62**:954–958.
9. Millero, F. 1986. The thermodynamics and kinetics of the hydrogen sulfide system in natural waters. *Mar. Chem.* **18**:121–147.
10. Møller, M. M., L. P. Nielsen, and B. B. Jørgensen. 1985. Oxygen responses and mat formation by *Beggiatoa* spp. *Appl. Environ. Microbiol.* **50**:373–382.
11. Nelson, D. C., B. B. Jørgensen, and N. P. Revsbech. 1986. Growth pattern and yield of a chemoautotrophic *Beggiatoa* sp. in oxygen-sulfide microgradients. *Appl. Environ. Microbiol.* **52**:225–233.
12. Nielsen, P. H., M. A. de Muro, and J. L. Nielsen. 2000. Studies on the in situ physiology of *Thiothrix* spp. present in activated sludge. *Environ. Microbiol.* **2**:389–398.
13. Otte, S., J. G. Kuenen, L. P. Nielsen, H. W. Paerl, J. Zopfi, H. N. Schulz, A. Teske, B. Strotmann, V. A. Gallardo, and B. B. Jørgensen. 1999. Nitrogen, carbon, and sulfur metabolism in natural *Thioploca* samples. *Appl. Environ. Microbiol.* **65**:3148–3157.
14. Revsbech, N. P. 1989. An oxygen microelectrode with a guard cathode. *Limnol. Oceanogr.* **34**:472–476.
15. Schulz, H. N., T. Brinkhoff, T. G. Ferdelman, M. Hernández Mariné, A. Teske, and B. B. Jørgensen. 1999. Dense populations of a giant sulfur bacterium in Namibian shelf sediments. *Science* **284**:493–495.
16. Schulz, H. N., B. Strotmann, V. A. Gallardo, and B. B. Jørgensen. 2000. Population study of the filamentous sulfur bacteria *Thioploca* spp. off the Bay of Concepción, Chile. *Mar. Ecol. Prog. Ser.* **200**:117–126.
17. Waldron, F. M. 1900. On the appearance and disappearance of a mud island at Walfish Bay. *Trans. S. Afr. Phil. Soc.* **11**:185–188.