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Nitrogen, Carbon, and Sulfur Metabolism in Natural *Thioploca* Samples

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Filamentous sulfur bacteria of the genus *Thioploca* occur as dense mats on the continental shelf off the coast of Chile and Peru. Since little is known about their nitrogen, sulfur, and carbon metabolism, this study was undertaken to investigate their (eco)physiology. *Thioploca* is able to store internally high concentrations of sulfur globules and nitrate. It has been previously hypothesized that these large vacuolated bacteria can oxidize sulfide by reducing their internally stored nitrate. We examined this nitrate reduction by incubation experiments of washed *Thioploca* sheaths with trichomes in combination with ^{15}N compounds and mass spectrometry and found that these *Thioploca* samples produce ammonium at a rate of 1 nmol min^{-1} mg of $protein^{-1}$. Controls showed no significant activity. Sulfate was shown to be the end product of sulfide oxidation and was observed at a rate of 2 to 3 nmol min^{-1} mg of $protein^{-1}$. The ammonium and sulfate production rates were not influenced by the addition of sulfide, suggesting that sulfide is first oxidized to elemental sulfur, and in a second independent step elemental sulfur is oxidized to sulfate. The average sulfide oxidation rate measured was 5 nmol min^{-1} mg of $protein^{-1}$ and could be increased to 10.7 nmol min^{-1} mg of $protein^{-1}$ after the trichomes were starved for 45 h. Incorporation of $^{14}CO_2$ was at a rate of 0.4 to 0.8 nmol min^{-1} mg of $protein^{-1}$, which is half the rate calculated from sulfide oxidation. [2-^{14}C]acetate incorporation was 0.4 nmol min^{-1} mg of $protein^{-1}$, which is equal to the CO_2 fixation rate, and no $^{14}CO_2$ production was detected. These results suggest that *Thioploca* species are facultative chemolithoautotrophs capable of mixotrophic growth. Microautoradiography confirmed that *Thioploca* cells assimilated the majority of the radiocarbon from [2-^{14}C]acetate, with only a minor contribution by epibiontic bacteria present in the samples.

Massive communities of *Thioploca* species occur as dense mats in the top sediment underlying the oxygen minimum zone of the continental shelf off the coast of Chile and Peru (17). Extending down to 5 to 10 cm into the sediment, the total biomass (including sheaths) of these colorless sulfur bacteria may be as high as 800 g (wet weight) m^{-2} (32), potentially covering several thousands of square kilometers along a 3,000-km stretch of coast.

Thioploca chileae and *Thioploca araucae* are the two dominant species in the mat, measuring 12 to 22 and 28 to 42 μm in diameter, respectively (32, 36). Both species produce 2- to 7-cm-long trichomes (filaments), each of which consists of a uniseriate row of many vacuolated cells. Morphologically and phylogenetically, they are similar to vacuolated *Beggiatoa* species (24, 37), and it has been suggested that their physiology might be similar as well. A chief difference between the genera is, however, that *Thioploca* produces characteristic bundles of usually 10 to 20 trichomes, surrounded by 10- to 15-cm-long sheaths up to 1.5 mm in diameter. Individual trichomes can glide independently within the sheaths and extend up to 3 cm into the water phase above the sediment (16). In general, *Thioploca* species and *Beggiatoa* species appear to occupy different niches, the former living in vertical and horizontal sheaths down to 10 cm in sediments that contain relatively little sulfide. In contrast, *Beggiatoa* species live in the top layer of sediments that have relatively high sulfide concentrations. Since their discovery by V. A. Gallardo (8, 9), it has been assumed that the *Thioploca* mats play a crucial role in balancing the sulfur cycle of their marine habitat by reoxidizing all, or at least a substantial portion, of the sulfide produced in the sediment. The sulfide results from high rates of bacterial sulfate reduction, up to 2.4 g of sulfur m^{-2} day^{-1} (6), driven by extremely high primary productivity (up to 9.6 g of carbon m^{-2} day^{-1}) over the continental shelf (7). Recently, *Thioploca* spp. have been identified off the coast of Namibia (10), where similar oceanographic conditions exist, i.e., upwelling, high primary productivity, and oxygen-depleted bottom water.

Given that the high remineralization rates of organic compounds result in the often-observed depletion of oxygen in the bottom water overlying the sea floor (7, 9, 32), the question arose as to which electron acceptor might be used for the reoxidation of all the sulfide produced in these sediments. When it was discovered that the vacuolated *Thioploca* species living in the mat were capable of accumulating up to 500 mM nitrate from the overlying water (containing ~25 μM [7]), it was hypothesized that *Thioploca* species would be able to use the nitrate as a terminal electron acceptor for sulfide oxidation (7). It was assumed that nitrate would be reduced to dinitrogen, although no experimental data was available to support this (7). The question was, therefore, still open as to whether

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dinitrogen gas or ammonium would be the product. This was particularly interesting in view of the finding by McHatton et al. (21) that vacuolated *Beggiatoa* species are also capable of accumulating and reducing nitrate and in view of conflicting observations by others with respect to the final product of nitrate reduction by the nonvacuolated *Beggiatoa alba*, i.e., ammonium or dinitrogen gas (35, 39).

So far, it has not been possible to cultivate *Thioploca* species in pure culture. The same is true for the vacuolated (nitrate accumulating) *Beggiatoa* species. Hence, little is known about their (eco)physiology, specifically, their sulfide and sulfur oxidation rates, abilities to respirate oxygen and/or nitrate, growth rates, or capabilities to grow autotrophically, heterotrophically, or mixotrophically. Clearly, this knowledge is essential for understanding the role of *Thioploca* species in their habitat.

McHatton et al. (21) studied partially purified cultures of naturally occurring populations of large vacuolated *Beggiatoa* species and showed that these organisms contain substantial activities of membrane-bound nitrate reductase, indicating that they may indeed be capable of using nitrate as the terminal electron acceptor. Significant activities of ribulose-1,5-bisphosphate carboxylase were also detected, evidencing that the vacuolated marine *Beggiatoa* species are capable of autotrophic growth. Using rinsed samples of *Thioploca* material from a mat, Ferdelman et al. (6) were able to demonstrate CO_2-fixing capacity in these preparations, indicating that *Thioploca* has an autotrophic potential.

Since *Thioploca* species live at high densities in the mats of the Chilean marine sediments and could be seen with the naked eye, we decided that it was possible to obtain samples of these organisms sufficiently pure to allow the performance of physiological experiments. We developed a simple method by which we handpicked individual sheaths with trichome bundles with forceps from the top 2 cm of sediments incubated under an N_2 atmosphere. After they had been collected and washed, cells were used for various experiments. By using radiolabeled and unlabeled substrates, a study was made of carbon, nitrogen, and sulfur metabolism. The observed activities were compared with data obtained from field measurements. The results indicate that *Thioploca* species are (metabolically) highly active under anoxic conditions and that they can play a significant role in the total oxidation of sulfide in the mat under anoxic conditions in the presence of nitrate. They appear to be facultative chemolithoautotrophs with a mixotrophic potential, meaning they can use sulfide or sulfur as an energy source for growth and CO_2 fixation and can use acetate under these conditions as an additional carbon source. Evidence presented in this study points to ammonium as the end product of nitrate reduction, although conversion to dinitrogen gas cannot be ruled out. Oxygen at approximately 10% air saturation did not inhibit the observed CO_2 fixation.

MATERIALS AND METHODS

Samples were collected in January and February 1997 on the continental shelf within the Bay of Concepción, central Chile, onboard the research vessel *Kay Kay*, and the laboratory work was performed at the Marine Biological Station of Dichato, both of the University of Concepción. Sampling was performed at 34 m of water depth at station 7 (32), at 36°36′5″S, 73°00′6″W. At this station, at the time of sampling, the percentage of organisms in the upper 2 cm of the sediment found to be *T. araucae* was 39 to 67%. The ratio of the biovolumes of *T. araucae* and *T. chileae* is approximately 70:30, and therefore, the majority of the community consisted, in biovolume, of *T. araucae*.

Collection. Sediment samples were obtained by a Rumohr corer with Plexiglas cores (inside diameter, 9.5 cm), stored at 4°C, and processed within 8 days of sampling. The top 1 to 2 cm was removed and placed on ice in an N_2-filled glove bag (Sigma-Aldrich, Zwÿndrecht, The Netherlands). *Thioploca* sheaths with bundles of trichomes (1 to 2 cm in length) were collected with forceps and transferred to synthetic medium (containing [per liter] 25 g of NaCl, 6 g of $MgSO_4 \cdot 7H_2O$, 1 g of $CaCl_2$, 0.5 g of K_2HPO_4, 0.1 g of KH_2PO_4, and 0.5 g of $NaHCO_3$ [pH 7]) or to synthetic medium without $NaHCO_3$, supplemented with 0.05% (wt/vol) thioglycolate and 1 mg of catalase per liter (Sigma-Aldrich). The latter medium (hereafter referred to as medium, unless stated otherwise) was found to give the best results. Media were sparged with N_2 for 30 min for anoxic conditions and subsequently stored at 4°C. Shortly before use, media were sparged again with N_2 for 10 min, unless stated otherwise, while kept cold. Under an N_2 atmosphere in a glove bag, the sheaths with trichome bundles were washed twice by transfer into fresh medium and were incubated in airtight 6-ml vials equipped with rubber septa (Exetainer; Labco, High Wycombe, United Kingdom). The medium in the vial was changed twice by decanting under an N_2 atmosphere, with minimal disturbance of the sheaths and trichome bundles. The incubation volume was adapted to the conditions of the experiment performed. Under anoxic conditions, substrates were injected through the rubber septum. Residual sediment, left after washing *Thioploca* trichome bundles, was used as a control. In these controls, the amount of sediment used was 5 to 10 times higher than the estimated contamination of sediment attached to washed sheaths with trichome bundles. Another control consisted of disrupted *Thioploca* sheaths with trichome bundles (equal to the amount used in the experiment) which had been disintegrated mechanically by a Potter-Elvehjem homogenizer (Fisher Scientific, Zoetermeer, The Netherlands). This control was necessary because observations under the fluorescence microscope had shown that the sheaths were covered with epibiontic bacteria, including sulfate-reducing filamentous bacteria of the genus *Desulfonema* (36, 40). After the Potter-Elvehjem treatment, it was observed under the fluorescent microscope that *Thioploca* trichomes were mechanically disrupted, while the majority of the epibiontic bacteria remained intact. Bundles consisting of sheaths with bundles of trichomes will hereafter in this paper be referred to as "trichome bundles," while sheaths with Potter-Elvehjem-treated bundles will hereafter be referred to as "disrupted trichome bundles."

The method of handpicking *Thioploca* sheaths with trichome bundles had a bias towards *T. araucae*, which led to a majority (80 to 90% in biovolume) of this species in the samples.

Incubation. For each experiment, approximately 100 *Thioploca* trichome bundles were collected in a final volume of 3.5 ml under an N_2 atmosphere in gas-tight vials, unless stated otherwise. Vials were incubated with substrates in a water bath at approximately 12°C. At specific time intervals, samples were taken with a syringe previously flushed with dinitrogen and analyzed for ammonium, nitrite, sulfide, thiosulfate, and sulfate.

Analytical procedures. Nitrite and ammonium in the supernatant were determined colorimetrically (as described in references 14 and 5, respectively). Intracellular nitrate concentrations were measured with a miniaturized version of the standard colorimetric method of Grasshoff et al. (13). Nitrate was measured in 100-μl extracts of rinsed and dried *Thioploca* trichomes. Trichomes 5 to 40 mm in length were dissected under the microscope with the help of forceps and needles. Length and width of these trichomes were measured and, after washing and drying, the filaments were resuspended in 50 μl of distilled water to measure the nitrate concentration. Biovolume was calculated from trichome length and width. An average nitrate concentration ($n = 27$) of 160 ± 150 mM was found. Protein was determined by the microbiuret method of Goa (11). The observed protein concentrations were in agreement with calculations for expected protein content. Where no protein measurements were available (in experiments where labeled compounds were used), a protein content of 315 ± 95 μg was assumed for 100 *Thioploca* sheaths with trichomes (which is based on an average of 34 protein measurements of *Thioploca* samples). Thiosulfate was derivatized with monobromobimane (4) and analyzed by reversed-phase high-performance liquid chromatography (28). Sulfide was determined either colorimetrically according to the method of Cline (2) or by the method described for thiosulfate determination. Standards for sulfide and thiosulfate were prepared in degassed sulfate-free medium ($MgCl_2$ instead of $MgSO_4$ and without thioglycolate and catalase). Sulfate was determined by nonsuppressed ion chromatography as described by Ferdelman et al. (6). Since high concentrations of chloride interfere with sulfate analysis by ion chromatography, chloride was removed from the samples by adding 40 mg of Ag^+-loaded cation exchange (Ag 50W-X8; Bio-Rad) per 150-μl sample and incubating for 2 h at room temperature. After centrifugation and filtering, the sample was analyzed. Standards were treated in the same way.

^{15}N experiments and mass spectrometry. Under anoxic conditions, a concentrated solution of $Na^{15}NO_3^-$ was added to gas-tight vials, each with 120 *Thioploca* bundles in 4.5 ml of medium. No direct protein measurements could be performed and, therefore, a total protein content of 378 ± 114 μg was assumed on the basis of the average protein content of 100 *Thioploca* bundles (see above). The headspace was changed with He, and at certain time intervals samples were taken for analysis. Total nitrite and ammonium concentrations were measured immediately after centrifugation. To determine the concentration of $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$, samples were removed with a syringe, centrifuged, sterilized by passing the supernatant through a 0.2-μm-pore-size filter (Dynagard; Microgon Inc., Laguna Hills, Calif.), acidified to pH 4 to 5, and stored at −20°C until the time of analysis. At the end of the experiment, 1 to 2% (final concentration) formaldehyde was added to the vial and pressure was equilibrated with He. Vials were stored at 4°C until the headspace could be analyzed for $^{15}N_2$. Analysis and mass spectrometry were performed at the Institute of Biological Sciences, University of Aarhus, Aarhus, Denmark. For determining concentrations and isotopic compositions of $^{15}NO_3^-$ and $^{15}NO_2^-$, samples were neutral-

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ized and incubated with denitrifying bacteria to convert these compounds to N_2 for mass spectrometry analysis (29). The labeling pattern of the obtained N_2 gives an indication of the ratios of labeled and unlabeled nitrate and nitrite present in the samples. However, the denitrifiers used can reduce both nitrate and nitrite. Therefore, the recovered N_2 is the product of the reduction of nitrate as well as nitrite present in the medium. Standards with known concentrations (120 μM) of $^{15}NO_3^-$ and $^{15}NO_2^-$ were included in the assay and confirmed that the conversion efficiency was consistent (standard deviation = 4%) and that residual nitrate and nitrite concentrations were insignificant.

To analyze the isotopic composition of the NH_4^+ formed, hypobromite was added for specific oxidation of ammonium to N_2 (30). The mass spectrometer measured singly and doubly labeled dinitrogen ($^{14,15}N_2$ and $^{15,15}N_2$) in excess of the natural background. From this, the recovery of added ^{15}N in the sampled N_2, NO_3^-, NO_2^-, and NH_4^+ was calculated. Dinitrogen is formed by random isotope pairing and, therefore, the ratio of labeled nitrogen recovered as $^{14,15}N_2$ versus the recovery as $^{15,15}N_2$ was used as a minimum estimate of the ^{14}N:^{15}N ratio in the source (26). If the source is isotopically uniform and constant, the estimate is correct. If several pools are involved, i.e., discrete intracellular nitrate pools in the incubation vial or nitrate and nitrite in the water samples, the true representation of unlabeled nitrogen cannot be much (at the most, 0.5 nmol) less but can be higher, depending on the pool sizes and isotopic variations.

$NaH^{14}CO_3$, [2-^{14}C]acetate, and [^{3}H]acetate incorporation experiments. Several vials were incubated with approximately 30 *Thioploca* bundles in 1.3 ml of medium, in the dark. On the basis of the average protein content of 100 *Thioploca* bundles (see above), the total protein for 30 bundles was assumed to be 94.5 ± 28.5 μg. One hundred micromolar $NaNO_3^-$, 25 μl of CO_2 gas (headspace was approximately 5 ml), and, for all experiments, 1 mM HCO_3^- were added to the medium. Under anaerobiosis, 0.005 nmol of [^{3}H]acetate (~500 μCi) or 0.034 μmol of a [^{14}C]acetate solution (~5 μCi) was added for the labeled-acetate experiments. For the labeled-bicarbonate experiments, 0.139 μmol (~10 μCi) or 1.85 μmol of a neutralized $NaH^{14}CO_3$ solution (~100 μCi for microautoradiography experiments) was added. Experiments with [^{14}C]bicarbonate were performed in the absence as well as in the presence of approximately 70 μM sulfide. At certain time intervals, a vial was opened and the supernatant was analyzed for NO_2^- and NH_4^+. The pellet of *Thioploca* bundles or debris from disrupted bundles was washed four times (by vigorous mixing and subsequent centrifugation) in medium containing 10 mM acetate (when incubated with labeled acetate) or containing 10% trichloroacetic acid (when incubated with $NaH^{14}CO_3$) and then added to 2.4 ml of H_2O and 7.5 ml of scintillation liquid (EcoLite [+]; ICN Biomedicals). This suspension was subsequently analyzed in a scintillation counter (Packard liquid scintillation analyzer model 1600 TR). When necessary, CO_2 was trapped by suspending a small cup filled with 100 μl of 2 M NaOH in the gas-tight vial. After the vial was opened, this solution was neutralized and added to scintillation liquid and counted. Experiments with [^{14}C]acetate took 3 h, experiments with $NaH^{14}CO_3$ required 4 h of incubation (to test the influence of oxygen, trichomes were incubated for 22 h), and trichomes used for microautoradiography were incubated for 4 h or 20 to 22 h.

Microautoradiography. Microautoradiography was performed on the experiments described above. Following incubation with [^{3}H]acetate or with $NaH^{14}CO_3$, bundles were washed six times with medium containing 1 mM acetate or 1 mM HCO_3^-, respectively. Individual sheaths were then sorted onto 25-mm hydroxyapatite Millipore filters (Millipore Corp., Bedford, Mass.) or left in 2 ml of medium containing 2% formaldehyde. Filters were subsequently washed in filter-sterilized (first through 0.45-μm-pore-size then through 0.2-μm-pore-size Gelman filters [Millipore]) medium with 1 mM phosphate buffer. After drying, the filters were stored at 4°C. At the end of the cruise, the filters and samples, stored in 2% formaldehyde, were analyzed. Some filters were stained with 2% (wt/vol) erythrosin-B (Sigma-Aldrich) and were subsequently destained by placing them face up on deionized H_2O-saturated pieces of gauze. Filters were air dried, attached to microscope slides, and optically cleared by fuming acetone (27). Cleared filters were prepared for microautoradiography by dipping in Kodak NTB-2 nuclear track emulsion. After exposure (1 to 3 weeks), autoradiographs were developed (Kodak D-19 developer), fixed, rinsed, and air dried prior to microscopic examination with a Nikon Labophot 2 phase-contrast microscope at ×200 to ×400 magnification. Photographs were recorded on either Ilford Pan-F fine-grain black-and-white or Kodachrome 200 color slide 35 mm film.

Calculations. (i) ^{15}N experiments: the ratio between added [^{15}N]nitrate and intravacuolar [^{14}N]nitrate. Addition of 100 μM [^{15}N]nitrate in 4.5 ml of medium yields 0.45 μmol of [^{15}N]nitrate. One hundred twenty *Thioploca* bundles have a protein content of approximately 0.38 ± 0.11 mg (see analytical procedures). Assuming that 50% of the dry weight is protein, and 24% of the wet weight is dry weight and knowing that 90% of the cell is vacuole (as measured in this study and by Maier et al. [20]), then the total wet weight of 120 bundles is 0.38 × 2 × [100/24] × [100/10] = 31.7 ± 9.17 mg, of which 28.5 ± 8.25 mg is vacuolar liquid. Assuming that 1 mg is equal to 1 μl of liquid in the vacuole, then the volume of all vacuoles in the bundles used for the experiments will be approximately 28.5 ± 8.25 μl. If all the added [^{15}N]nitrate is transported into the vacuoles, then this would lead to a concentration of the label of 15.8 ± 4.57 mM (0.45 μmol in 28.5 μl). Since the vacuoles are filled with an average of 160 mM (see Materials and Methods) unlabeled nitrate, the labeled nitrate will be diluted to 9.9% ± 2.85%. If *Thioploca* trichome bundles were damaged and all internal nitrate were released, then approximately 5.6 μmol (160 mM in 28.5 μl) would be released into 4.5 ml of medium. This would lead to an increase in nitrate concentration of 1.2 mM, i.e., a 12-fold increase, which would be visible during measurement of the ^{15}N:^{14}N ratio of the external nitrate pool.

(ii) Sulfide oxidation: the ratio between observed sulfide reoxidation rates and specific activity of *Thioploca*. Sulfate reduction rates measured in sediments at station 7 at the time of sampling were approximately 30 mmol m^{-2} day^{-1} (26 to 37 mmol m^{-2} day^{-1} [34]). If all sulfide produced from this reduction was subsequently oxidized by the *Thioploca* mats, then the mats should be able to oxidize sulfide at the same rate, which is equal to 20.8 μmol m^{-2} min^{-1}. Schulz et al. (32) estimated the wet biomass of trichomes without sheaths to be 50 to 120 g m^{-2}. Assuming an average of 85 g (wet weight) m^{-2} and knowing that 90% of the biovolume is taken up by the central vacuole, the active cytoplasm weighs approximately 8.5 g (wet weight) m^{-2}. This active cytoplasm is then responsible for the sulfide oxidation rate as stated above, which would give a specific rate of 20.8/8.5 = 2.4 μmol min^{-1} g of wet weight^{-1}. Assuming that 24% of the wet weight is dry weight and that 50% of the dry weight is protein, the sulfide oxidation rate in vivo should be 2.4 × (100/24) × 2 = 20.4 nmol min^{-1} mg of protein^{-1}. In analogy, Ferdelman et al. (6) found an in vivo sulfate reduction rate of approximately 17.5 mmol m^{-2} day^{-1} for station 7. This reduction rate corresponds to a sulfide oxidation rate by *Thioploca* of 12 μmol m^{-2} min^{-1}. Making the same assumptions as above, this oxidation rate is equal to 11.8 nmol min^{-1} mg of protein^{-1}.

RESULTS

Cultivation and survival. *Thioploca* bundles, consisting of 10 to 20 trichomes in a sheath, were collected from the top 2 cm of the sediment with forceps and cleaned by several transfers through medium. In developing the method, there were three parameters to be considered. Firstly, motility of *Thioploca* trichomes under a microscope was a measure of viability (19, 31). Secondly, it was observed during the initial experiments that high nitrite concentrations (50 to 100 μM), in addition to high nitrate concentrations, were obtained within 1 h of anoxic incubation of the bundles, suggesting lysis of the cells. Thirdly, in previous studies of *Thioploca* and large *Beggiatoa* species, it was observed that these organisms are highly sensitive to oxygen (16, 22) and that catalase is required in the growth medium of *Beggiatoa* species (1). These considerations led to several improvements of the final cleaning procedure. To avoid contact with oxygen, all steps of the method (collection, washing, and incubation) were performed under a dinitrogen atmosphere. Sparging of the medium during incubation for anoxic conditions was avoided, because this mechanically affected the trichomes. To keep the growth medium anoxic, thioglycolate was added as a reducing agent and catalase was also included in the synthetic medium. The endogenous ammonium production rate did not increase after thioglycolate was included in the medium, suggesting that this compound was not used as a carbon source. Survival experiments, as monitored under the microscope (31), showed that the medium highly improved survival and that trichomes did not show a decrease in motility over 2 days of incubation. From similar survival experiments, it was further concluded that *Thioploca* cells could get damaged when transferred through a liquid-gas interface. To avoid this damage, washing was performed twice by draining off approximately 80% of the medium, such that the trichomes were still in the liquid, and then fresh medium was added.

Further improvement of the method was obtained by keeping the sediment on ice during collection and by washing the trichomes and omitting bicarbonate in the medium, since removal of CO_2 during sparging of the medium caused an increase in pH. In all subsequent experiments these cleaned *Thioploca* trichomes were used.

N metabolism. Inside *Thioploca* cells, intravacuolar nitrate concentrations measured up to 500 mM, with an average of 160 ± 150 mM (n = 27). *Thioploca* trichome bundles, incubated in medium without addition of NO_3^-, produced NO_2^- and NH_4^+. The average NH_4^+ production (with internal sulfur

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available but no added external electron donor) of eight independent measurements was 1.0 ± 0.3 nmol min^{-1} mg of $protein^{-1}$, whereas NH_4^+ production by the controls (disrupted trichome bundles or NO_3^--supplemented sediment) was 0.07 ± 0.03 nmol min^{-1} mg of $protein^{-1}$ and 0.03 ± 0.01 nmol min^{-1} mg of $protein^{-1}$, respectively. Nitrite production was negligible (<0.1 nmol min^{-1} mg of $protein^{-1}$) in most experiments but was sometimes observed at a maximum production rate of 1 nmol min^{-1} mg of $protein^{-1}$.

^{15}N-labeling experiments. To determine whether *Thioploca* reduces NO_3^- to NH_4^+ or to N_2, experiments were performed by using $^{15}NO_3^-$. After addition of $^{15}NO_3^-$, total NO_2^- and total NH_4^+, as well as $^{15}NO_3^-$/$^{15}NO_2^-$ and $^{15}NH_4^+$, were monitored over time (the ^{15}N-labeling method does not differentiate between labeled NO_3^- and NO_2^-; see Materials and Methods). At the end of the experiment, total $^{15}N_2$ and the ratio between unlabeled and (singly or doubly) labeled N_2 were determined. Figure 1A shows that 95% of the externally available NO_3^- or NO_2^- originated from the supplied $^{15}NO_3^-$. During the course of the experiment, the specific labeling of the extracellular nitrate pool remained 95%, indicating that the trichomes were not damaged and did not release $^{14}NO_3^-$ (see calculations in Materials and Methods). Figure 1A shows that nearly all of the nitrate was taken up linearly during the course of the experiment in approximately 3.5 h. As calculated in Materials and Methods, if all of the $^{15}NO_3^-$ were taken up by *Thioploca* trichomes, the label would be diluted inside the vacuoles to 9.9% ± 2.85% (see calculations in Materials and Methods). The NH_4^+ produced during the experiment (1.8 ± 0.4 nmol min^{-1} mg of $protein^{-1}$) was 44% (at 85 min) and 48% (at 145 and 215 min) labeled (Fig. 1B). This difference in specific labeling between the externally available nitrate pool and the NH_4^+ produced indicates that the internal nitrate of *Thioploca* trichomes contributes substantially to the total NH_4^+ production. However, the amount of label is not diluted as much as would be expected if all the labeled nitrate were first taken up in the vacuole and subsequently reduced. Therefore, it seems that in the cytoplasm the [^{15}N]nitrate is readily reduced before it reaches the vacuole. Figure 1C shows that at the end of the experiment N_2 had also been produced, but the amount was only 15% (nanomoles of nitrogen per nanomole of nitrogen) of the total amount of nitrogen compounds produced. The specific labeling of the N_2 was substantially higher than that of NH_4^+, suggesting that epibionts might be responsible for this production, although the amount of unlabeled N_2 is a minimum estimate (see Materials and Methods). This implies that, although N_2 appeared to not be a major product of the washed *Thioploca* sample, the present data cannot completely rule out that *Thioploca* can reduce nitrate to N_2, i.e., denitrify, in addition to the observed full reduction of nitrate to ammonium.

Sulfur metabolism. To measure sulfide oxidation rates, *Thioploca* trichomes were incubated in medium. After addition of approximately 50 μM sulfide to the vials, sulfide, nitrite, and ammonium concentrations were observed over time. An ammonium production rate by the *Thioploca* suspension of approximately 1.9 nmol min^{-1} mg of $protein^{-1}$ was observed, whereas the controls (sediment samples and samples treated in a Potter-Elvehjem homogenizer) showed activities of 0.02 and 0.04 nmol of NH_4^+ min^{-1} mg of $protein^{-1}$, respectively (Table 1). The sulfide consumption rate decreased with decreasing sulfide concentrations, but the average maximum rate was approximately 4.2 nmol min^{-1} mg of $protein^{-1}$, while the controls showed a 10- to 200-fold lower consumption rate. Since the control, which contained sediment, represented an overestimate of the amount of sediment attached to the trichomes,

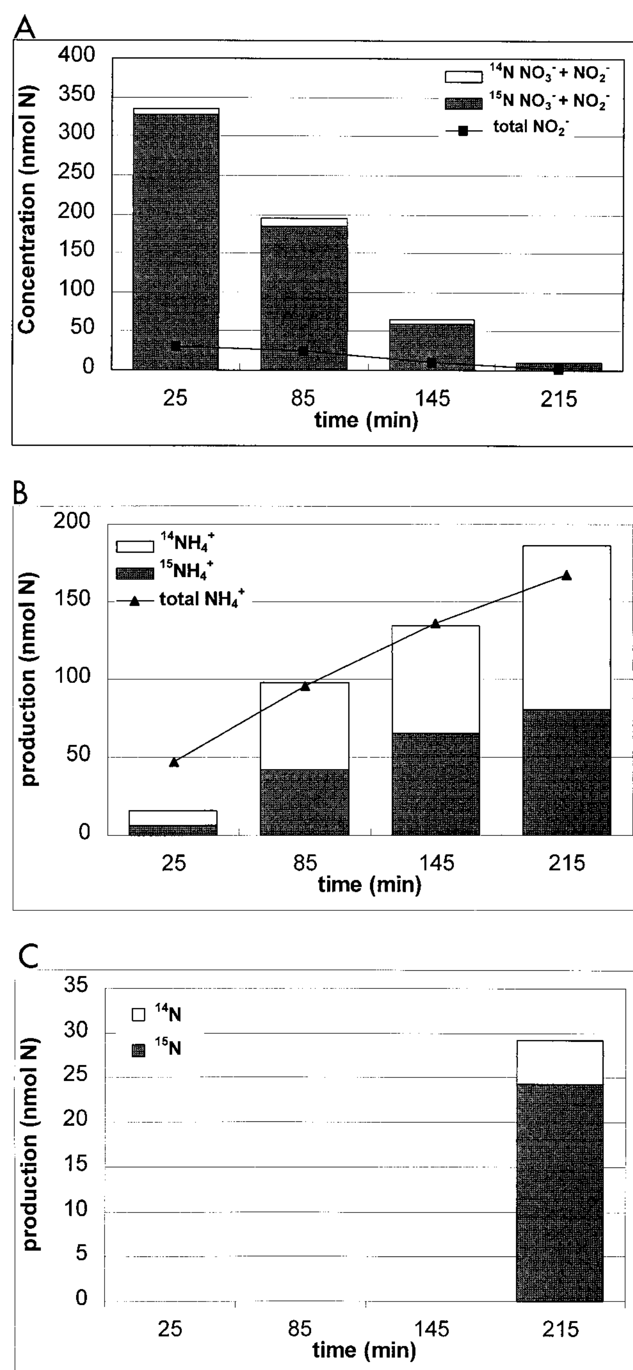

FIG. 1. Distribution of label during ammonium and dinitrogen production by *Thioploca* trichome bundles after incubation in medium with [^{15}N]nitrate under a helium headspace. (A) ^{14}N- and ^{15}N-labeled nitrate and nitrite in the growth medium; (B) ^{14}N- and ^{15}N-labeled ammonium in the growth medium; (C) total amount of ^{14}N and ^{15}N derived from dinitrogen species in the headspace ($^{14,14}N_2$, $^{14,15}N_2$, $^{15,14}N_2$, $^{15,15}N_2$). Symbols: ▲, total ammonium measured colorimetrically; ■, nitrite measured colorimetrically.

the contribution of the sediment to the total activity in live *Thioploca* experiments was negligible. Trichomes, which had been collected and incubated 2 days before the experiment was performed, showed continued motility under the microscope, an ammonium production rate of 3.2 nmol min^{-1} mg of pro-

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TABLE 1. Specific rates of ammonium production and sulfide consumption by *Thioploca* trichrome bundles incubated in medium

Sample	NH_4^+ production rate (nmol min^{-1} mg of $protein^{-1}$)	HS^- consumption rate (nmol min^{-1} mg of $protein^{-1}$)
Thioploca sheaths with trichomes	1.9	4.2
Thioploca sheaths with trichomes (2-day-old culture)	3.2	5.5
Disrupted *Thioploca* sheaths with trichomes	0.04	<0.02
Sediment (with 100 μM NO_3^-)	0.02	0.5

tein^{-1}, and a sulfide consumption rate of 5.5 nmol min^{-1} mg of protein^{-1}, after addition of sulfide to the incubation medium. The cells appear to reduce their metabolic activity when no external substrate is present (NH_4^+ production rate is approximately 1 nmol min^{-1} mg of protein^{-1}) but are able to respond quickly when substrate is encountered again (NH_4^+ production increases to 3.2 nmol min^{-1} mg of protein^{-1}). An internal nitrate concentration of 160 mM would be sufficient for approximately 200 h (given our estimate that 90% of the cell is vacuole and that 1 mg of vacuolar liquid is equal to 1 μl), with an NH_4^+ production rate of ± 1 nmol min^{-1} mg of protein^{-1}. This experiment indicates that trichomes are still active and motile after 2 days and that the internal NO_3^- is sufficient for at least 2 days of normal metabolism without external supply of fresh substrate. In an experiment where two different concentrations of sulfide were added to *Thioploca* suspensions (Fig. 2), a small accumulation of thiosulfate, which was higher when the sulfide concentration was higher, was observed. This thiosulfate accumulation suggested that this compound may be a by-product or an intermediate in sulfide oxidation, and therefore, *Thioploca* might be able to oxidize thiosulfate to sulfate. To investigate this possibility, approximately 100 μM thiosulfate was added to *Thioploca* trichome bundles incubated in sulfate-free medium with $MgCl_2$ instead of $MgSO_4$ and without thioglycolate and catalase, to avoid interference with analytical measurements. In these experiments (results not shown) only a slight thiosulfate consumption was observed.

Freshly harvested cells contain high concentrations of elemental sulfur (200 nmol mm^{-3}) and nitrate (160 mM), which could influence the observed oxidation rates. In line with the calculations made above for consumption of internal nitrate during starvation, it can be calculated that the internal sulfur would be sufficient for approximately 170 h. Thus, it was possible that partially starved cells would show higher oxidation rates. Therefore, suspensions of 50 *Thioploca* trichome bundles were sulfur starved for 45 h by incubation in 4.5 ml of sulfate-free medium in the presence of 50 μM nitrate. After 45 h, approximately 135 μM sulfide or 70 μM thiosulfate was added to the suspensions and ammonium, sulfide, thiosulfate, and sulfate concentrations were monitored over time (results not shown). During anoxic sulfur starvation, productions of NH_4^+ and SO_4^{2-} by *Thioploca* trichome bundles were approximately 1 and 2 to 3 nmol min^{-1} mg of protein^{-1}, respectively. In the control with disrupted trichome bundles, NH_4^+ and SO_4^{2-} production levels were 0.1 and 0.45 nmol min^{-1} mg of protein^{-1}, respectively. This activity was ten times lower than the activity of the trichome bundles, indicating that epibiontic bacteria are not responsible for the observed sulfate production. Subsequent sulfide addition (135 μM) to these sulfur-starved trichome bundles led to an initial sulfide consumption rate of 10.7 nmol min^{-1} mg of protein^{-1}, which was the largst oxidation rate observed, while ammonium and sulfate production did not increase significantly. There was no production of sulfite during these experiments; however, accumulation of thiosulfate was observed in the disrupted control (up to 21 μM) during starvation. This control contained sulfur compounds released from the ruptured *Thioploca* trichome bundles, suggesting that epibiontic bacteria may be responsible for thiosulfate accumulation. After addition of sulfide to intact, starved trichome bundles, thiosulfate accumulation also occurred (up to 10 μM). After addition of thiosulfate to sulfur-starved *Thioploca* trichome bundles, only a low thiosulfate consumption rate was measured, which was equal to the rate observed previously. Measurements of this consumption rate

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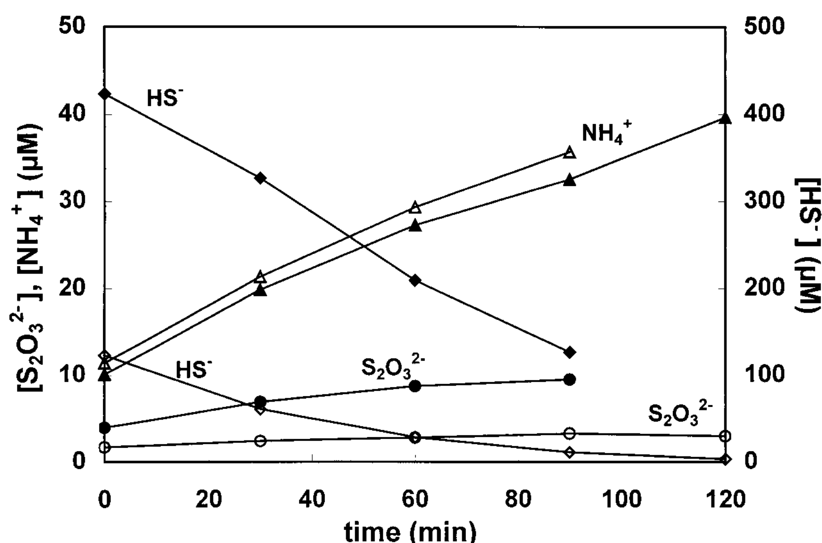

FIG. 2. Thiosulfate production by *Thioploca* trichome bundles incubated anoxically in medium with two different initial sulfide concentrations. Open symbols, 100 μM initial sulfide concentration; closed symbols, 400 μM initial sulfide concentration. Symbols: triangle, ammonium; circle, thiosulfate; diamond, sulfide.

TABLE 2. Specific rates of NH_4^+ production and ^{14}C incorporation by *Thioploca* trichome bundles incubated in medium after addition of [^{14}C]acetate or $NaH^{14}CO_3$

Sample	NH_4^+ production rate (nmol min^{-1} mg of protein^{-1})	^{14}C incorporation rate (nmol min^{-1} mg of protein^{-1})
Thioploca trichomes + ~5 μCi of [^{14}C]acetate	4.0	0.37
Thioploca trichomes + ~10 μCi of $NaH^{14}CO_3$	1.3	0.5–0.8
Thioploca trichomes + ~10 μCi of $NaH^{14}CO_3$ + 70 μM Na_2S	1.7	0.3–0.5
Thioploca trichomes + ~100 μCi of $NaH^{14}CO_3$	1.2	0.25–0.35
Thioploca trichomes + ~100 μCi of $NaH^{14}CO_3$ + 2% O_2	0.65	0.36–0.48
Disrupted trichomes + ~3 μCi of [^{14}C]acetate	<0.01	<0.01
Disrupted trichomes + ~100 μCi of $NaH^{14}CO_3$	<0.01	0.01

also showed high variability. Addition of thiosulfate had no effect on ammonium or sulfate production.

Carbon metabolism. In order to gain some insight into the carbon source used by *Thioploca* for its cell material, experiments were performed with radioactively labeled acetate and bicarbonate additions (Table 2) in combination with microautoradiography.

(i) $NaH^{14}CO_3$. Addition of $NaH^{14}CO_3$ to a *Thioploca* suspension resulted in a linear incorporation rate of 0.4 to 0.8 nmol min^{-1} mg of protein^{-1} and an NH_4^+ production rate of approximately 1.3 to 1.7 nmol min^{-1} mg of protein^{-1}. Addition of sulfide (ca. 70 μM) did not have a significant effect on the incorporation rate. The presence of low concentrations of oxygen (ca. 10% air saturation) also did not have a significant effect on the rates of $NaH^{14}CO_3$ fixation. Control experiments with disrupted trichome bundles showed a ^{14}C fixation rate of only 0.01 nmol min^{-1} mg of protein^{-1}, which is 1 to 3% of the rates observed in intact *Thioploca* suspensions. Intact bundles obtained from these experiments were used for microautoradiography. Uptake of ^{14}C appeared to be largely dominated by *Thioploca* trichome bundles, since there was no significant uptake of label in epibiontic microbial cells associated with the sheaths. Microautoradiography and control experiments with disrupted trichome bundles both show that the measured uptake rate of ^{14}C by the *Thioploca* suspension primarily represents the activity of the trichome bundles and not of epibionts. Differences in intensity of labeling among trichomes were observed, but no differences were observed that could be due to possible differences in the physiology of the two major species in the sample (*T. araucae* and *T. chileae*). Among individual trichomes, labeling was homogeneously distributed along their entire length, and labeling was concentrated along the transverse walls (Fig. 3), suggesting the presence of a vacuole.

(ii) [^{14}C]- and [^3H]acetate. After addition of [^{14}C]acetate to *Thioploca* trichome bundles, an acetate uptake rate of approximately 0.4 nmol min^{-1} mg of protein^{-1} and an NH_4^+ production rate of 4 nmol min^{-1} mg of protein^{-1} were observed. Unaccounted loss of label was less than 10%. $^{14}CO_2$ production was not significant (less than 2% of acetate incorporation), indicating that under these conditions (i.e., in the presence of internal sulfur) acetate was not used as a significant energy source. Control experiments with disrupted trichome bundles showed an incorporation rate of less than 0.01 nmol min^{-1} mg of protein^{-1}, which was less than 2% of the activity of intact *Thioploca* trichome bundles.

Bundles incubated with [^3H]acetate and showing similar activities, as described above, were examined by microautoradiography. Results indicate acetate uptake by trichomes as well as by bacteria associated with the sheath (Fig. 4). However, taking into account the volume ratio between trichomes and attached bacteria, uptake of label was largely dominated by *Thioploca* trichomes. This indicated that measured uptake

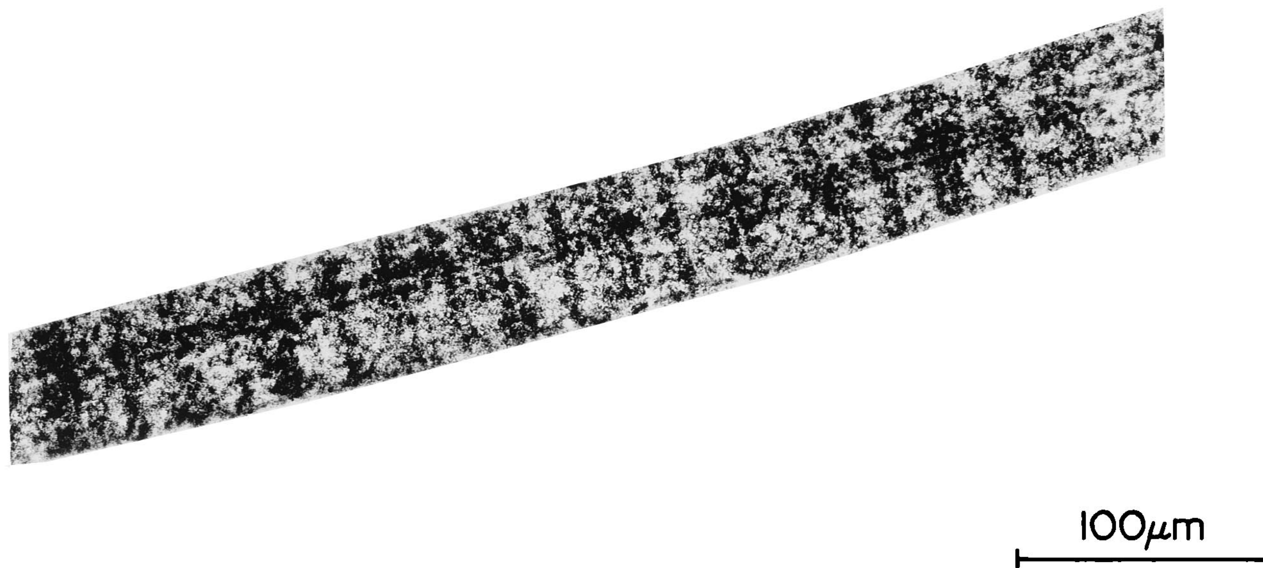

FIG. 3. High-magnification microautoradiogram of a single *Thioploca* trichome incubated with $NaH^{14}CO_3$.

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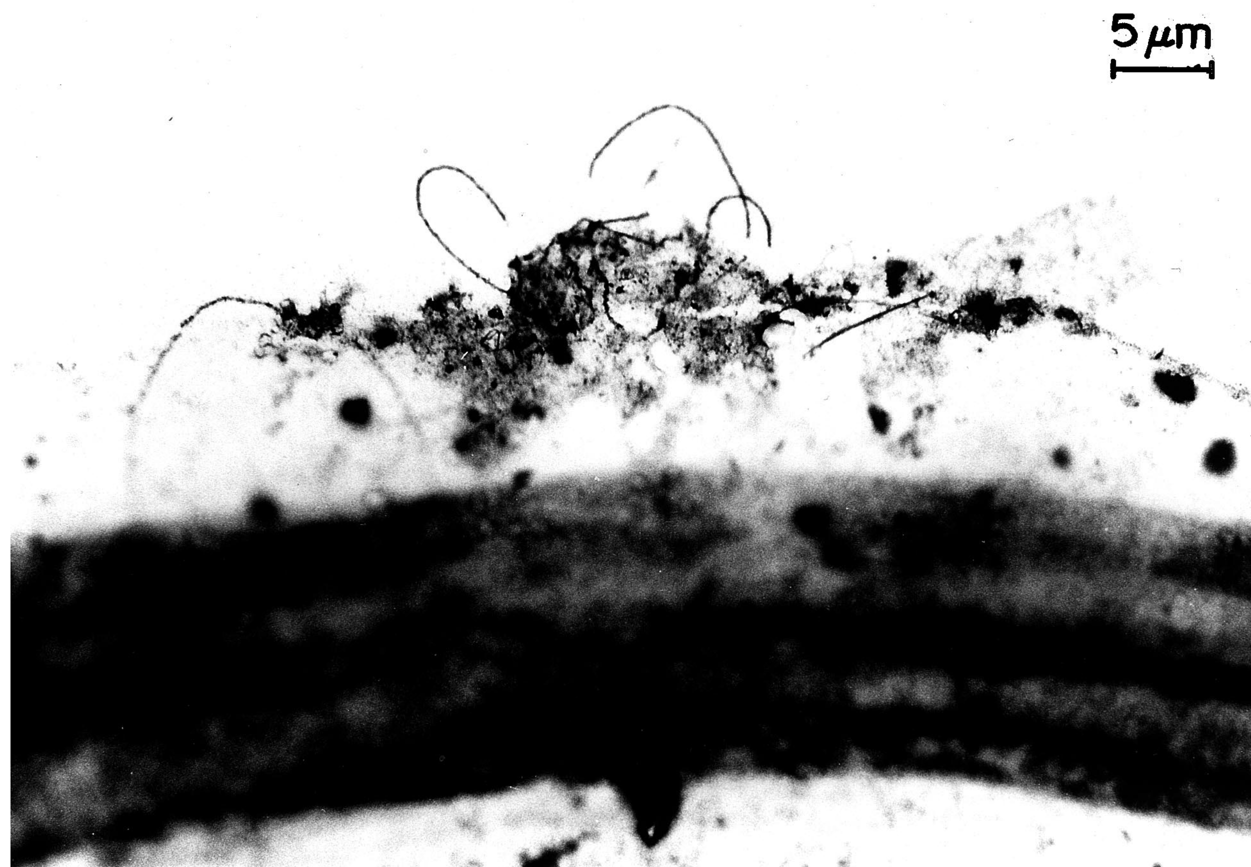

FIG. 4. Low-magnification microautoradiogram of a *Thioploca* sheath with trichomes incubated with [^{3}H]acetate, showing ^{3}H uptake by trichomes and associated bacteria. The heavily labeled trichomes are out of focus to show uptake by bacteria situated on the sheath. This image has been selected for its high concentration of epibionts and is not representative of the overall results from the microautoradiography.

rates of label were primarily due to *Thioploca* trichomes. No differences were observed between the two species of *Thioploca* present. Labeling with the soft β emitter ^{3}H gives a higher resolution than labeling with ^{14}C and, therefore, more clearly shows the difference between uptake of label by epibionts and by *Thioploca* trichomes. Figure 5 shows, more clearly than with ^{14}C label, that the ^{3}H label is situated along the transverse cell walls. This reflects the presence of a large central vacuole, leaving the cytoplasm concentrated along the cell walls. The results with ^{3}H labeling showed uniformity in cell to cell labeling along the entire length of a trichome, as described above for ^{14}C labeling, as well as differences in cellular labeling between individual trichomes. Uniform trichome labeling was observed immediately after addition of the labeled acetate and increased in intensity with time, indicating accumulation of label, reflecting measured uptake rates.

DISCUSSION

Filamentous sulfur bacteria of the genus *Thioploca* occur along the continental shelf off the coast of Chile and Peru. High sulfate reduction rates in *Thioploca* mats have been reported (6). *Thioploca* species are able to store internally high concentrations of sulfur globules and nitrate. It is assumed that these vacuolated *Thioploca* species use their internally stored nitrate as a terminal electron acceptor for sulfide and sulfur oxidation (7). The product of nitrate reduction, however, was still unknown. Also, *Thioploca* trichome bundles have been shown to take up both CO_2 and acetate, but quantitative data were lacking (19). Therefore, this study was undertaken to investigate carbon, nitrogen, and sulfur metabolism in *Thioploca* species.

A method was developed to collect and clean individual sheaths with bundles of trichome bundles from the top 2 cm of the sediment. After being collected and washed under an N_2 atmosphere, trichomes were still motile and could be used for physiological experiments. In the early stages of method development, high cellular or extracellular nitrite and nitrate concentrations were observed, possibly as a result of lysis of the cells. However, after adjustments (anoxic conditions, medium supplemented with thioglycolate and catalase, low temperature, and avoiding transfer through the gas-liquid interface) these nitrite and nitrate accumulations were no longer observed. *Thioploca* trichome bundles incubated for 2 days still showed activity comparable to activities measured immediately after incubation (Table 1), indicating that trichome bundles were able to survive and remain physiologically intact in the synthetic medium.

Nitrogen metabolism. During experiments performed with intact *Thioploca* trichome bundles, without addition of external substrate, an ammonium production rate of approximately 1 nmol min^{-1} mg of protein^{-1} was observed. Since in these experiments the only available substrates were internally stored sulfur and nitrate in *Thioploca* trichome bundles, it is highly unlikely that epibiontic bacteria were responsible for this NH_4^+ production. Experiments using [^{15}N]nitrate resulted

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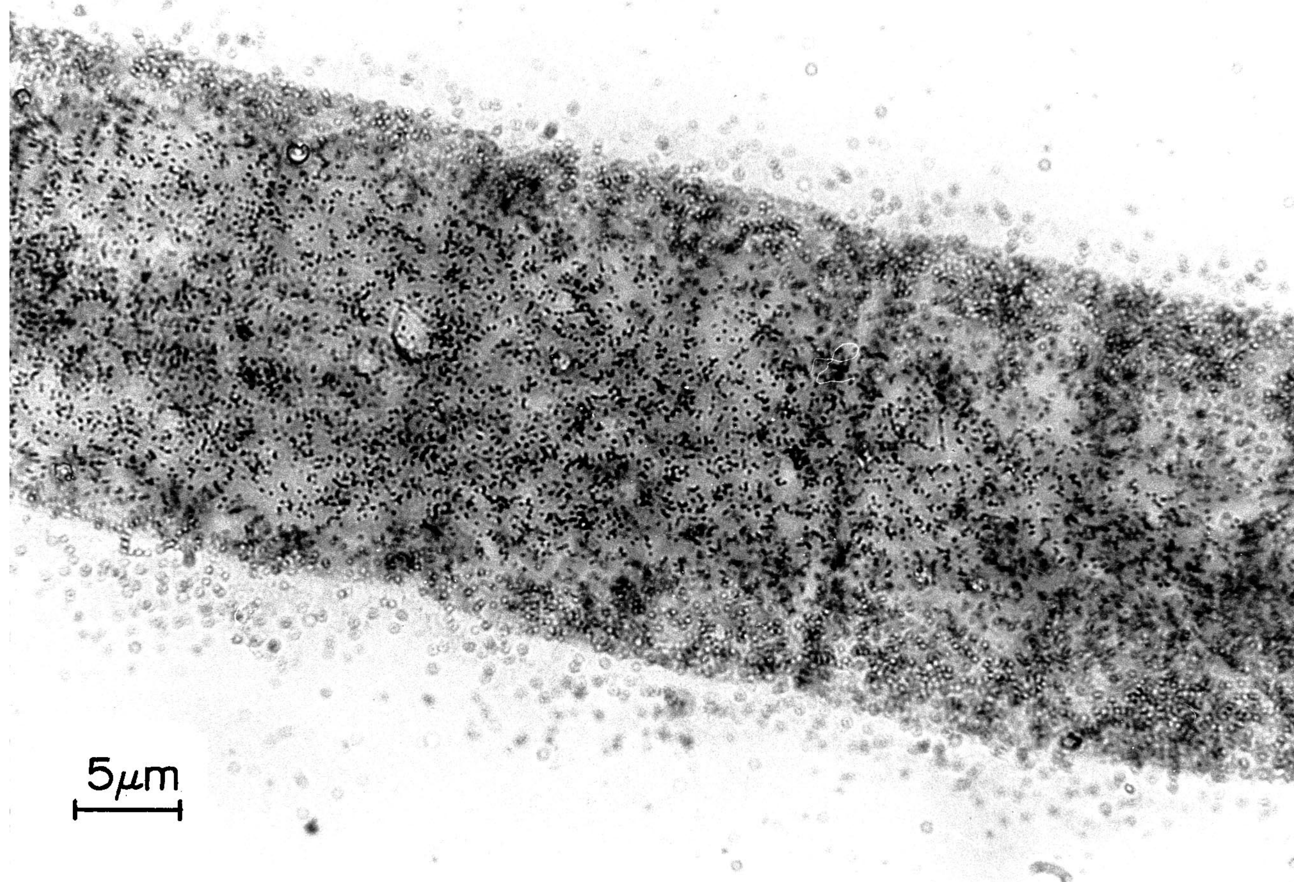

FIG. 5. High-magnification microautoradiogram of *Thioploca* trichome incubated with [^{3}H]acetate.

in uptake of all the labeled nitrate in approximately 3.5 h. The specific label of the external NO_3^- pool remained 95% and was, therefore, not diluted during the course of the experiment, indicating that trichome bundles were not damaged and leaking NO_3^-. Analysis showed an increase in NH_4^+ production (1.8 ± 0.4 nmol min^{-1} mg of protein^{-1}) immediately after addition of labeled NO_3^-. The specific label of NH_4^+ produced was maximally 48%. This indicates that NH_4^+ is produced from a different NO_3^- pool than the external pool, since the external pool was more heavily labeled. The only other source of NO_3^- is the internal NO_3^- of *Thioploca* trichome bundles, which is not available to epibiontic bacteria. This indicates that *Thioploca* species reduce NO_3^- to NH_4^+. Another argument is that there was no electron donor for NO_3^- reduction available in these experiments, except the internally stored sulfur.

If all the NO_3^- were taken up by *Thioploca* trichome bundles, this would lead to an increase in NO_3^- of 15.8 ± 4.57 mM within the vacuole (see calculations in Materials and Methods). Since the average NO_3^- concentration in the vacuoles was found to be 160 mM, this would correspond to a dilution of the $^{15}NO_3^-$ to a specific labeling ($^{15}NO_3^-$:$^{14}NO_3^-$) of 9.9% ± 2.85% (see calculations in Materials and Methods). If this NO_3^- pool were subsequently reduced, then labeling of the NH_4^+ would be much lower than 48%. The fact that the produced NH_4^+ is more heavily labeled suggests that during transport of the labeled NO_3^- across the membrane into the thin layer of the cytoplasm, it is readily reduced. If the transport rate of nitrate from the vacuole into the cytoplasm is in the same order of magnitude, then this would explain why the actual specific labeling of the cytoplasm is near 48%.

N_2 was also detected in the headspace and was more heavily labeled than the NH_4^+ produced. However, since the amount of unlabeled N_2 was a minimum estimate (see Materials and Methods), the specific labeling of the produced N_2 can actually be lower than shown in Fig. 1C, suggesting that the produced N_2 may also have a different specific labeling than the external pool of NO_3^-. Therefore, on the basis of these data, one cannot completely exclude the possibility that *Thioploca* can also reduce NO_3^- to N_2. The amount of N_2 produced, however, was approximately 15% of the amount of NH_4^+ produced, emphasizing that under the conditions tested, reduction of NO_3^- to NH_4^+ is the preferred pathway in *Thioploca*, and this pathway is probably used for energy conservation. Conservation of energy from NO_3^- reduction to NH_4^+ has also been found in *Sulforospirillum deleyianum*, which uses sulfide as an electron donor (3), and in *Campylobacter* species (33), where H_2 was used as an electron donor. The ecological implications of the finding that *Thioploca* prefers to produce NH_4^+ are significant, since this means that nitrate reduction by *Thioploca* does not lead to nitrogen loss in this vast ecosystem along the entire coast of Chile and Peru.

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Sulfur metabolism. After addition of sulfide to *Thioploca* trichome bundles in a particular experiment, a sulfide oxidation rate of approximately 4.2 nmol min^{-1} mg of $protein^{-1}$ was observed in the absence of external nitrate. The NH_4^+ production was 1.9 nmol min^{-1} mg of $protein^{-1}$, resulting in a ratio of 2.2 between sulfide oxidized and NH_4^+ produced. If the sulfide were oxidized to elemental sulfur and NO_3^- were reduced to NH_4^+, then the expected ratio of sulfide to ammonium would be 4. If sulfide were oxidized to sulfate then a ratio of 1 would be expected. The observed ratio suggests that the sulfide is oxidized to both sulfur and sulfate, since there was no significant accumulation of other (intermediate) sulfur species (i.e., sulfite and thiosulfate). Analogous to observations in marine *Beggiatoa* (23), it is likely that the immediate product of sulfide oxidation is elemental sulfur, which is stored in *Thioploca* as globules. The elemental sulfur is then oxidized to SO_4^{2-} in a second, independent step, as suggested by Fossing et al. (7). In experiments without addition of sulfide, sulfate production was observed at a rate of 2 to 3 nmol min^{-1} mg of $protein^{-1}$, which must have originated from internal elemental sulfur. In the presence of sulfide, the SO_4^{2-} production rate did not increase significantly, suggesting that sulfide is oxidized to sulfur and that further oxidation of sulfur to SO_4^{2-} occurs independently of the presence of sulfide. In these two experiments, the ratio of SO_4^{2-} to NH_4^+ produced was approximately 1.5 in the absence and approximately 1.7 in the presence of sulfide. If NO_3^- is reduced to NH_4^+ and sulfur is oxidized to SO_4^{2-}, then a ratio of 1.3 is expected. This is in agreement with the observed ratio in the absence of sulfide, indicating, again, that *Thioploca* trichome bundles reduce most NO_3^- to NH_4^+ under the conditions tested. It was also observed that addition of different concentrations of sulfide (100 μM and 400 μM) did not result in a significant increase in NH_4^+ production (Fig. 2). This reconfirms that oxidation of sulfide, and subsequently sulfur, occurs independently. The observed ratios indicate that net sulfur accumulation will occur when external sulfide is present. Addition of sulfide led to a small accumulation of thiosulfate ($S_2O_3^{2-}$) in the medium, suggesting that $S_2O_3^{2-}$ may be an intermediate in sulfur oxidation to sulfate. However, addition of $S_2O_3^{2-}$ to trichome bundles showed only a very low consumption of $S_2O_3^{2-}$. Starvation of the trichome bundles for 45 h in the presence of NO_3^- did not enhance this consumption rate. Accumulation of $S_2O_3^{2-}$ during starvation of disrupted trichome bundles indicates that *Thioploca* cells may not be responsible for the observed accumulation in previous experiments. At present, due to variations in the measurements, it cannot be determined whether or not *Thioploca* produces $S_2O_3^{2-}$ as an intermediate.

Sulfate reduction rates measured in sediments from station 7 at the time of sampling were approximately 30 mmol m^{-2} day^{-1} (34). If all sulfide produced from this reduction were subsequently oxidized by the *Thioploca* mats then *Thioploca* cells should be able to oxidize sulfide with a rate of 20.4 nmol min^{-1} mg of $protein^{-1}$ (see Materials and Methods). In comparison, Ferdelman et al. (6) measured an average SO_4^{2-} reduction rate of 17.5 mmol m^{-2} day^{-1}, indicating that *Thioploca* should be able to oxidize sulfide with a rate of 11.8 nmol min^{-1} mg of $protein^{-1}$ (see Materials and Methods). The average sulfide oxidation rate observed during our experiments was 5 nmol min^{-1} mg of $protein^{-1}$, which increased to 10.7 nmol min^{-1} mg of $protein^{-1}$ after starvation. Compared to the above-mentioned reduction rates, this oxidation rate observed in *Thioploca* could be responsible for 25 to 91% of the observed SO_4^{2-} reduction rates measured in the sediments. This indicates that *Thioploca* species may be able to oxidize the majority of the sulfide produced in the sediment of the continental shelf. These data are in agreement with observations by Ferdelman et al. (6), who found an oxidation capacity for *Thioploca* of 35% of the sulfide production in the sediment.

Carbon metabolism. Addition of [^{14}C]bicarbonate resulted in an incorporation rate of 0.4 to 0.8 nmol min^{-1} mg of $protein^{-1}$. The presence of sulfide did not increase the incorporation rate significantly. The measured SO_4^{2-} production rate (generated from internal sulfur) was 2 to 3 nmol min^{-1} mg of $protein^{-1}$, which is equivalent to an average of 1.3 nmol min^{-1} mg of dry $weight^{-1}$, assuming that 50% of dry weight is protein. From these data we can predict the CO_2 fixation rate, assuming that 12.5% of the electrons produced go to CO_2 fixation (assuming a yield of 8 g (dry weight) · mol of $sulfide^{-1}$ [23, 38]). The oxidation of sulfur to SO_4^{2-} produces six electron equivalents. Given the fact that CO_2 reduction to biomass (dry weight) requires four electron equivalents, the predicted rate of CO_2 fixation would be $0.125 \times (6/4) \times 1.3 = 0.24$ nmol min^{-1} mg^{-1} (dry weight). This rate is equivalent to 0.49 nmol min^{-1} mg of $protein^{-1}$ (assuming that 50% of the dry weight is protein), which is the rate observed, suggesting that *Thioploca* species can grow autotrophically by using internally stored sulfur and NO_3^- for energy generation. Results obtained with microautoradiography confirm earlier qualitative experiments by Maier and Gallardo (19) and indicate that the CO_2 fixation measured can be attributed to *Thioploca* trichome bundles and not to epibiontic bacteria. Ferdelman et al. (6) measured a CO_2 fixation rate in cleaned *Thioploca* suspensions of 2,400 ± 700 nmol day^{-1} g^{-1} (wet weight). Assuming that the wet weight of trichomes is 10% of the wet weight of sheaths and trichomes (32), that 10% of the wet weight of trichomes is cytoplasm, that 24% of the wet weight of the cytoplasm is dry weight, and that 50% of the dry weight is protein (see calculations in Materials and Methods), then the fixation rate was estimated to be 1.4 ± 0.4 nmol min^{-1} mg of $protein^{-1}$. This rate is approximately three times as high as the rate observed in our study.

Experiments performed with [^{14}C]acetate in the absence of sulfide resulted in an uptake rate of approximately 0.4 nmol min^{-1} mg of $protein^{-1}$. Microautoradiography showed that epibiontic bacteria also incorporated acetate, but the majority of the label (>50%) was taken up by trichomes. Labeling experiments performed with *Thiobacillus neapolitanus* showed that obligate autotrophs are able to incorporate acetate via an incomplete trichloroacetic acid cycle, lacking the enzyme α-ketoglutarate dehydrogenase (18), resulting in an acetate incorporation rate of 20 to 30% of the CO_2 fixation rate. However, for the *Thioploca* trichome bundles the acetate uptake rate was approximately equal to the CO_2 fixation rate, which strongly suggests that *Thioploca* species are facultative chemolithoautotrophs, as previously shown for a marine *Beggiatoa* strain (15) and as has also been suggested for the large vacuolated *Beggiatoa* spp. from the Guaymas Basin (25). Production of $^{14}CO_2$ was not observed after the addition of [2-^{14}C]acetate, suggesting that acetate, under these conditions, was used only as a source for cell carbon, since total oxidation of acetate for energy would release $^{14}CO_2$. Since *Thioploca* has internally stored sulfur, which is available as an energy source, it would be most beneficial, strategically, to use acetate as the primary carbon source. This economic use of energy and carbon sources is typical for mixotrophic growth (12).

Labeling experiments with bicarbonate and acetate followed by microautoradiography showed localization of the label along the transverse walls, indicating the presence of the central vacuole.

The ecophysiological experiments presented here indicate that *Thioploca* is a facultative chemolithoautothroph, capable of fixing CO_2 and assimilating available acetate when sulfur or sulfide is present as an energy source. This use of acetate as a

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carbon source when other substrates are present as an energy source is typical behavior for organisms capable of mixotrophic growth. In spite of its ability to rapidly respond to fluctuations in both NO_3^- and sulfide, its metabolic strategy seems to be geared toward continuous, but extremely slow, growth which is apparently unaffected by such fluctuations. Indeed, the large reservoir of both NO_3^- (average 160 mM) and sulfur (200 nmol mm^{-3}) indicates a turnover time for NO_3^- and sulfur of 8 to 10 days. Based on the observed rate of autotrophic CO_2 fixation, *Thioploca* would grow with a doubling time of 69 to 139 days under the laboratory conditions tested (0.4 to 0.8 nmol of CO_2 min^{-1} mg of $protein^{-1}$ is equal to 0.4 to 0.8 nmol of carbon min^{-1} mg (dry weight) of $carbon^{-1}$, assuming that 50% of the dry weight is carbon. One milligram of carbon is equal to 0.08 mmol of carbon, and thus, it would take 69 to 139 days to incorporate this amount. Assuming that *Thioploca* can grow mixotrophically on acetate, this doubling time could be increased to 26 to 52 days. Although this may be an underestimate, such a rate coincides with the observed increase in biomass (wet weight) of 1 g m^{-2} day^{-1} as has been observed for station 6 by H. N. Schulz (31). This increase would lead to a doubling time of approximately 70 days, assuming an average of 85 g (wet weight) m^{-2} for trichomes without sheaths (see Materials and Methods). In general, however, we should remember that samples used in this study were mixed populations and, therefore, differences in activity between the two species used may occur.

In spite of its low growth rate, the evidence presented here shows that *Thioploca* is one of the major players in sulfur and nitrogen cycling of the sediment along the west coast of South America.

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