APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1991, p. 1758–1763
0099-2240/91/061758-06$02.00/0

Vol. 57, No. 6

Methylated Sulfur Compounds in Microbial Mats: In Situ Concentrations and Metabolism by a Colorless Sulfur Bacterium

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Received 23 January 1991/Accepted 5 April 1991

The concentrations of the volatile organic sulfur compounds methanethiol, dimethyl disulfide, and dimethyl sulfide (DMS) and the viable population capable of DMS utilization in laminated microbial ecosystems were evaluated. Significant levels of DMS and dimethyl disulfide (maximum concentrations of 220 and 24 nmol cm^3 of $sediment^{-1}$, respectively) could be detected only at the top 20 mm of the microbial mat, whereas methanethiol was found only at depth horizons from 20 to 50 mm (maximum concentration of 42 nmol cm^3 of $sediment^{-1}$). DMS concentrations in the surface layer doubled after cold hydrolysis of its precursor, dimethylsulfoniopropionate. Most-probable-number counts revealed 2.2×10^5 cells cm^3 of $sediment^{-1}$, in the 0- to 5-mm depth horizon, capable of growth on DMS as the sole source of energy. An obligately chemolithoautotrophic bacillus designated strain T5 was isolated from the top layer of the marine sediment. Continuous culture studies in which DMS was the growth-limiting substrate revealed a maximum specific growth rate of 0.10 h^{-1} and a saturation constant of 90 μmol $liter^{-1}$ for aerobic growth on this substrate.

Microbial decomposition of sulfur-containing amino acids and the osmolyte dimethylsulfoniopropionate (DMSP) are the major processes by which volatile organic sulfur compounds are produced (19). Despite a growing interest in production and consumption of volatile sulfur compounds with respect to atmospheric exchange mechanisms (2), relatively little is known of microbial transformations of these compounds in sediments. Although the contribution of marine coastal sediments to the global sulfur flux might be of minor importance (2, 33), turnover rates of methylated sulfur compounds in these systems are expected to be very high (18). The presence of dimethyl sulfide (DMS) was detected in microbial mats (5), whereas the production of methanethiol (MSH) could be measured during anoxic incubations (43). In anoxic marine sediment slurries, dissimilatory sulfate-reducing bacteria and methanogenic bacteria were found to be involved in the metabolism of methylated sulfur compounds (18, 19, 21–23). Anoxygenic phototrophic bacteria have been reported to use DMS as electron donor for photosynthesis (42).

The aerobic metabolism of DMS has been described for methylotrophic *Hyphomicrobium* spp. (7, 34) and *Thiobacillus thioparus* (14, 15). From a variety of marine, estuarine, and freshwater sediments, dimethyl disulfide (DMDS)-oxidizing microbes, probably thiobacilli, could be isolated. However, none of these isolates was able to metabolize DMS (30).

Marine microbial mats develop under a variety of environmental parameters in the supralittoral areas of shelf seas and in salt ponds. These laminated microbial ecosystems are generally subject to extreme diel fluctuations in oxygen and hydrogen sulfide (38, 39) and are characterized by the presence of a layer of cyanobacteria close to the sediment surface, under which a distinct layer of anoxygenic phototrophs can be found (32). Because of the coinciding presence of oxygen and reduced sulfur compounds (38), the upper 10 mm of the microbial mat also provides the specific niche for colorless sulfur bacteria (thiobacilli) (24). Many of these organisms can grow autotrophically when oxidizing reduced sulfur compounds by using oxygen, iron, or nitrate as the terminal electron acceptor while fixing carbon dioxide. The utilization of DMS as electron donor for growth has also been reported for this type of organism (15, 16). We describe here a field study in which the importance of methylated sulfur compounds and the potential population of organisms metabolizing this substrate in microbial mats are evaluated. Also, the growth characteristics on DMS of a pure culture isolated from the microbial mat are presented.

MATERIALS AND METHODS

Field experiments. In situ concentrations of methylated sulfur compounds and viable counts of the DMS-utilizing and chemolithotrophic populations were determined in the upper 50 mm of a microbial mat located on the North Sea island of Texel (55°08′N, 4°50′E). Profiles of oxygen and sulfide were assessed in situ with needle electrodes (38). Because of the nature of these electrodes, the observed oxygen values could represent a slight underestimation at low O_2 concentrations. Sediment samples were taken at three locations by using stainless steel cores (2.54-cm inner diameter) which were stoppered at both ends after the sampling. Cores for DMDS, DMS, and MSH determination were immediately sliced under a flow of N_2 and fixed in filter-sterilized seawater to which glutaraldehyde was added to stop biotic activity. The slurries of 10 ml thus obtained (1:1 sediment and fixative, final concentration 0.5% [vol/vol] glutaraldehyde, pH 7.8) were incubated in 26-ml glass vials with Teflon-lined crimp seal caps and stored on ice, and the headspace was analyzed within 24 h after the sampling. Subsequently, the samples were subjected to cold hydrolysis (5 mol of NaOH per liter, final concentration) at 0°C for 40 min, after which the concentration of DMS in the headspace was measured again. From the difference thus obtained, the concentration of DMSP-like precursors of DMS was evaluated (40). Samples for most-probable-number (MPN) incubations were kept on ice and processed within 1 h after

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collection. The viable population of chemolithoautotrophs was determined after incubation in a liquid mineral medium supplemented with thiosulfate (5 mmol liter^{-1}) and the pH indicator bromocresol blue to visualize a decrease in pH caused by sulfate formation. The viable population of DMS utilizers was measured similarly on agar plates containing bromocresol blue and a DMS-saturated headspace, which was replenished daily with fresh air and DMS. Growth was determined in three replicates after 4 weeks of incubation at room temperature and scored by the method of De Man (8). Colonies from the highest positive dilutions were checked for growth on DMS in liquid medium.

Isolation and culture conditions. Mineral medium consisted of the following (in grams per liter): NaCl (25.0), NH_4Cl (0.2), $CaCl_2 \cdot 2H_2O$ (0.225), KCl (0.2), $MgCl_2 \cdot 6H_20$ (0.2), anhydrous KH_2PO_4 (0.02), and anhydrous Na_2CO_3 (2.0), supplemented with vitamin B_{12} (20 μg liter^{-1}), $FeSO_4 \cdot 7H_2O$ (1 mg liter^{-1}), trace element solution (1 ml liter^{-1}) of Widdel and Pfennig (41), and as the only reduced sulfur source, either $Na_2S_2O_3 \cdot 5H_2O$ (2.48 g liter^{-1}) or DMS (0.083 g liter^{-1}). The pH of the medium was adjusted to 7.5 with HCl or NaOH. The top 5 mm of the microbial mat was used as the inoculum for enrichment cultures on thiosulfate. Colony isolation obtained an obligately autotrophic *Thiobacillus*-like strain designated T5, the purity of which was repeatedly checked by serial dilution and colony isolation. Substrate characterization of the strain was carried out in batch cultures, and growth kinetics on DMS in continuous culture (pH stat) at 25°C (3) were studied. In the latter case, the medium was pumped at equal rates from two carboys, each containing a double-strength solution. All nutrients were present in one carboy (pH 5) except carbonate and the growth-limiting substrate DMS, which were pumped from the second carboy. The latter was kept at an alkaline pH to increase the stability of DMS (29). All solutions were kept under an atmosphere of oxygen-free nitrogen. Because of an increasing gas phase in the reservoir vessels during the experiment, a loss of DMS to the headspace was anticipated. However, a distribution coefficient (ratio of the concentration in the liquid phase to the concentration in the gas phase) of 15.6 was found, which was similar to the value reported earlier (23). Taking this into consideration, a maximum loss of 2% DMS to the headspace was expected. Repeated monitoring of the concentration of the growth-limiting substrate showed no significant decrease in the DMS concentration in the liquid phase of the alkaline reservoir bottle. Also, the actual concentration of DMS entering the culture vessel (S_R, DMS_{in}) and the concentration in the culture effluent (s, DMS_{out}) were frequently determined to allow calculation of the sulfur balance. The dilution rate was maintained as described for the individual experiments.

Analytical procedures. DMS, DMDS, and MSH were determined by headspace analysis on a Packard 427 gas chromatograph equipped with a Porapak R (80/100 mesh) column and flame ionization detection (19, 23); retention times of MSH and DMS were 170 and 390 s, respectively, but DMDS eluted only after 60 min. Peak areas were obtained with an Shimadzu C-R6A integrator. All glassware was routinely cleaned in boiling NaOH (1 mol liter^{-1}) to remove absorbed organic sulfur compounds (20). Polythionates, elemental sulfur, thiosulfate, and polysulfides were determined colorimetrically after cyanolysis (28, 39), sulfate was measured by high-performance liquid chromatography (HPLC) (12), and nitrite was measured colorimetrically after reaction with sulfanilamide (4). Protein was measured by using the Folin phenol reagent (26) following extraction with methanol to remove elemental sulfur (37).

Calculation of growth kinetics. The relation between the specific growth rate (μ) and the concentration of the limiting substrate (s) is described by the Monod equation:

$$\mu = \mu_{max} \times \frac{s}{s + K_s} \quad (1)$$

The maximum specific growth rate (μ_{max}) and saturation constant (K_s) for growth on DMS as the limiting substrate were calculated by applying the direct linear plot (10) on continuous culture data collected at four different steady states (39). For each steady state, the negative of the determined values of the residual s, as the abscissa, and the μ, as the ordinate, were connected. The lines obtained for individual steady states intersect in a common point with coordinates K_s and μ_{max}.

Reagents. DMS and dimethyl sulfoxide (purity, >99%) were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany; DMDS and MSH (purity, >99 and >98%, respectively) were obtained from Fluka Chemie AG, Buchs, Switzerland. All other chemicals were analytical grade.

RESULTS

Field experiments. The depth profile of the methylated sulfur compounds measured in 10-mm strata in the upper 50 mm of the microbial mat showed a peak for both DMS and DMDS in the top layer, whereas MSH could be detected only from 20- to 50-mm depths (Fig. 1). DMS peaked in the top 10 mm of the mat (average concentration of 220 nmol cm^3 of sediment^{-1}), where amounts of DMDS and MSH were much lower (24 and 0 nmol cm^3 of sediment^{-1}, respectively). Values for the three replicate cores showed corresponding concentrations for all methylated sulfur compounds (Fig. 1). Clearly, of the three sulfur species directly measured in the sediments, DMS was present in the highest concentration. Upon cold hydrolysis, the DMS concentration increased to 450 nmol cm^3 of sediment^{-1} in the top layer, indicating the presence of DMSP-like precursors of DMS.

MPN counts on a mineral medium with either DMS or thiosulfate as the sole energy source revealed maximum populations of 2.2×10^5 and 2.0×10^9 cells cm^3 of sediment^{-1}, respectively, in the top 5 mm of the mat (Fig. 2A and C). The location with the highest number of chemotrophic DMS utilizers coincided with the location with highest total concentration of DMS. Depth profiles of viable counts for both substrates demonstrated that the majority of the chemotrophs were present in the zone of the sediment where oxygen could be detected (Fig. 2B) (39). Microscopy revealed that the surface of the mat consisted mainly of *Microcoleus chthonoplastes* and other cyanobacteria, whereas decaying remains of the macroalgae *Ulva* sp. and *Enteromorpha* sp. were also observed.

Experiments with pure cultures. Strain T5 showed rapid autotrophic growth in the presence of oxygen with either thiosulfate or DMS as the sole source for energy. The motile cells (0.5 by 2.5 μm) stained gram negative. Autotrophic growth on hydrogen sulfide, polysulfides, elemental sulfur, thiocyanate, MSH, DMDS, and dimethyl sulfoxide as reduced sulfur sources could also be demonstrated. Anaerobic growth with nitrate as the terminal electron acceptor was observed only for a short period, during which nitrite was

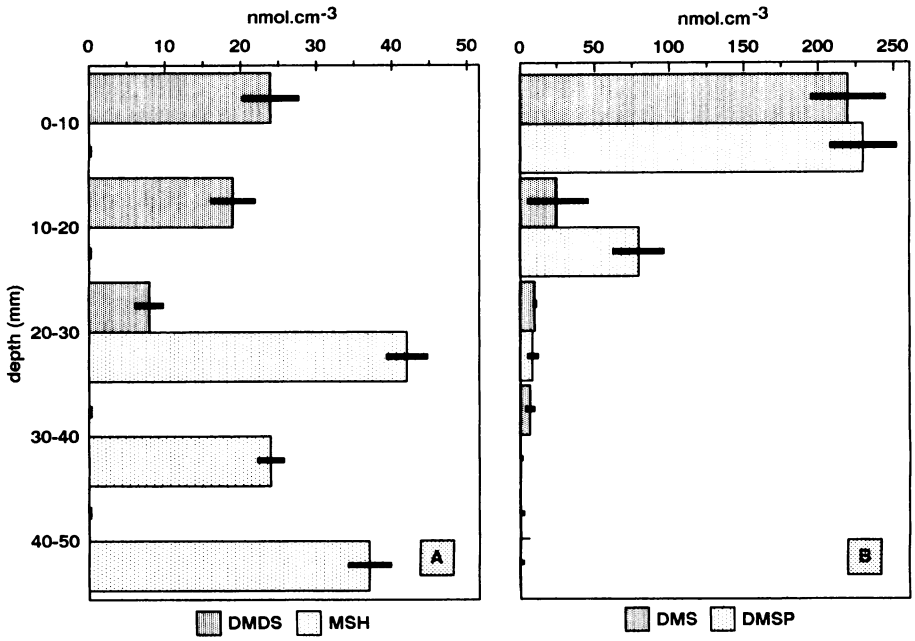

FIG. 1. Depth profile of the concentration of (A) DMDS and MSH and (B) DMS and DMSP in nanomoles per cubic centimeter of sediment, measured in a microbial mat on Texel. Bars indicate average values and the range of three replicate samples.

formed. Growth occurred in a pH range from 4.0 to 9.5; the final pH typically was 3.5 to 4.0. On the basis of these characteristics, the isolate resembled the obligately autotrophic *Thiobacillus thioparus* (25).

Continuous culture experiments. The isolate described above was grown in continuous culture, during which the oxygen concentration was kept at a constant level of 65.3 μmol liter^{-1} (9). After at least five volume changes of the culture, a steady state was reached as judged from a constant protein concentration, indicating that the μ equaled the dilution rate. Under steady-state conditions, the concentrations of various sulfur species were measured (Table 1).

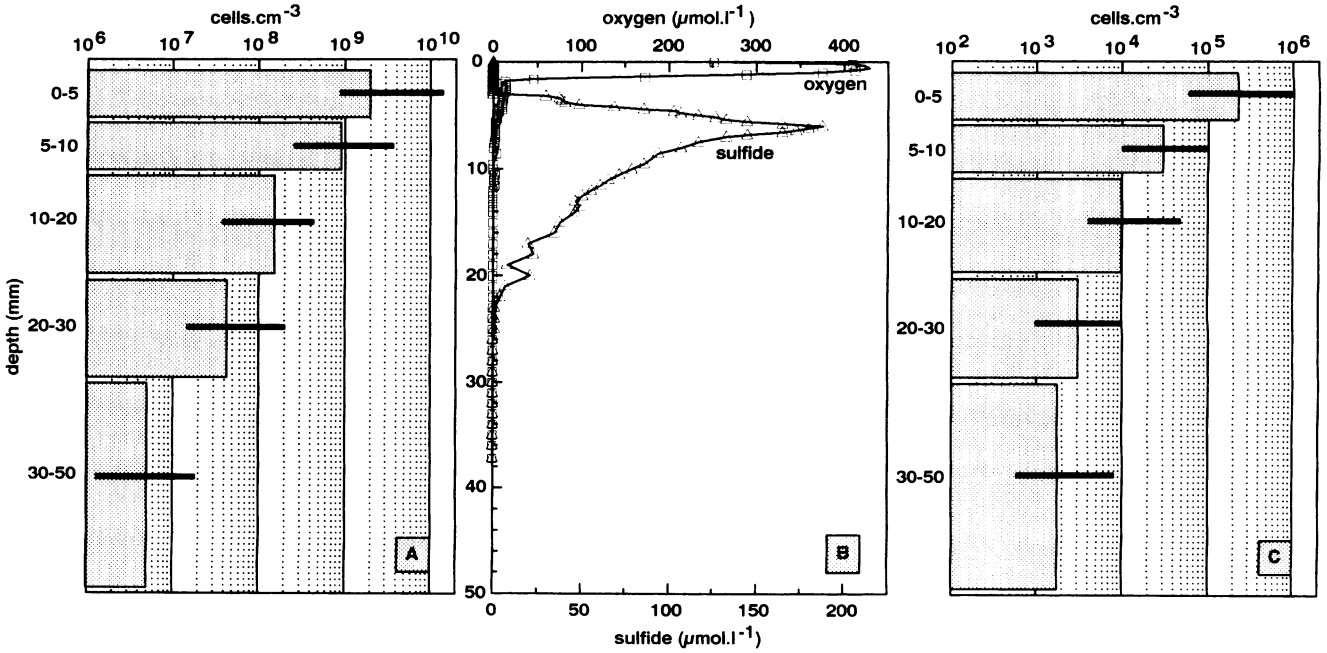

FIG. 2. MPN counts of chemolithotrophic bacteria utilizing (A) thiosulfate and (C) DMS. Error bars indicate 95% confidence limits. (B) Profiles of oxygen and hydrogen sulfide measured with a combined needle electrode (sediment temperature was 23°C, light intensity was 1,800 microeinsteins m^{-2} s^{-1}) (37).

TABLE 1. Steady-state data obtained from DMS-limited continuous cultures of strain T5

Dilution rate (h^{-1})	DMS concn in reservoir (μmol liter^{-1})	DMS concn in culture (μmol liter^{-1})	SO_4^{2-} concn in culture (μmol liter^{-1})	δDMS[a] (μmol liter^{-1})	Protein concn in culture (mg liter^{-1})	Y_{DMS}[b] (mg mmol^{-1})
0.043	ND[c]	61	ND	ND	4.77	ND
0.063	1,360[d]	160	1,110	1,200	16.67	13.89
0.077	1,170[d]	438	718	730	14.70	20.14
0.088	1,330[d]	920	423	410	8.00	19.51

[a] δDMS, amount of DMS used by the culture ($DMS_{in} - DMS_{out}$).
[b] Y_{DMS}, yield of the culture in milligrams of protein per millimoles of DMS oxidized.
[c] ND, not determined.
[d] The actual concentration in the alkaline carboy was twice as high; see Materials and Methods.

Besides sulfate, no other sulfur compounds could be detected. Also, the DMS concentration in the culture effluent was monitored twice daily to allow calculation of the sulfur balance (Table 1), which clearly demonstrated that sulfate was the major product of DMS oxidation. With the obtained steady-state values for μ and s, the μ_{max} on DMS was graphically determined to be 0.1 h^{-1}, and the accompanying K_s was determined to be 90 μmol liter^{-1}. Batch experiments on thiosulfate (data not shown) indicated a μ_{max} of approximately 0.31 h^{-1}. Data for protein concentration revealed the yield for strain T5 on DMS to be 13.89 to 20.14 mg of protein mmol^{-1} of DMS (Table 1).

DISCUSSION

When evaluating concentrations of methylated sulfur compounds in sediment ecosystems, several aspects of sampling should be taken into consideration. First of all, Howes et al. (13) reported an increase in DMS concentration when cutting and centrifuging sediments as opposed to directly collecting pore water. The increase was caused by release of DMS due to damaging of *Spartina* roots. In the microbial mat samples of the present study, no plant roots were present. Although DMSP has been reported to occur in cultures of *Microcoleus lyngbyaceus* (40) and mats dominated by *Microcoleus chthonoplastes* (5), cutting the sediment is unlikely to yield strongly elevated values, since the 0-to-10-mm layer was analyzed as an entity and these organisms were dominant in the top 5 mm of the mat only. Second, Kiene et al. (20) found sorption of amended thiols (>90%) to anoxic sediment slurries. It can be speculated that the sorption and desorption could occur with DMS as well (18a). Finally, fixing with glutaraldehyde during slurry preparation can yield slightly higher DMS concentrations abiotically formed from its precursors (23). However, this is expected to be a minor process. Sampling, as described in this study, yields total concentrations and does not imply complete availability to organisms.

With surface layers of salt marsh sediments, Kiene (18) reported total DMS concentrations of 300 nmol cm^3 of sediment^{-1} after cold hydrolysis (i.e., DMS plus DMSP), whereas Howes et al. (13) found 0.1 to 20 nmol of DMS ml^{-1} of pore water, depending on the sampling procedure. Andreae (1) detected only 0.12 nmol of DMS ml^{-1} of pore water in deep-sea sediments of the Peru upwelling area, and Sørensen (31) reported 0.1 nmol of DMS ml^{-1} of pore water in a Danish estuary. Compared with these values, the total concentration of 450 nmol of DMS plus DMSP cm^3 of sediment^{-1} in the surface layer of the eutrophic microbial mat sediments examined in this study is relatively high but not unexpected. The depth profile of DMS in the microbial mat displayed a sharp decrease with increasing depth, similar to results in earlier reports (13, 18). The concentration of DMSP in the mat was found to be 80 to 230 nmol cm^3 of sediment^{-1}. This compatible solute may originate from a *Microcoleus* sp. and detritus, but it may also come from a *Phaeocystis* sp. and an *Emiliania* sp. (36), an *Ulva* sp., and an *Entermorpha* sp. (6) which were washed onto the mat at high tide. MSH could be detected only in strata deeper than 20 mm, where anoxic conditions prevailed and hydrogen sulfide was present, which is similar to observations by Sørensen (31). At shallower depth horizons, DMDS which could have been formed by abiotic oxidation of MSH or microbial metabolism (18) was found.

Viable counts of thiosulfate-utilizing chemolithotrophs revealed a maximum population density of 2.0×10^9 cells cm^3 of sediment^{-1} in the oxic zone of the mat. Lower numbers were observed in the anoxic layers, where these organisms may utilize various reduced sulfur compounds (i.e., hydrogen sulfide, thiosulfate, or polysulfide [39]) with nitrate as the terminal electron acceptor. Nitrate was present in the mat at significant levels (>100 μM) (10a).

The MPN counts for DMS-utilizing chemolithotrophic organisms demonstrated a significant population of 2.2×10^5 cells cm^3 of sediment^{-1} (0.6×10^5 to 9.6×10^5, applying 95% confidence limits). Assuming that the same metabolic type of organism is growing in both thiosulfate- and DMS-containing mineral media, the viable populations found for these substrates indicate that only a minor fraction of the thiosulfate-utilizing chemolithoautotrophic population is able to grow on DMS as well. However, because of the toxicity of relatively high concentrations of DMS (34), the transfer of samples into a DMS-saturated headspace during incubation could have affected the outcome of the enumeration in a negative way. Because of the pitfalls associated with the MPN technique, the numbers reported here should be interpreted as an indication of the presence of a potential DMS-oxidizing population.

Various anaerobic and aerobic organisms that metabolize DMS have been described. Under anoxic conditions, DMS-producing and -consuming processes are reasonably well described (18): both methanogens and sulfate reducers play a predominant role here, and DMS could very well be the substrate that supports simultaneous sulfate reduction and methane production in sediments (23, 27). Also, purple sulfur bacteria are capable of DMS oxidation (42). Therefore, the presence of blooms of anoxygenic phototrophs might contribute significantly to DMS consumption in microbial mats.

Aerobic metabolism of methylated sulfur compounds has been described for methylotrophic *Hyphomicrobium* spp., which employ the serine pathway for dimethyl sulfoxide and DMS utilization, and for *Thiobacillus thioparus*, a chemo-

lithoautotroph which fixes carbon dioxide via the Calvin cycle during which DMS serves as electron donor (15). The proposed overall DMS oxidation reaction for the latter organism is:

$$CH_3SCH_3 + 8H_2O \rightarrow 2CO_2 + H_2SO_4 + 20H \quad (2)$$

Accordingly, oxidation of 1 mmol of DMS to sulfate would generate 20 mmol of electrons. However, if the initial step of DMS oxidation involved O_2, as proposed for the *Hyphomicrobium* sp. (7), complete oxidation would liberate 16 mmol of electrons. Direct comparison of yield values reported in literature is unavailable because of the use of different parameters. The maximum yield for strain T5 was 20.1 mg of protein $mmol^{-1}$ of DMS. Assuming 70% protein of the dry weight as reported for other thiobacilli (11), this value corresponds with a DMS yield of 28.7 mg of dry weight $mmol^{-1}$. For *Thiobacillus thioparus* TK-m, a yield of 21 mg of dry weight $mmol^{-1}$ has been reported (16). In another study (15), the same author found a yield of 12 to 13 g of total organic carbon (TOC) mol^{-1} of DMS. When applying a dry weight value of 49% TOC (11), this corresponds to a yield of 24.5 to 26.5 mg of dry weight $mmol^{-1}$ of DMS, which is similar to the value for strain T5. For the *Hyphomicrobium* sp., a slightly lower yield (19.1 mg of dry weight $mmol^{-1}$) has been reported (35).

To facilitate comparisons among different organisms and different substrates, the yield in milligrams of dry weight per millimoles of electrons should be taken. Assuming that 80% of the electrons are used for energy generation by the chemolithoautotrophic thiobacilli (17) and 20 mmol of electrons are liberated per mmol of DMS oxidized according to equation 2, the growth yield for the *Thiobacillus*-like strain T5 described in this study is 1.44 mg of dry weight $mmol^{-1}$ of electrons. This value fits well within the range of 0.65 to 1.84 calculated from the data of Kelly (17). The yield of an anoxygenic phototroph, tentatively identified as a *Thiocystis* sp., derived from the increase in protein when supplying DMS as a cosubstrate was 7.14 mg of dry weight $mmol^{-1}$ of DMS (42). However, the proposed DMS metabolism generates only 2 mmol of electrons per mmol of DMS when oxidized to dimethyl sulfoxide. Thus, when growing on DMS, the purple sulfur bacterium was able to yield 3.57 mg of dry weight $mmol^{-1}$ of electrons, which is considerably higher than the yield of thiobacilli on this substrate. This may indicate that colorless sulfur bacteria use more than 80% of the substrate for energy generation.

The affinity [defined as $\mu/(K_s + s)$ with s decreasing to zero, i.e., the initial slope of the Monod curve] for growth on DMS of strain T5, as determined at steady states in continuous cultures, was 1.11 h^{-1} $mmol^{-1}$ liter. The μ_{max} of *Thiobacillus thioparus* TK-m on DMS, as estimated from published data (15), was 0.06 h^{-1}. The apparent K_m of 45 $\mu mol\ liter^{-1}$ (15) was derived from oxygen uptake studies. These values result in an affinity of 1.33 h^{-1} $mmol^{-1}$ liter, which is very similar to the 1.11 value of our isolate in this study. The μ_{max} and K_s values for *Hyphomicrobium* strain EG on DMS, as determined by oxygen uptake, were reported to be 0.08 h^{-1} and 3 $\mu mol\ liter^{-1}$ (35), resulting in an affinity of 26.67 h^{-1} $mmol^{-1}$ liter. However, this value represents a substrate oxidation rate and also includes respiration and thus is conceivably higher. Despite a slightly lower yield, it is obvious that the methylotroph can oxidize DMS more efficiently than our isolate. Clearly, further studies on the occurrence of high-affinity colorless sulfur bacteria and hyphomicrobia in marine environments are necessary.

The ecological relevance of DMS as a potential substrate for growth in the microbial mat is still obscure. Potential candidates for DMS consumption in these highly fluctuating environments are colorless sulfur bacteria, anoxygenic phototrophic bacteria, sulfate-reducing bacteria, methanogenic bacteria, and possibly other methylotrophic bacteria. In these laminated ecosystems, much higher concentrations of hydrogen sulfide, as well as an array of other reduced sulfur compounds, commonly are observed (38). However, high standing concentrations of sulfur compounds do not imply high turnover rates of these compounds, and future studies should reveal the actual importance of DMS and other methylated sulfides in the oxic zone of marine sediment ecosystems.

ACKNOWLEDGMENTS

This work was supported by the Foundation for Fundamental Biological Research (BION) grant 437.022Bo.

The assistance of Willem Stolte during isolation of the strain is gratefully acknowledged. The generous hospitality of Thomas Cappenberg and Cees Hordijk at the Limnological Institute during HPLC measurements is much appreciated.

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