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A New Mechanism for the Aerobic Catabolism of Dimethyl Sulfide

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Aerobic degradation of dimethyl sulfide (DMS), previously described for thiobacilli and hyphomicrobia, involves catabolism to sulfide via methanethiol (CH_3SH). Methyl groups are sequentially eliminated as HCHO by incorporation of O_2 catalyzed by DMS monooxygenase and methanethiol oxidase. H_2O_2 formed during CH_3SH oxidation is destroyed by catalase. We recently isolated *Thiobacillus* strain ASN-1, which grows either aerobically or anaerobically with denitrification on DMS. Comparative experiments with *Thiobacillus thioparus* T5, which grows only aerobically on DMS, indicate a novel mechanism for aerobic DMS catabolism by *Thiobacillus* strain ASN-1. Evidence that both organisms initially attacked the methyl group, rather than the sulfur atom, in DMS was their conversion of ethyl methyl sulfide to ethanethiol. HCHO transiently accumulated during the aerobic use of DMS by *T. thioparus* but not with *Thiobacillus* strain ASN-1. Catalase levels in cells grown aerobically on DMS were about 100-fold lower in *Thiobacillus* strain ASN-1 than in *T. thioparus* T5, suggesting the absence of H_2O_2 formation during DMS catabolism. Also, aerobic growth of *T. thioparus* T5 on DMS was blocked by the catalase inhibitor 3-amino-1,2,4-triazole whereas that of *Thiobacillus* strain ASN-1 was not. Methyl butyl ether, but not $CHCl_3$, blocked DMS catabolism by *T. thioparus* T5, presumably by inhibiting DMS monooxygenase and perhaps methanethiol oxidase. In contrast, DMS metabolism by *Thiobacillus* strain ASN-1 was unaffected by methyl butyl ether but inhibited by $CHCl_3$. DMS catabolism by *Thiobacillus* strain ASN-1 probably involves methyl transfer to a cobalamin carrier and subsequent oxidation as folate-bound intermediates.

Dimethyl sulfide (DMS) is a major contributor to total sulfur emission, from land and the ocean, to the atmosphere (2), and its production and consumption have been studied in environmental samples, as well as in cultures (19, 30). Aerobic and anaerobic bacteria degrade DMS, but its metabolism by facultative aerobes has been neglected, even though this aspect has ecological importance and is of biochemical interest.

Aerobes that catabolize DMS are either hyphomicrobia or thiobacilli (11). The aerobic metabolic pathway for DMS has been best documented in hyphomicrobia (9, 29). DMS is oxidized by an NADH-dependent monooxygenase to methanethiol and formaldehyde: $CH_3SCH_3 + O_2 + NADH + H^+ = CH_3SH + HCHO + H_2O + NAD^+$. Methanethiol oxidases have been purified from *Hyphomicrobium* strain EG (28) and *Thiobacillus thioparus* TK-m (15), and they catalyze another O_2-dependent transformation: $CH_3SH + O_2 + H_2O = HCHO + H_2S + H_2O_2$.

H_2S is oxidized to sulfate and protection against toxic H_2O_2 is afforded by high catalase activities in both hyphomicrobia and thiobacilli (26, 29).

O_2-independent mechanisms exist for the catabolism of DMS and CH_3SH, since methanogenic bacteria grow on these C_1 sulfur compounds (14, 21, 25). Fluctuating oxic-anoxic conditions are common in many environments, and it should be advantageous for bacteria to degrade DMS by similar biochemical mechanisms in the presence or absence of O_2, thus avoiding processes of adaptation and induction.

We compared the mechanisms for DMS catabolism by aerobic *T. thioparus* T5 (32) and a newly described bacterium, *Thiobacillus* strain ASN-1 (33), which grows on DMS either aerobically or anaerobically, with nitrate as the electron acceptor. We concluded, mainly on the basis of inhibitor studies, that the mechanism for aerobic degradation of DMS by the facultative organism is different from that previously observed for hyphomicrobia and thiobacilli. Catabolism of DMS and CH_3SH probably involves initial removal of the methyl groups by transmethylation reactions. In this novel mechanism, O_2 functions only as an electron acceptor and does not participate as a substrate in the destruction of methyl groups. The mechanism can therefore proceed either aerobically or anaerobically.

MATERIALS AND METHODS

Medium and growth of cultures. *Thiobacillus* strain ASN-1 was isolated from a marine sediment on Sapelo Island, Ga. (33). *T. thioparus* T5 was previously isolated from a marine microbial mat on the Frisian Island of Texel, The Netherlands (32). The medium for both organisms contained (in grams per liter) NaCl (25.0), NH_4Cl (0.2), $CaCl_2 \cdot 2H_2O$ (0.225), KCl (0.2), $MgCl_2 \cdot 6H_2O$ (0.2), KH_2PO_4 (0.02), and Na_2CO_3 (2.0) supplemented with $FeSO_4 \cdot 7H_2O$ (1 mg/liter), the trace element solution (1 ml/liter) of Widdel and Pfennig (36), and vitamin B_{12} (20 μg/liter). Growth substrates were $Na_2S_2O_3 \cdot 5H_2O$ (1.24 or 2.48 g/liter) and DMS (0.37 or 0.73 ml/liter). Nitrate (KNO_3, 0.505 or 2.02 g/liter) or nitrite ($NaNO_2$, 0.345 g/liter) was added as the electron acceptor for anaerobic growth of *Thiobacillus* strain ASN-1. The pH of the medium was adjusted to 7.5 with HCl or NaOH.

Cultures were grown at 25°C without shaking in 100- or 150-ml batches in 250-ml Erlenmeyer flasks (aerobic growth) or in completely filled serum bottles (150 ml) with Teflon-

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lined septa (anaerobic growth). Growth was monitored by measurements with a Klett-Summerson colorimeter and by determining cell protein.

Cell suspension experiments. Cells were harvested in the late exponential phase of growth by centrifugation at 10,000 × *g* for 10 min at 5°C. Cells were washed twice by resuspension and centrifugation in medium lacking a substrate. Oxygen uptake was measured in a 5-ml chamber incubated at 30°C by using a Clark-type electrode (5). Oxygen uptake rates were determined after adding substrates with or without addition of potential inhibitors. The compounds tested as inhibitors were methyl butyl ether (MBE), 2 or 0.5 mM; dibutyl ether, 2 mM; $CHCl_3$, 0.5 mM; and chloramphenicol, 75 μg/ml. Catalase activity was measured as oxygen produced after addition of H_2O_2 to cell suspensions (10). The concentration of the H_2O_2 solution was calculated from its A_{240} by using a molar extinction coefficient of 39.58 (1). DMS, ethyl methyl sulfide (EMS), and CH_3SH consumption rates in cell suspensions were measured by headspace analysis in 14-ml serum vials that contained 2 ml of a cell suspension and were sealed with butyl rubber stoppers. Consumption rates were corrected for abiotic reactions and sorption to the glass and stopper.

Analytical methods. Gas samples were assayed for alkyl sulfides by gas chromatography with flame ionization detection (Shimadzu GC-14A; Shimadzu Corp., Kyoto, Japan) and a column (1.4 m [length] by 3 mm [inside diameter]) of 40/60 Carbopak BH T 100 (Supelco, Inc., Bellefonte, Pa.) at 110°C with a carrier gas (N_2) flow rate of 60 ml/min (6). Peak areas were recorded on a Shimadzu CR601 integrator. DMS was calibrated either directly with DMS diluted in water or methanol or by alkaline decomposition of dimethylsulfoniopropionate (35); CH_3SH and ethanethiol were calibrated by reduction of dimethyl disulfide and diethyl disulfide with 0.5 mM tributylphosphine (24); EMS was calibrated directly with dilutions of EMS in water or methanol.

Thiosulfate was measured colorimetrically after cyanolysis (17). Sulfide was measured colorimetrically by the methylene blue method (31). HCHO was quantified by a colorimetric method using alkaline 4-amino-5-hydrazino-3-mercapto-1,2,4-triazole (3). Sulfate was measured by high-performance liquid chromatography using a buffer of 1.8 mM Na_2CO_3 and 2.1 mM $NaHCO_3$ (flow rate, 1.5 ml/min) on an anion-exchange column (Dionex Ionpac AS4A; 250 mm [length] by 4 mm [inside diameter]) with conductivity detection. Protein was determined by the bicinchoninic acid assay (27) after extraction of elemental sulfur, when present, with methanol.

Chemicals. Chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., or Aldrich Chemical Co., Milwaukee, Wis.

RESULTS

Thiobacillus strain ASN-1 grew on DMS or thiosulfate with oxygen or nitrate as the electron acceptor (Fig. 1). Cells of *Thiobacillus* strain ASN-1 rapidly consumed DMS, CH_3SH, and EMS in the presence of oxygen, nitrate, or nitrite (Table 1); nitrate and nitrite functioned only with anaerobically grown cells. *T. thioparus* T5 grown on DMS used DMS and CH_3SH at lower rates and EMS at much lower rates than did *Thiobacillus* strain ASN-1 (Table 1). *T. thioparus* T5 grown on DMS metabolized EMS aerobically with nearly quantitative accumulation of ethanethiol when tributylphosphine was added to reduce diethyl disulfide produced by chemical oxidation (Fig. 2). *Thiobacillus* strain ASN-1 also initially demethylated EMS and, as for *T. thioparus* T5, ethanethiol transiently appeared during aerobic incubations. Anaerobic demethylation required nitrate, and ethanethiol initially persisted better under these conditions, presumably because anoxia retarded its rate of abiotic oxidation (Fig. 3). However, ethanethiol was eventually metabolized unless chloramphenicol was present to prevent protein synthesis (Fig. 3). In the absence of chloramphenicol, ethanethiol was completely degraded by *Thiobacillus* strain ASN-1 with nearly full recovery of the sulfur as sulfate; for 0.35 mM EMS initially present in the experiment shown in Fig. 3, 0.31 mM sulfate was formed. HCHO was detected during aerobic oxidation of DMS by *T. thioparus* T5, but HCHO was not formed from DMS by *Thiobacillus* strain ASN-1 (Fig. 4).

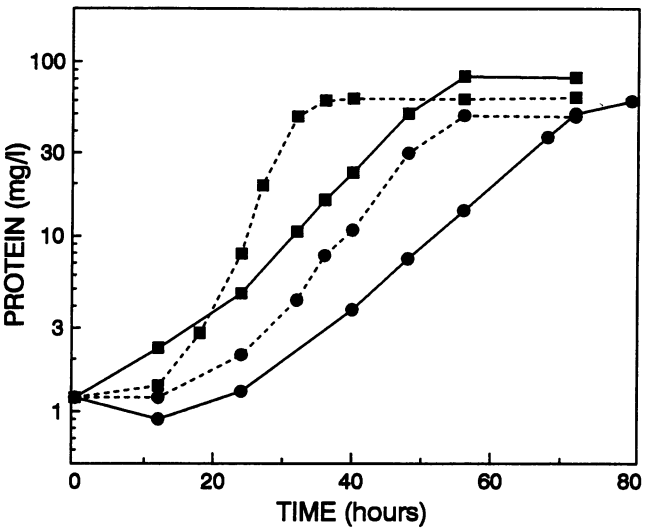

FIG. 1. Aerobic and anaerobic growth of *Thiobacillus* strain ASN-1 on 5 mM DMS or 10 mM thiosulfate. Symbols: —●—, DMS plus 20 mM nitrate; -■-, DMS plus O_2; - -●- -, thiosulfate plus nitrate; - -■- -, thiosulfate plus O_2.

The catalase inhibitor 3-amino-1,2,4-triazole (ATA) (8), at a concentration of 1 mM, prevented the growth of *T. thioparus* T5 on DMS but had no effect upon its growth on thiosulfate (Fig. 5B). ATA was without effect on the aerobic

TABLE 1. Consumption rates of methylated sulfides by cell suspensions of *Thiobacillus* strain ASN-1 and *T. thioparus* T5[a]

Substrate	Utilization (nmol · min^{-1} · mg of $protein^{-1}$)	
	Thiobacillus strain ASN-1	*T. thioparus* T5
Methanethiol	58 (O_2) 70 (NO_3^-)[b] 72 (NO_2^-)[b]	43 (O_2)
DMS	65 (O_2) 49 (NO_3^-)[b] 60 (NO_2^-)[b]	47 (O_2)
EMS	40 (O_2) 35 (NO_3^-)[b]	10 (O_2)

[a] Cells were grown on DMS. The electron acceptor for cell suspension experiments is shown in parentheses.
[b] Cells were grown anaerobically with nitrate.

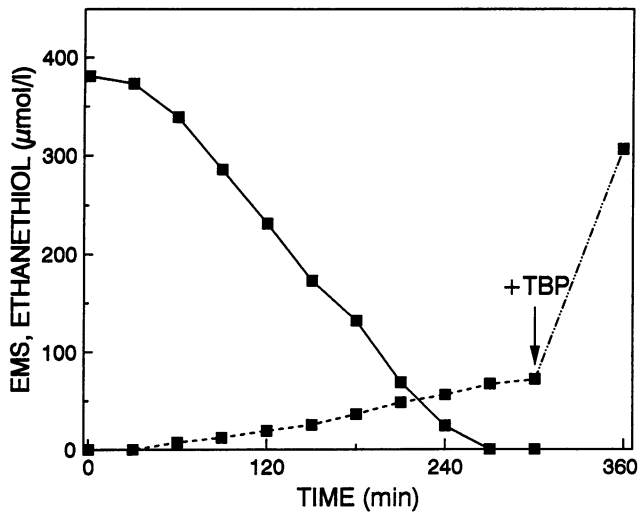

FIG. 2. Consumption of EMS by *T. thioparus* cells (140 μg of protein ml^{-1}) grown on DMS. Tributylphosphine (0.5 mM) was added at 300 min (arrow) to reduce diethyl disulfide formed by chemical oxidation of ethanethiol. Symbols: —■—, EMS consumed; - -●- -, ethanethiol produced.

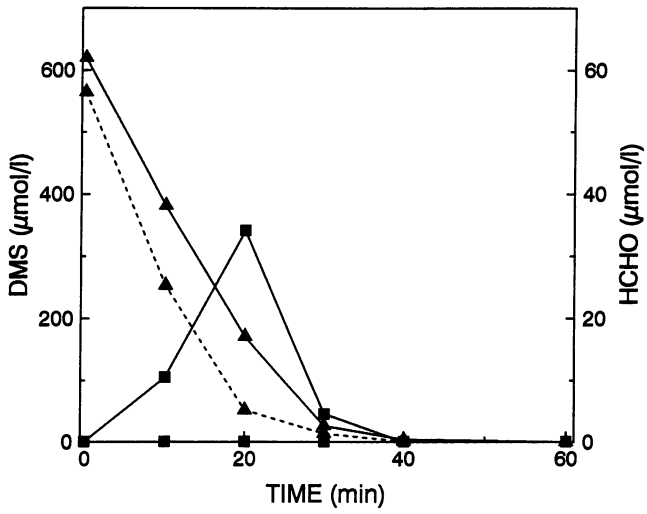

FIG. 4. HCHO formation during aerobic degradation of DMS. *T. thioparus* (—), 430 μg of protein ml^{-1}; *Thiobacillus* ASN-1 (- - -), 380 μg of protein ml^{-1}; ▲, DMS; ■, HCHO.

growth of *Thiobacillus* strain ASN-1 on thiosulfate or DMS (Fig. 5A).

H_2O_2 was rapidly decomposed by DMS-grown cells of *T. thioparus* T5 but not by thiosulfate-grown strain T5 cells or *Thiobacillus* strain ASN-1 grown aerobically on DMS or thiosulfate (Fig. 6). Catalase levels were low in *Thiobacillus* strain ASN-1, about 0.02 μmol of H_2O_2 consumed min^{-1} mg of $protein^{-1}$. The high catalase activity, about 2.0 μmol of H_2O_2 consumed min^{-1} mg of $protein^{-1}$, in DMS-grown *T. thioparus* T5 was over 95% inhibited by 1 mM ATA (Fig. 6B).

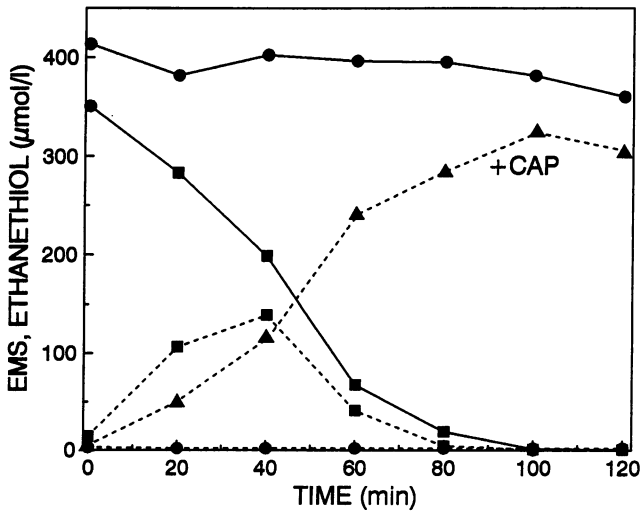

FIG. 3. Nitrate-dependent consumption of EMS and ethanethiol production by *Thiobacillus* strain ASN-1 cells (130 μg of protein ml^{-1}) grown anaerobically on DMS. Symbols: -●-, EMS (no nitrate); -■-, EMS plus 5 mM nitrate; - -●- - ethanethiol production without nitrate; - -■- -, ethanethiol production with nitrate; - -▲- -, ethanethiol with nitrate and chloramphenicol (CAP; 75 μg ml^{-1}).

Utilization of DMS by induced cells of *T. thioparus* T5 was about 33% inhibited by 0.5 mM MBE and nearly 100% inhibited by 2 mM MBE (Fig. 7). However, aerobic consumption of DMS by *Thiobacillus* strain ASN-1 was insensitive to MBE (2 mM) (Fig. 7) but was blocked by CH_3Cl (0.5 mM) (Fig. 8). In contrast to *Thiobacillus* strain ASN-1, $CHCl_3$ had no impact on DMS utilization by *T. thioparus* T5 (Fig. 8). The same pattern of inhibition by MBE and $CHCl_3$ was evident when oxygen uptake rates, rather than DMS disappearance, were measured. Oxygen uptake stimulated by DMS (178 nmol of O_2 min^{-1} mg of $protein^{-1}$) in *Thiobacillus* strain ASN-1 was unaffected by 0.5 mM MBE but promptly and fully inhibited by 0.5 mM $CHCl_3$. In contrast, with *T. thioparus* T5, DMS-stimulated oxygen uptake (118 nmol of O_2 min^{-1} mg of $protein^{-1}$) was immediately blocked by 2 mM MBE but continued without a rate change when 0.5 mM $CHCl_3$ was added. Dibutyl ether (2 mM), in contrast to MBE, had no effect on DMS consumption and associated oxygen uptake by DMS-grown cells of *T. thioparus* T5. $CHCl_3$ inhibited CH_3SH consumption in *Thiobacillus* strain ASN-1 as effectively as it blocked DMS use; CH_3SH use dropped from 58 to 5 nmol min^{-1} mg of $protein^{-1}$ when 0.5 mM $CHCl_3$ was added. Neither MBE (2 mM) nor $CHCl_3$ (0.5 mM) affected rates of substrate use or oxygen uptake during thiosulfate metabolism by cells of thiosulfate-grown organisms.

DISCUSSION

EMS was metabolized by both organisms with removal of the methyl group to yield ethanethiol. With *Thiobacillus* strain ASN-1, ethanethiol was metabolized with extensive degradation to sulfate and probably CO_2. *Hyphomicrobium* strain EG (28) and *T. thioparus* E6 (26) were reported to consume ethanethiol, but ethanethiol was not metabolized by *T. thioparus* T5 in our experiments. Instead, it was abiotically oxidized to diethyl disulfide, which could be reduced by tributylphosphine to recover ethanethiol (Fig. 2).

The results for EMS metabolism point to an initial attack of the methyl group rather than the sulfur atom to yield dimethyl sulfoxide, as occurs both aerobically and anaero-

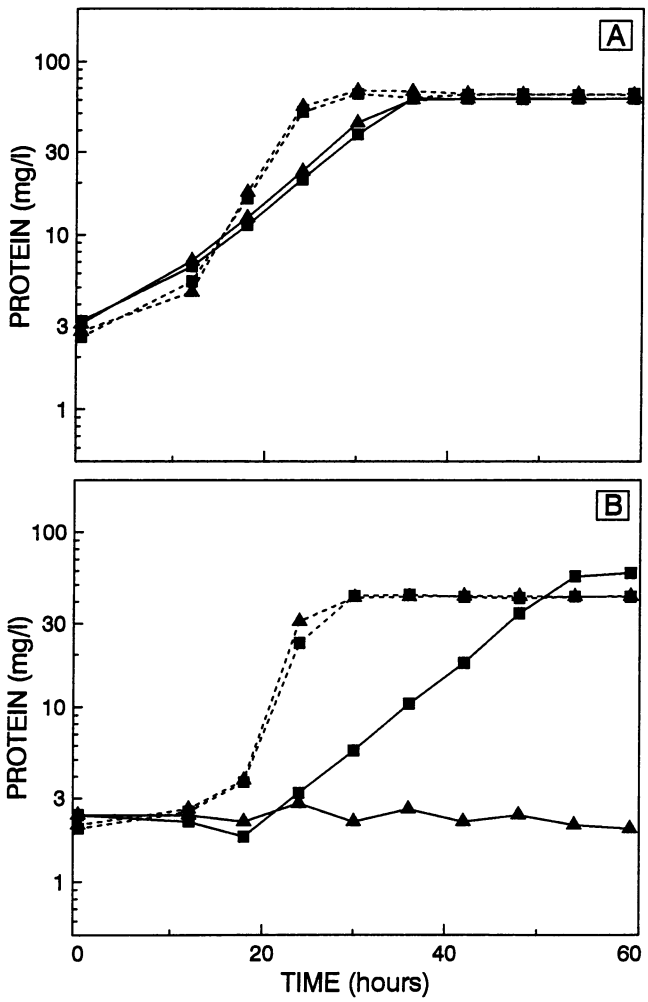

FIG. 5. Effect of ATA (1 mM) on aerobic growth of *Thiobacillus* strain ASN-1 (A) and *T. thioparus* T5 (B) on DMS (3.5 mM strain for ASN-1; 4.7 mM for strain T5) or thiosulfate (10 mM). Symbols: —■—, DMS; —▲—, DMS plus ATA; - -■- -, thiosulfate; - -▲- -, thiosulfate plus ATA.

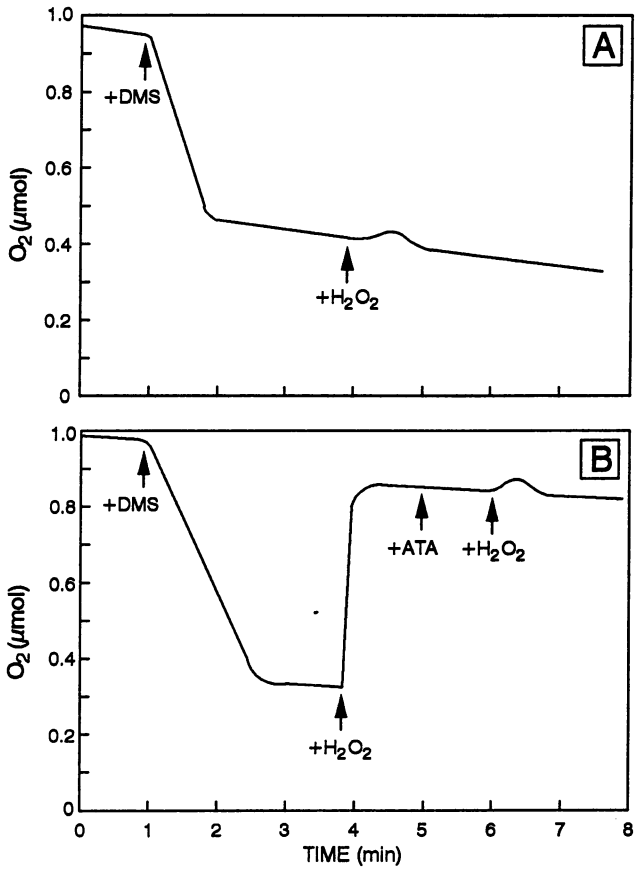

FIG. 6. DMS oxidation and catalase activity determined polarographically in cells grown aerobically on DMS. (A) *Thiobacillus* strain ASN-1 (610 μg of protein ml^{-1}), addition of 0.10 μmol of DMS at 1 min and 0.10 μmol of H_2O_2 at 4 min. (B) *T. thioparus* T5 (540 μg of protein ml^{-1}), addition of 0.15 μmol of DMS at 1 min, 0.10 μmol of H_2O_2 at 4 min, and 5 μmol of ATA at 5 min.

bically with some microorganisms (34, 39, 40) and sometimes involves an oxygenase (16). HCHO production from DMS by *T. thioparus* T5 indicates the functioning of the oxygenase-oxidase pathway in this organism. However, the lack of HCHO formation by *Thiobacillus* strain ASN-1 suggests the absence of the oxygenase-oxidase mechanism.

Higher levels of catalase occurred in *Hyphomicrobium* strain EG when it was growing on DMS or dimethyl sulfoxide than when it was growing on methylamine (29). Similarly, the catalase inhibitor ATA severely retarded the growth of *T. thioparus* E6 on dimethyl disulfide (metabolically equivalent to CH_3SH) but not on thiosulfate (26). *T. thioparus* T5 responded in a similar fashion in our experiments, because growth on DMS (but not on thiosulfate) was blocked completely by ATA, even though the cells had a high catalase level i.e., about 2.0 μmol of H_2O_2 decomposed min^{-1} mg of $protein^{-1}$, which is similar to the activity of 3.4 μmol min^{-1} mg^{-1} of protein present in *T. thioparus* E6 grown on DMS (26). The pathway using O_2 as a substrate for elimination of methyl groups therefore operated in *T. thioparus* T5. In contrast, the aerobic growth of *Thiobacillus* strain ASN-1 on DMS was not affected by ATA and the cells had low catalase levels even when grown on DMS.

The sensitivity of *Thiobacillus* strain ASN-1, but not *T. thioparus* T5, to $CHCl_3$ and the inhibition by MBE of DMS metabolism in *T. thioparus* T5, but not in *Thiobacillus* strain ASN-1, indicate that different pathways of DMS metabolism operate in these strains. $CHCl_3$ inhibits C_1 metabolism by reaction with cobalamin carriers and thus blocks methyltransferase-catalyzed reactions employing this cofactor (37). Our data suggest that MBE probably inhibits DMS monooxygenase and perhaps methanethiol oxidase, whereas $CHCl_3$ inhibits a pathway which involves methyltransferase(s). MBE probably interferes with the metabolism of methylated sulfides because of its structural similarity to methylated thioethers, since dibutyl ether was without effect.

Thiol *S*-methyltransferases are common in many organisms, and they catalyze the sequential methylation of sulfide to yield CH_3SH and then DMS (12). Methoxylated aromatic compounds, probably originating from lignin catabolism in natural environments, were recently identified as methyl donors for the formation of methylated sulfides in anoxic, sulfidic sediments (13) and in a culture of an acetogenic bacterium growing on syringate (4). The acetogen, called strain TMBS 4 and tentatively identified as a *Pelobacter* species (4), possesses a corrinoid-dependent methyltrans-

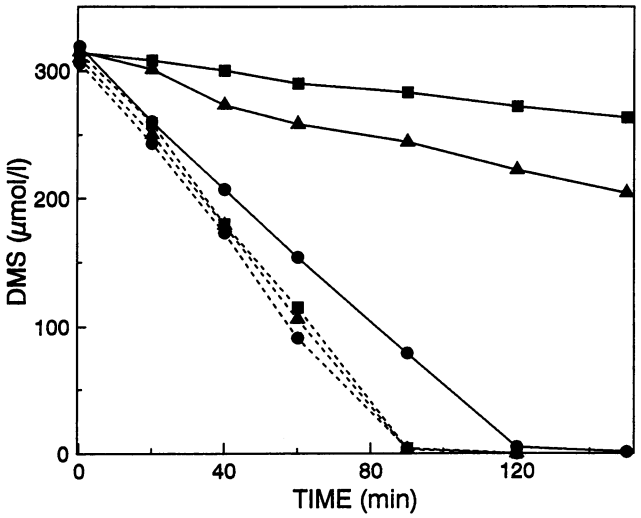

FIG. 7. Effect of MBE on DMS consumption by cells aerobically grown on DMS. —, *T. thioparus* T5 (68 μg of protein ml^{-1}); – – –, *Thiobacillus* strain ASN-1 (56 μg of protein ml^{-1}). Symbols: ●, no MBE; ▲, 0.5 mM MBE; ■, 2 mM MBE.

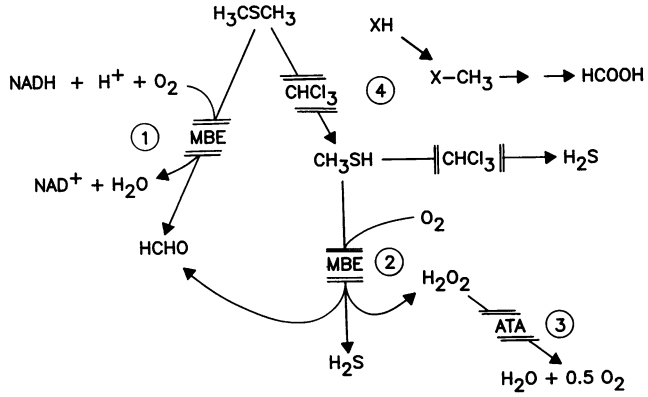

FIG. 9. Outline of oxygenase-oxidase and methyl transfer pathways for catabolism of methylated sulfides. Reactions 1 and 2 are DMS monooxygenase and methanethiol oxidase which are inhibited by MBE. H_2O_2 from the methanethiol oxidase reaction is removed by catalase (reaction 3), and this is inhibited by ATA. Reaction 4 encompasses methyl transfer mechanisms that employ a cobamide carrier (X) and are inhibited by $CHCl_3$. Oxidation of the bound methyl group probably occurs after transfer from cobalamin to tetrahydrofolate and thence oxidation to HCOOH. HCHO from the oxygenase-oxidase pathway is oxidized by dehydrogenases to HCOOH and then CO_2.

ferase (23). Demethylation of 3,4,5-trimethoxybenzoate by intact cells was inhibited by propyl iodide, another established inhibitor of cobamide-dependent systems (7, 38).

An ecological implication of metabolism of DMS by methyl transfer, rather than oxygenases, is for facilitated consumption under fluctuating oxygen concentrations (hyperoxic through anoxic), which are typically encountered in intertidal sediments (35). Evidence suggests that a methyl transfer system is more common in natural environments than is the oxygenase-oxidase pathway. $CHCl_3$ is an effective inhibitor of DMS utilization in natural samples (18, 20, 22). The inhibition by $CHCl_3$ of DMS consumption in seawater (20) suggests that bacteria using a methyltransferase pathway are the dominant DMS consumers in that environment. A similar conclusion arises from experiments with slurries of coastal sediments in which DMS consumption was blocked by $CHCl_3$ (22). MBE may provide a tool for detecting the occurrence of the O_2 incorporation pathway in natural environments. A possible example might be for bacteria associated with oxygenic phototrophs that synthesize dimethylsulfoniopropionate and periodically generate high levels of O_2 (35), i.e., the source of *T. thioparus* T5 (32). Oxygen consumption by oxygenases, as well as by respiration, might provide additional protection from O_2.

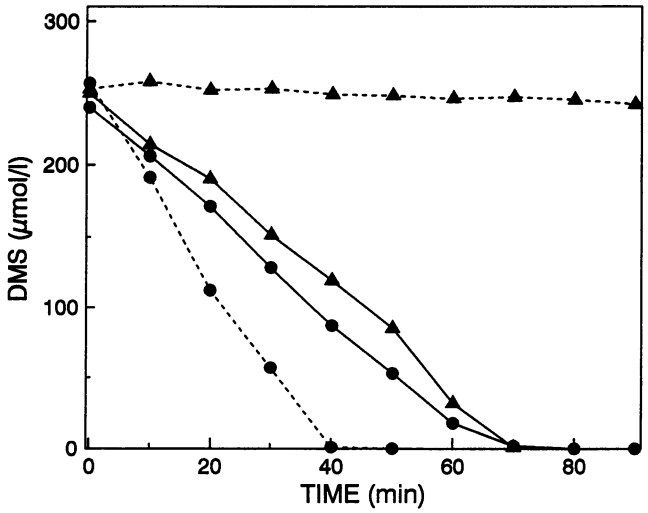

FIG. 8. Effect of $CHCl_3$ on DMS consumption by cells grown aerobically on DMS. —, *T. thioparus* T5 (70 μg of protein ml^{-1}); – – –, *Thiobacillus* strain ASN-1 (102 μg of protein ml^{-1}). Symbols: ●, no $CHCl_3$; ▲, 0.5 mM $CHCl_3$.

In conclusion, at least two mechanisms for aerobic DMS degradation exist (Fig. 9). The first one, previously described for hyphomicrobia and thiobacilli, involves direct incorporation of molecular oxygen into the methyl groups of DMS and CH_3SH in reactions catalyzed by DMS monooxygenase and methanethiol oxidase. These enzymes appear to be inhibited by the structural analog MBE, and growth requires removal by catalase of the H_2O_2 produced by methanethiol oxidase, the organisms are therefore sensitive to the catalase inhibitor ATA during growth on DMS. Methyl transfer reactions are not involved in this catabolic route, and so $CHCl_3$ does not inhibit aerobic growth on methylated sulfides. The other mechanism, previously undescribed, does not use molecular oxygen as a substrate but operates via a methyltransferase which is inhibited by $CHCl_3$ but not by MBE. Because molecular oxygen is not a substrate, this catabolic route also permits growth on DMS with alternative electron acceptors, such as nitrate or nitrite. $CHCl_3$ inhibition implicates a cobamide carrier which probably transfers the methyl group to tetrahydrofolate with subsequent oxidation of the bound C_1 unit to formate.

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