Microbial cycling of volatile organic sulfur compounds in anoxic environments


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Abstract Microbial cycling of volatile organic sulfur compounds (VOSC) is investigated due to the impact these compounds are thought to have on environmental processes like global temperature control, acid precipitation and the global sulfur cycle. Moreover, in several kinds of industries like composting plants and the paper industry VOSC are released causing odor problems. Waste streams containing these compounds must be treated in order to avoid the release of these compounds to the atmosphere. This paper describes the general mechanisms for the production and degradation of methanethiol (MT) and dimethyl sulfide (DMS), two ubiquitous VOSC in anaerobic environments. Slurry incubations indicated that methylation of sulfide and MT resulting in MT and DMS, respectively, is one of the major mechanisms for VOSC in sulfide-rich anaerobic environments. An anaerobic bacterium that is responsible for the formation of MT and DMS through the anaerobic methylation of H2S and MT was isolated from a freshwater pond after enrichment with syringate as a methyl group donating compound and sole carbon source.

In spite of the continuous formation of MT and DMS, steady state concentrations are generally very low. This is due to the microbial degradation of these compounds. Experiments with sulfate-rich and sulfate-amended sediment slurries demonstrated that besides methanogens, sulfate-reducing bacteria can also degrade MT and DMS, provided that sulfate is available. A methanogen was isolated that is able to grow on DMS as the sole carbon source. A large survey of sediments slurries of various origin demonstrated that both isolates are commonly occurring inhabitants of anaerobic environments.

Keywords Dimethyl sulfide; methanethiol; methanogen; sulfide methylation; volatile organic sulfur compounds

Introduction

Several environmental problems (acid precipitation, global warming and odor problems) are related to SO2, H2S and methylated sulfur compounds (Lovelock et al., 1972; Andreae, 1990; Derikx et al., 1990). Release of these compounds from chemical and biotechnological processes should therefore be avoided. Industries that release large amounts of volatile (organic) sulfur compounds (V(O)SC) are composting plants, municipal wastewater treatment plants (Konig et al., 1980), sludge treatment plants, paper and textile industries (Silvelä and Sundmann, 1975). Two commonly occurring VOSC are methanethiol (MT) and dimethyl sulfide (DMS). Several mechanisms for formation and degradation of MT and DMS are reported in the literature (Table 1). In anaerobic environments MT and DMS can be derived from the degradation of sulfur-containing amino acids like S-methylmethionine, methionine and S-methyl-cysteine (Kadota and Ishida, 1972) and from the anaerobic methylation of sulfide (Finster et al., 1990). According to the latter mechanism, microbes transfer the methyl groups of methoxylated aromatic compounds on sulfide and MT resulting in the formation of MT and DMS, respectively. Other possible mechanisms for MT and DMS formation under anaerobic conditions are dimethylsulfoxide (DMSO) reduction and dimethyl disulfide (DMDS) reduction. MT can also be formed due to the aerobic methylation of sulfide by various bacteria as a method of detoxification of sulfide.
Despite the continuous formation of MT and DMS, steady-state concentrations in anaerobic environments are generally very low. This is due to anaerobic degradation of DMS and MT that can be mainly attributed to the activity of methanogenic Archaea. During methanogenic conversion, DMS is reduced to form methane and MT, and the latter is subsequently disproportionated resulting in the formation of methane, carbon dioxide and H₂S. The excess of reduction equivalents that is released during this disproportionation of MT is used for the initial reduction of DMS. In this way, 2 moles of DMS are stoichiometrically converted to 3 moles of methane and 1 mole of carbon dioxide. However, also sulfate-reducing bacteria, anoxygenic phototrophs and denitrifying bacteria have been reported to be able to degrade MT and DMS under anaerobic conditions. A sulfate-reducing bacterium growing on DMS has been isolated from a thermophilic digestor (Tanimoto and Bak, 1994). The strain, which belongs to the genus *Desulfotomaculum*, oxidizes DMS to carbon dioxide forming 2.5 moles of sulfide per mole of DMS (Table 1). The present study was performed in order to elucidate the dominant mechanisms for VOSC production and degradation in anaerobic environments and to identify the microbes responsible.

**Methods**

**Sediment slurry incubations.** Sediment samples were collected from various freshwater systems in The Netherlands. The sediment samples were transferred by suction to anaerobic bottles as described previously (Lomans *et al.*, 1997). The sampling and dispensing of the sediment slurries was done with new and sterilized equipment to avoid cross contamination of the samples in the MPN counting and ARDRA. The sediment slurries collected were used for sediment incubation experiments, MPN series and molecular detection of DMS producing and degrading microorganisms. Sediment slurries for incubation experiments were prepared and dispensed in anaerobic bottles as described previously (Lomans *et al.*, 1997). Additions were made from pH neutral stock solutions prepared in distilled water. These additions included bromoethanesulfonic acid (BES, an inhibitor of methanogens, final concentration 2.5 mM), sodium tungstate (inhibitor of sulfate reducing
bacteria, final concentration 2.0 mM), and syringate (final concentration 0.1 mM). The sedi-
ment slurries (duplicates or triplicates) were incubated in the dark without shaking at
30°C. Sterilized sediment slurries (121°C, 20 min) served as abiotic controls.

**Enrichment, isolation and cultivation procedure.** Enrichment of the DMS-degrading iso-
late, *Methanomethylovorans hollandica* strain DMS1T (Lomans et al., 1999) was done in
an anaerobic chemostat fed with low concentrations of DMS (20–250 nmol per ml head-
space) via the N₂/CO₂ (80/20, vol/vol) gas stream through the system (Figure 1). After
enrichment, strain DMS1 was isolated by the deep agar method. The MT/DMS-producing
bacterium, *Parasporobacterium paucivorans* strain SYR1, was isolated in similar way on
medium supplemented with syringate and sulfide. Batch cultivation was carried out in
either 60 or 120 ml serum bottles filled with 25 or 50 ml of medium, respectively. The
defined sulfide-reduced and bicarbonate-buffered medium of Widdel and Bak (1992) was
slightly modified and used for isolation.

**MPN counting.** The number of bacteria involved in the cycling of MT and DMS as
determined by MPN series. Since degradation of methoxylated aromatic compounds has
been shown to result in the formation of VOSC in freshwater sediments, the dominant
microorganisms involved in the degradation of this compound were studied by MPN series
with anaerobic medium supplemented with syringate. The type and number of the
MT/DMS consuming bacteria was determined by MPN series with the anaerobic medium
described by Widdel and Bak (1992) supplemented with DMS as was used for isolation of
*Methanomethylovorans hollandica* (Lomans et al., 1999). Analysis of the type and number
of anaerobic methylo trophic bacteria was done by MPN series with the same medium
supplemented with a mixture of methanol, TMA and DMS. Screening of the highest
positive dilution was done by the measurement of MT/DMS formation/disappearance,
methane formation and turbidity.

**Amplified Ribosomal DNA Restriction Analysis (ARDRA).** The presence of *M. hollandica-
like organisms was determined by molecular analysis of the total DNA isolated from the
sediment slurries using PCR-primers specific for the amplification of the 16S rRNA gene
of Archaea, followed by restriction analysis with the restriction enzyme HindIII. After gel
electrophoresis this procedure resulted in a *M. hollandica* specific band pattern.

**Chemical analysis.** Specific determination of sulfur compounds (H₂S, MT and DMS) in
gas samples was done on a Packard 438A gas chromatograph equipped with a flame photo-
metric detector and a Carbopack B HT100 (40/60 mesh) column as described by Derikx et
al. (1990) and Lomans et al. (1997).

Gas samples (0.5–1.0 ml) were analyzed for methane, MT and DMS on a Hewlett
Packard (5890) gas chromatograph equipped with a flame ionization detector and a
Porapak Q (80/100 mesh) column (12, 20).

**Results and discussion**

**Anaerobic methylation of sulfide.** In sulfide-rich sediments, formation of MT and DMS
was significantly higher as compared to sulfide-poor sediments (data not shown).
Moreover, addition of sulfide to sulfide-poor sediments slurries resulted in a strong stimu-
lation of the MT formation rate (Figure 1a). In sulfide-rich sediments, MT/DMS formation
was significantly stimulated upon addition of methoxylated aromatic compounds like
syringate and 3,4,5-trimethoxybenzoate and syringate. Moreover high in situ MT/DMS
concentrations appeared to coincide with high sulfide concentrations (data not shown).
These results therefore indicate that anaerobic methylation of sulfide is a major mechanism for MT/DMS formation in sulfide/organic-rich anaerobic environments.

**Anaerobic degradation of DMS.** Although many sediments had been found to have high aerobic DMS degradation capacities (4.95 nmol DMS per ml of sediment slurry · h⁻¹), DMS is mainly degraded anaerobically (0.32–0.3 nmol DMS per ml of sediment slurry · h⁻¹) due to the lack of oxygen. The origin of anaerobic degradation of DMS and MT was studied by sediment incubation experiments. Inhibition of sediment slurries with BES resulted in a strong accumulation of MT, indicating that MT is degraded by methanogens (Figure 1b). Also BES inhibition of DMS amended sediment slurries clearly demonstrated that DMS is mainly degraded by methanogens (Figure 1c). These experiments therefore revealed that methanogenic conversion is the major mechanism for MT/DMS degradation in anaerobic environments.

**Effect of sulfate on the degradation of endogenous DMS and MT.** Competition between sulfate reducing bacteria and methanogenic bacteria for DMS has been described to occur under conditions where sulfate is present. In order to find out whether a similar competition occurs for the conversion of MT/DMS, incubation experiments were performed with sulfate-rich sediment slurries and slurries amended with sulfate. Degradation of endogenously produced MT was analyzed by the inhibition with BES, molybdate or BES plus molybdate. In sulfate-poor sediment slurries (IV), accumulation of MT was obtained after addition of BES (Table 2). In sulfate-rich sediment slurries (I–III), however, MT accumulation was only found upon addition of both BES and molybdate.

In incubation experiments with MT- and DMS-amended slurries, the inhibition of MT

| Table 2 Effect of sulfate concentrations on the formation of methane and the degradation of MT of various sediment slurries prepared from freshwater ditches at Zegveld, The Netherlands |
|---|---|---|---|---|---|
| Sample | [SO₄²⁻] (µM) | Control | BES | Molybdate | BES + |
| | | CH₄ | MT | CH₄ | MT | CH₄ | MT |
| I | 1,783 | 4.2 | 0 | 2.6 | 0 | 108 | 0 | 12 | 0.21 |
| II | 1,780 | 1.3 | 0 | 0.6 | 0 | 55 | 0 | 23 | 0.20 |
| III | 1,825 | 9.8 | 0 | 3.4 | 0 | 92 | 0 | 17 | 0.19 |
| IV | 549 | 175 | 0 | 36.5 | 0.46 | 105 | 0 | 27 | 0.12 |

* Rates of methane and MT formation were calculated from time courses of duplicate bottles, without addition or with addition of BES (25 mM), molybdate (20 mM) or BES (25 mM) plus molybdate (20 mM) and the formation rates are given in pmol/mg organic matter · h⁻¹.
and DMS degradation by BES could be reversed by the addition of sulfate. This effect of sulfate was only present under N₂ atmosphere and absent under H₂ atmosphere. These results point to a syntrophic metabolism between methanogens and sulfate reducers in which reduction equivalents derived from the oxidation of DMS (methanogen) are scavenged by the sulfate reducing bacterium.

**MPN counts.** Numbers for syringate-utilizing, DMS-degrading and methanol/TMA/DMS-degrading microbes were determined by MPN counts. Counts from MPN series with mixed substrate series differed dramatically between the various sediments, ranging from \(2.3 \times 10^1\) to \(9 \times 10^5\) bacteria per ml sediment slurry. Microscopic analysis revealed that the cultures existed of one type of methanogenic Archaea, which was morphologically similar to *M. hollandica*, previously isolated from the Dekkerswald sediment (Lomans et al., 1999). Numbers of syringate-utilizing and DMS-degrading microbes were generally lower (\(2.3\times10^1\)–\(2.3\times10^5\) bacteria/ml slurry and \(0.4\times10^1\)–\(2.3\times10^5\) bacteria/ml).

**Amplified Ribosomal DNA Restriction Analysis (ARDRA).** Restriction enzyme analysis of the Archaea-specific PCR product mixture with HindIII followed by polyacrylamide gel electrophoresis (PAGE) resulted in a band pattern specific for *M. hollandica* (687 and 187bp). This band pattern, although in different intensities, was present in the samples of all sediments (lane 6–15) (Figure 2).

**Conclusion**
The unique combination of sediment incubation experiments, enrichment and isolation of microbial strains, MPN series and molecular genetic detection provided convincing evidence to define anaerobic methylation of sulfide and MT and the anaerobic methanogenic degradation of MT and DMS as the major mechanisms in the cycling of MT and DMS in sulfide-rich anaerobic environments. It was illustrated that the degree of MT/DMS formation depends on the amount of sulfide and methyl-donating compounds (e.g. syringate) present in the sediment. The concentration of sulfide in its turn is determined mainly by the concentration of sulfate and iron (Figure 3). Furthermore, it illustrated

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**Figure 2** ARDRA of the total DNA isolated from the various sediments (1–11). The PCR product generated with two Archaea specific primers (1AFOR and ARC915) was digested with restriction enzyme, HindIII. The band pattern with a 187 and 874 product appeared to be specific for *Mm. hollandica*. Loading of the samples (from left to right); Marker, Low Molecular Weight Marker; A, *Mm. hollandica* strain DMS1; B, *Ms. Barkeri*; C, *M. thermoautotrophicum*; D, *Methanosphaera curviculi*; 1–11, sediment 1 to 11 (For sediment origin see Table 1). Sediment 11 is a sandy organic-poor sediment, which is used as a negative control since it is unlikely to contain large amounts of methanogenic Archaea.
that the isolated DMS-degrading methanogen, *M. hollandica*, is a commonly occurring inhabitant of these anaerobic freshwater environments. It was also elucidated that besides methanogens, sulfate reducing bacteria can also take part in MT/DMS degradation provided that sulfate is present. A recently performed study on an UASB reactor treating MT as major organic compound revealed that methanogens similar to *M. hollandica* were also present in this reactor. These recent findings illustrate that processes as they occur in these reactors are similar, although intensified, to that in nature and are therefore a nice example of how naturally occurring processes can be used to our own benefit.

**References**


