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Anaerobic microbial degradation of methylated sulfur compounds

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

1996

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van der Maarel, M. J. E. C. (1996). *Anaerobic microbial degradation of methylated sulfur compounds*. s.n.

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Chapter 8

Dimethylsulfide (DMS) is one of the most abundant organic sulfur compounds in the marine environment and is thought to play an important role in the formation of cloud condensation nuclei through its atmospheric oxidation products methanesulfonic acid and sulfuric acid. DMS also forms a crucial link in the global sulfur cycle because it is the main sulfur species that is transported from marine to terrestrial environments. The precursor of DMS in marine environments is the osmolyte dimethylsulfoniopropionate (DMSP), which is produced by a variety of micro- and macroalgae, e.g. *Phaeocystis* sp. and *Emiliania huxleyi*. Processes such as senescence, grazing of zooplankton, or lysis of the algae result in the liberation of DMSP. Most of the free DMSP is likely degraded in the oxic water phase of the oceans and a variety of aerobic DMSP-degrading bacteria have been isolated in recent years. However, at the start of this thesis project less was known about the microorganisms which are involved in the conversion of DMSP in anoxic environments such as intertidal sediments and the gut of marine animals.

The degradation of DMSP in anoxic marine environments had only been studied in sediment slurry experiments at the start of this thesis project (Kiene and Visscher 1987; Kiene and Taylor 1988). These experiments showed that DMSP can be degraded via a cleavage to DMS and acrylate, via a demethylation to 3-mercaptopropionate (MPA), with 3-S-methylmercaptopropionate (MMPA) as a possible intermediate, or via an initial demethylation coupled to the production of methanethiol. A strain of *Clostridium propionicum*, which had been isolated from river mud, was the only pure culture of a DMSP-cleaving bacterium (Wagner and Stadtman 1962). This thesis describes studies that provide a better insight into the nature and properties of the microorganisms that are involved in the degradation of DMSP in anoxic intertidal sediments. In Chapter 1 some general information on DMS(P) as well as the anaerobic microbial degradation of DMS(P) is given.

A DMSP-demethylating, sulfate-reducing bacterium (strain WN) was isolated from an enrichment culture of 10^5 times diluted anoxic sediment of the Wadden Sea (Chapter 3). Strain WN was tentatively identified as a strain of *Desulfobacterium autotrophicum*, based on its substrate utilisation and hybridisation with a *Desulfobacterium*-specific 16S rRNA probe. However, phylogenetic analysis of the 16S rRNA gene sequence revealed

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that strain WN is more closely related to the genus *Desulfobacter*. Determination of the cellular fatty acid composition and measurement of certain enzyme activities lead to the conclusion that strain WN taxonomically has an intermediate position between the genera *Desulfobacterium* and *Desulfobacter*. It was also found that *Db. vacuolatum* (DSM 3385), *Db. niacini* (DSM 2650), and *Db. niacini* strain PM4 (Chapter 2) are able to demethylate DMSP to MMPA. *Eubacterium limosum* strain PM31, which is salt-tolerant, also grows on the demethylation of DMSP, albeit very slowly and with a very low growth yield. Substrate progress curves in cell suspensions indicated that strain WN and *Db. niacini* strain PM4 have a high affinity for DMSP (K_m approximately 10 μM).

The product of the demethylation of DMSP, MMPA, turned out to be a good substrate for the growth of *Methanosarcina* sp. strain MTP4 (DSM 6636), *Ms. siciliae* (DSM 3028), and *Ms. acetivorans* (DSM 2834), which all originate from marine environments and are also able to utilize DMS and methanethiol (Chapter 4). The methanogenic conversion of MMPA to MPA was also studied in sediment slurries. Addition of 500 μM MMPA resulted in the production of methanethiol, which was subsequently converted to 417 μM methane. In the presence of the antibiotics ampicillin, vancomycin, and kanamycin (20 $\mu\text{g/ml}$ each), 275 μM methane was formed from 380 μM MMPA; no methanethiol was formed during these incubations. Only methanethiol was formed from MMPA when 2-bromoethanesulfonic acid (25 mM) was added to a sediment suspension. These results indicate that in anoxic intertidal sediments, DMSP is not only a precursor of a negative effector of global warming, but can also result in the production of a potent greenhouse gas.

The finding that certain marine *Methanosarcina* strains are able to metabolize MMPA raised some interesting biochemical questions, which are discussed in Chapter 4. It also raised the question what the possible role of MMPA and other algal metabolites can be in the formation of methane in open ocean water. In most oceans a supersaturation of methane is found at certain depths that still do contain oxygen. Since methanogenesis is a strict anaerobic process, this phenomenon has been called the oceanic methane paradox (Kiene 1991). Active methanogenesis has been reported to occur in the Mediterranean Sea (Marty 1993) and several strains of methanogens have been isolated

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from open ocean water (Cynar and Yayanos 1991; Sieburth 1993). Two possible hypotheses have been put forward to explain the oceanic methane paradox: (i) formation of methane in the gut of zooplankton that graze on phytoplankton and subsequent leakage from the faecal pellets of this zooplankton (Marty 1993; de Angelis and Lee 1994; Karl and Tilbrook 1994); (ii) formation of methane inside reduced microzones of particles derived from decaying algal matter (Sieburth 1993). Compounds derived from algae, such as glycine betaine and DMSP, could through their degradation products trimethylamine, DMS, and MMPA indirectly be good substrates for these oceanic methanogens. It is not known whether the isolates that have been obtained from ocean water are able to grow on DMS or MMPA.

A new DMSP-cleaving strain (W218) was isolated from enrichments with DMSP and yeast extract as substrates and anoxic Wadden Sea sediment as inoculum (Chapter 5). After the cleavage of DMSP to DMS and acrylate, strain W218 did not ferment the acrylate but used it as an electron acceptor. Besides acrylate it can also reduce sulfate, thiosulfate, and sulfite. Strain W218 was identified as a new species of the genus *Desulfovibrio*, derived from its phylogenetic position based on 16S rRNA gene sequence analysis and physiological characteristics, and was named *Desulfovibrio acrylicus*. The reduction of the acrylate from DMSP by *Dv. acrylicus* was a complete surprise. We expected to find a fermentation of acrylate, as had been described for the DMSP-cleaving strain of *C. propionicum* (Wagner and Stadtman 1962). The reduction of acrylate is energetically more favourable than the reduction of sulfate, as already pointed out in Chapter 5. At limiting electron donor concentrations this thermodynamic advantage may be turned into a competitive advantage for *Dv. acrylicus* and may explain why a sulfate-reducing bacterium is also able to reduce acrylate in an environment where sulfate is present in high concentrations (up to 28 mM). The ability to reduce acrylate was not found among a variety of other sulfate-reducing bacteria.

The enzyme which is responsible for the cleavage of DMSP to DMS and acrylate has received considerable attention in the last three years and has been purified and characterized from the red alga *Polysiphonia paniculata* (Nishiguchi and Goff 1995), the facultative anaerobe *Alcaligenes* sp. strain M3A (de Souza and Yoch 1995a), and the sul-

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fate-reducing bacterium *Dv. acrylicus* strain W218 (Chapter 6). Also the general characteristics of the DMSP lyase of *Phaeocystis* sp. have been studied in cell extracts (Stefels and van Boekel 1993). Strain W218 has a very active DMSP-cleaving enzyme (9.8 U/mg protein in cell extracts), which was purified 390-fold and had a K_m for DMSP of 0.45 mM and a V_{max} of 2593 U/mg at 30°C (Chapter 6). It is only active with DMSP as a substrate and is most effectively inhibited by dimethylsulfoniobutyrate ($K_i = 0.25$ mM).

The general picture that is emerging from these data is that the algae have a DMSP lyase with a high affinity, but a low maximum specific activity for DMSP. Bacteria on the contrary have DMSP lyases with kinetic properties that are opposite to the algal DMSP lyases. However, when considering the turnover numbers, the DMSP lyase from *Dv. acrylicus* is able to convert more DMSP per unit of time than the *P. paniculata* and the *Alcaligenes* DMSP lyase ($k_{cat} = 127/s, 25/s,$ and $20/s,$ respectively). The V_{max}/K_m ratio, which is indicative for the efficiency of the enzyme, indicates that the *Dv. acrylicus* DMSP lyase ($K_m/V_{max} = 0.17$) is as efficient as the *P. paniculata* DMSP lyase ($K_m/V_{max} = 0.28$), but much more efficient than the *Alcaligenes* DMSP lyase ($K_m/V_{max} = 3.4$). Comparison of such kinetic properties indicates that the three DMSP lyases which have been purified and characterized so far are different from each other and are probably not closely related. Future analysis of their gene sequences will have to show whether this assumption is true or not.

The localization of the DMSP lyase in *Dv. acrylicus* has not been determined. Because the K_m of the purified *Dv. acrylicus* enzyme for DMSP is in the same order as the apparent K_m of whole cells, it can be speculated that this DMSP lyase is present in the periplasmic space and not intracellularly. That would be different from the DMSP lyase of *Pseudomonas doudoroffii* which is believed to be localized intracellularly, because the apparent K_m of cell extracts (1.82 mM DMSP) is much higher than the K_m value of whole cells (less than 20 μ M DMSP). It has been suggested that in *P. doudoroffii* DMSP is transported by a DMSP binding protein into the cell (de Souza and Yoch 1995b).

It had been reported in the literature that sulfate-reducing bacteria are not able to reduce dimethylsulfoxide (DMSO) to DMS (Kiene and Capone 1988). This conclusion

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was based on sediment slurry experiments to which DMSO and molybdate, a specific inhibitor of sulfate reduction, had been added. Because inhibitor studies have to be interpreted with caution, pure cultures of sulfate-reducing bacteria were tested for their ability to reduce DMSO. *Db. niacini* and several different strain of the genus *Desulfovibrio*, all from marine or saline environments, were found to reduce DMSO to DMS (Chapter 7). In *Dv. desulfuricans* strain PA 2805 DMSO was reduced simultaneously with sulfate and the reduction of DMSO was not inhibited by molybdate.

It is not yet clear whether the microorganisms which have been studied in this thesis or the metabolic pathways these microorganisms use, such as acrylate reduction, are abundantly present in nature. Novel molecular ecological techniques can be useful to get more information on such questions, for instance the use of species- or group-specific 16S rRNA probes either for slot-blot hybridization (Lee and Fuhrman 1990; Raskin et al. 1994) or for in-situ hybridization using fluorescent dyes (Amann et al. 1992). Once gene sequences for the different bacterial DMSP lyases or the acrylate-reducing enzyme are known, more specific probes can be developed for use in denaturing gradient gel electrophoresis (Wawer and Muyzer 1995) or reversed sample genome probing (Voordouw et al. 1991). These techniques have been applied successfully to study e.g. the diversity of *Desulfovibrio* species in microbial mats (Wawer and Muyzer 1995) or in oil field samples (Voordouw et al. 1991). The use of such molecular ecological techniques in future studies should also reveal whether the strains that have been studied in this thesis are ecologically relevant or not.

The results presented in this thesis underline the physiological potential of sulfate-reducing bacteria as a group in total. At the moment more than 100 different substrates have been identified for sulfate-reducing bacteria (Hansen 1993). It has recently been shown that certain sulfate-reducing bacteria are able to reductively degrade halogenated compounds (DeWeerd et al. 1990) or trinitrotoluene (Boopathy et al. 1993), to reduce uranium VI to uranium IV (Lovley and Phillips 1992), and are also involved in the degradation of polycyclic aromatic hydrocarbons (Coates et al. 1996). In the course of this thesis project it became clear that the conversion of DMSP to MMPA by sulfate-reducing bacteria may find biotechnological applications in the production of natural aromatic

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compounds. A patent application on the MMPA production by sulfate-reducing bacteria has been filed in collaboration with a Dutch biotechnology company (Hansen and van der Maarel 1994).

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