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‘Microbial demethylation of dimethylsulfoniopropionate and methylthiopropionate’

van Michael Jansen

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MICROBIAL DEMETHYLATION OF DIMETHYLSULFONIOPROPIONATE AND METHYLTHIOPROPIONATE

Michael Jansen

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RIJKSUNIVERSITEIT GRONINGEN

MICROBIAL DEMETHYLATION OF DIMETHYLSULFONIOPROPIONATE AND METHYLTHIOPROPIONATE

Proefschrift

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Michael Jansen

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Promotor:	Prof. Dr. L. Dijkhuizen
Co-promotor:	Dr. Ir. T.A. Hansen

Beoordelingscommissie:	Prof. Dr. J.A.M. de Bont
	Prof. Dr. D.B. Janssen
	Prof. Dr. B. Poolman

Hierbij wil ik graag alle mensen bedanken die op de een of andere manier een bijdrage hebben geleverd aan dit proefschrift.

Michael

Voor mijn moeder

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1

Introduction

Dimethylsulfoniopropionate: an interesting precursor of natural flavor compounds ?

Production of natural flavors. Flavor compounds play an important role in the manufacturing of food. Traditionally, mixtures of synthetically produced flavors are used for flavoring of, for example, candy, snacks, and certain dairy products. Nowadays, the use of naturally produced flavors is becoming increasingly important, because consumers tend to prefer natural compounds for health reasons (Armstrong and Yamazaki 1986). With the aid of extraction techniques it is possible to obtain flavors directly from plant material, but these methods are time-consuming and expensive, because the most interesting flavors are present in only very low concentrations. A more recent method to produce flavors is based on a biotechnological approach where natural precursors, isolated mainly from plant material, can be converted in a bioreactor with the aid of enzymes and/or microorganisms to the desired flavor (Tyrell 1990). The difference in price between a natural and a synthetic compound can be very large; synthetic vanillin costs $ 12 /kg, when extracted from vanilla pods the price is $ 4000 /kg (Feron et al. 1996). This large difference in price clearly demontrates that other methods for the production of natural flavors are needed. Some examples of important flavors that can be produced with a biotechnological approach are given below.

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is one of the most common flavor chemicals; it is used in a broad range of flavors. The consumption of vanillin is estimated to be 12000 tons/year; only 50 tons are in the form of natural vanillin from the pods of *Vanilla planifolia* (Lomascolo et al. 1999). A lot of research is done in order to produce vanillin that can be labelled as natural. Production of vanillin is possible from ferulic acid or vanillic acid by several fungi; the preferred organism *Pycnoporus cinnabarinus* produces only 40 –90 mg vanillin/l in 5 to 7 days (see, for example, Gross et al. 1993 or Lesage-Meessen et al. 1999). Production of vanillin is also possible from eugenol, a cheap constituent of clove oil, for example by a *Pseudomonas* strain (Washisu et al. 1993).

Various conversions are known which lead to γ-decalactone (the peach-like flavor) with the aid of fungi or bacteria (Krings and Berger 1998). A number of patents describe the fermentation of castor oil or other substrates to γ-decalactone (see, for example, Farbood and Willis 1985; Farbood et al. 1990; Cheetham et al. 1993; Kumin and Munch 1998). Castor oil from seeds of *Ricinus communis* in which approximately 85% of the fatty acids are ricinoleic acid, one of the most important precursors, is a good substrate for *Yarrowia lipolytica* (Farbood and Willis 1985). Also other microorganisms, such as *Candida, Sporobolomyces* and *Rhodotorula* species carry out this fermentation in rich media and produce γ-decalactone in yields ranging from approximately 0.3 – 1.0 g γ-decalactone/l in 7 days (Cheetham et al. 1993). Fermentation of ricinoleic acid to γ-decalactone by *Saccharomyces cerevisiae* results in 2.0-3.75 g γ-decalactone per kg fermentation broth (Boog et al. 1998). Unlike some of the other organims mentioned, *Saccharomyces cerevisiae* is an acceptable organism for making food grade products.

Sulfur-containing flavors and dimethylsulfoniopropionate. There are many examples of sulfur-containing flavors present in food. Often, these sulfur-containing compounds are present as natural flavors in food and contribute already in low concentrations to its total flavor. Dimethylsulfide (CH_3-S-CH_3) and methanethiol (CH_3-SH) are naturally present in several types of food or drinks, like raw milk (Patton et al. 1956; Law and Sharpe 1978), beer (Walker and Simpson 1993), cheese, garlic, and tea (Schreier and Drawert 1975). High concentrations of dimethylsulfide results in an unpleasant, cabbage like odor. Esters of methylthiopropionate (MTPA; or methylmercaptopropionate) and mercaptopropionate (MPA; Fig. 1) can be used as a flavoring ingredient (Hansen and van der Maarel 1998; Hansen et al. 1998). For example, ethyl-mercaptopropionate provides a pleasant grape flavor (Kolor 1982). The use of the methyl ester of MTPA as flavoring ingredient results in a fruity flavor (van der Maarel and Hansen 1998). Methyl and ethyl esters of MTPA naturally occur in pineapple fruits. These compounds can be used in, for example, baked goods, frozen dairy, meat products, soft candy and non-alcoholic beverages in low ppm concentrations (Fenaroli 1995).

An interesting precursor for several sulfur-containing flavors is beta-dimethylsulfoniopropionate (DMSP; formerly known as dimethyl-beta-propiothetin). DMSP is mainly present in the marine environment where it acts as an osmolyte in many algae (Reed 1983; Keller 1988) and in some higher plant species (see, for example, Dacey et al. 1987). DMSP can be used as feeding stimulator for fishes and shellfishes to promote their appetites (Nakajima and Itoh 1992). Tatsuya and coworkers (1988) suggested that DMSP, obtained from marine algae with a high DMSP content, can be used as a natural flavor precursor for food and drink. The major reason for scientific interest in DMSP during the past 15 years stems from an interest in dimethylsulfide, which is one of the products after DMSP is cleaved (Fig. 1). In the last five years, this interest in DMSP has resulted in two symposia (The First International Symposium on DMSP and Related Sulfonium Compounds, held in Mobile, Alabama, USA in June 1995, and the Second International Symposium on Biological and Environmental Chemistry of DMS(P) and related compounds, held in Haren, The Netherlands in August 1999), and in a number of Ph.D projects at the University of Groningen. Examples are: "Anaerobic microbial degradation of methylated sulfur compounds", by M.J.E.C van der Maarel (1996), "The smell of the sea. Production of dimethylsulfoniopropionate and its conversion into dimethylsulphide by the marine phytoplankton genus *Phaeocystis*", by J. Stefels (1997), and "Microbial production and consumption of dimethyl sulfide (DMS) in intertidal sediment ecosystems", by H.M. Jonkers (1999).

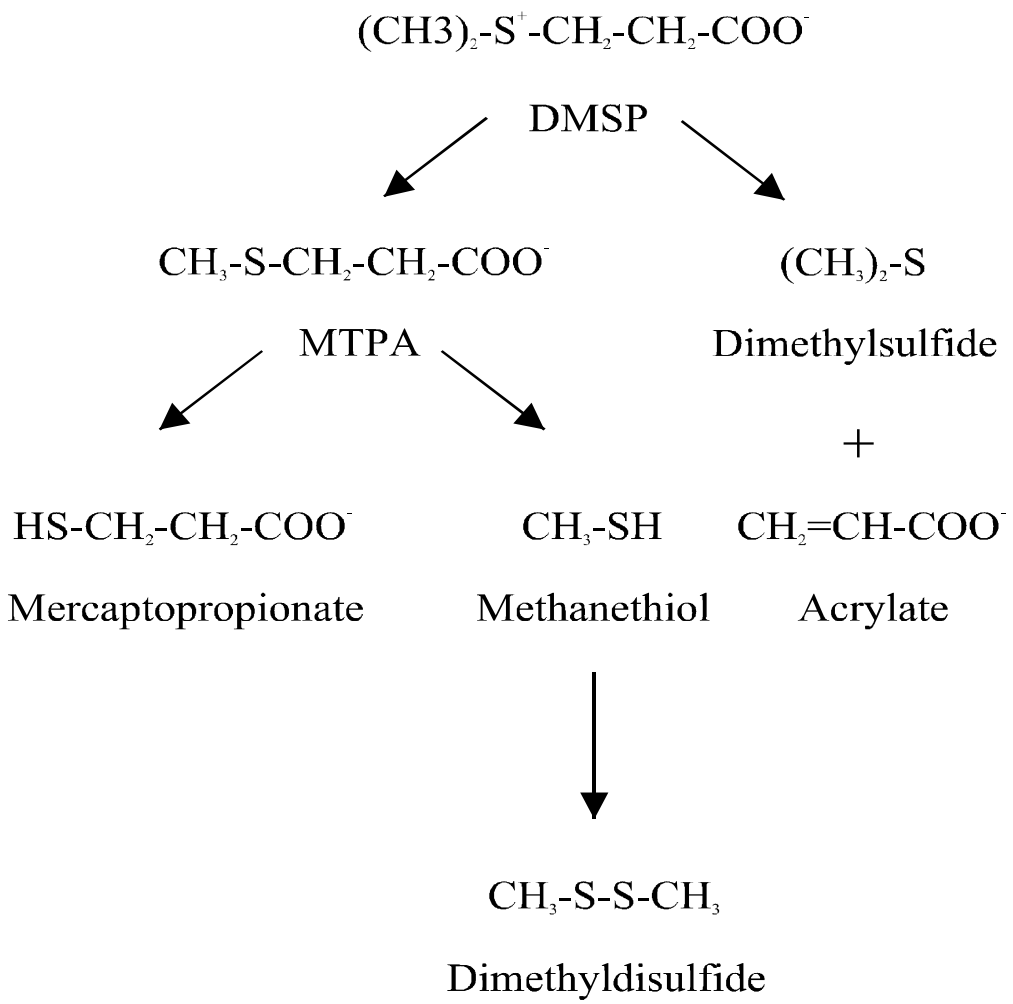

Fig. 1 Pathways for the microbial degradation of dimethylsulfoniopropionate

DMS(P) and the environment. Almost thirty years ago it became clear that dimethylsulfide is quantitatively the most important compound involved in the transport of sulfur from the ocean to land; it therefore plays an important role in the global sulfur cycle (Lovelock et al. 1972). This was not the only reason for the increased interest in dimethylsulfide. It has also been suggested that the emision of dimethylsulfide has an important effect on the climate of the earth (Bates et al. 1987; Charlson et al. 1987). When dimethylsulfide reaches the atmosphere it can be oxidized by radicals, such as hydroxyl radicals, resulting in dimethylsulfoxide and acidic products (methanesulfonic acid, sulfur dioxide, sulfuric acid) which act as cloud condensation nuclei and decrease the amount of sunlight reaching the earth's surface. This directly affects the climate of the planet. Another important effect of the oxidation products is their contribution to the acidity of rain water (Charlson and Rodhe 1982).

Biosynthesis of DMSP. DMSP is present mainly in the marine environment where it acts as an osmolyte in many different macro- and microalgae (Reed 1983; Keller 1988), but also in some higher plant species, such as *Spartina anglica* (van Diggelen et al. 1986), *Spartina alterniflora* (Dacey et al. 1987), *Wollastonia biflora* (Hanson et al. 1994) and sugarcane (Paquet et al. 1994). The pathway of DMSP formation in algae was elucidated recently by Gage et al. (1997). With in vivo isotope labelling, DMSP formation from methionine was shown to occur in the green

macroalga *Enteromorpha intestinalis*. Methionine conversion involved the following steps: transamination, reduction, S-methylation, and oxidative decarboxylation. The intermediates in this pathway to DMSP are: 4-methylthio-2-oxobutyrate, 4-methylthio-2-hydroxybutyrate and 4-dimethylsulfonio-2-hydroxybutyrate (Fig 2A). The compound 4-dimethylsulfonio-2-hydroxybutyrate was also identified in three diverse phytoplankton species, which indicates that the same pathway exists in other algal classes.

In plants the biosynthesis of DMSP is different (Fig 2B). In *Wollastonia biflora* leaves the first step was a methylation of methionine to *S*-methylmethionine catalyzed by the enzyme *S*-adenosylmethionine:methionine *S*-methyltransferase (Hanson et al. 1994). *S*-Methylmethionine is converted further in one step to DMSP-aldehyde, via a coupled transamination-decarboxylation reaction (Rhodes et al. 1997). In *Spartina alterniflora*, *S*-methylmethionine is not degraded in one step to DMSP-aldehyde, but via the intermediate DMSP-amine (Kocsis et al. 1998). Evidence that DMSP-aldehyde was an intermediate in the biosynthesis of DMSP in *Wollastonia biflora* was obtained by James and coworkers (1995) in three ways. In pulse-chase experiments with [^{35}S]methylmethionine, DMSP-aldehyde labeled as an intermediate and other possible intermediates did not. When adding [^{35}S]methylmethionine with other unlabeled compounds, only DMSP-aldehyde promoted [^{35}S]DMSP-aldehyde formation, while the other possible intermediates had no trapping effects. Plants without DMSP accumulation did not form [^{35}S]DMSP-aldehyde from [^{35}S]methylmethionine.

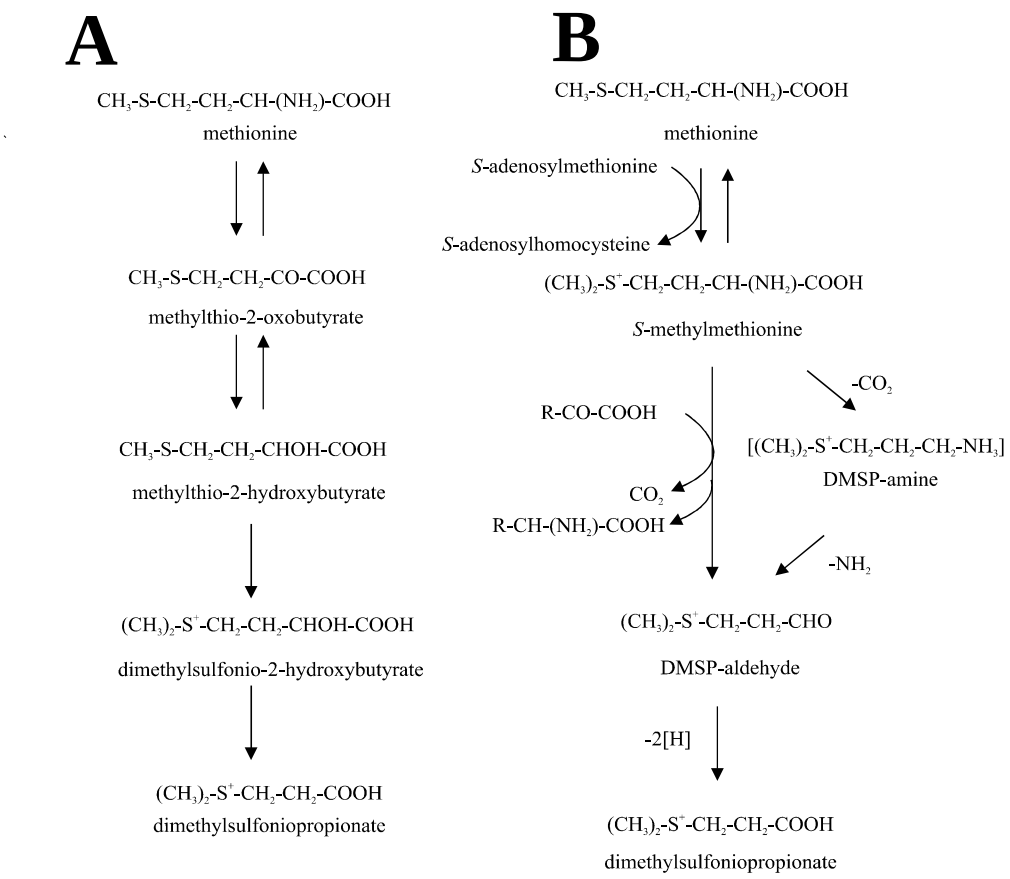

Fig. 2 Biosynthesis of dimethylsulfoniopropionate in *Enteromorpha intestinalis* (A) and *Wollastonia biflora* and *Spartina alterniflora* (B). Adapted from McNeil et al. (1999); see text for details

Microbial degradation of DMSP

DMSP is released from algae by lysis due to senescence (Stefels and van Boekel 1993), grazing (Dacey and Wakeham 1986) or viral activity (Bratbak et al. 1993, 1995). Most likely, a major part of the DMSP is degraded by bacteria. Kiene and coworkers were the first to describe the degradation pathways for DMSP in anoxic marine sediments (Kiene and Taylor 1988a and b; Kiene et al. 1990), and a few years later the same pathways turned out to be true for oxic environments as well (Taylor and Gilchrist 1991). The two different routes for degradation of DMSP are shown in Fig. 1. One possible route is the cleavage of DMSP, the other route involves a demethylation step. The microorganisms involved in the degradation of DMSP will be described in more detail below.

Cleavage of DMSP. The cleavage of DMSP to dimethylsulfide, acrylate and a proton (Fig. 1) is catalyzed by DMSP lyase (dimethylpropiothetin dethiomethylase EC 4.4.1.3). This enzyme has been detected in both aerobic and anaerobic bacteria, algae and even some fungi (see, for example, Ledyard et al. 1993; van der Maarel et al. 1996c; Stefels and Dijkhuizen 1996; Bacic et al. 1998). Examples of aerobic organisms catalyzing this cleavage reaction are numerous: they include strain LFR (Ledyard et al. 1993), *Pseudomonas doudoroffi*, *Alcaligenes* sp. strain M3A (De Souza and Yoch 1995a), strain MD 14-50 (Diaz et al. 1992), strain ML-D (Diaz and Taylor 1996), *Fusarium lateritium* (Bacic and Yoch 1998). Recently, Gonzalez et al. (1999) showed that fifteen marine, aerobic strains were able to produce DMS from DMSP, including isolates that were obtained without any selection for sulfur metabolism.

Already in 1962, Wagner and Stadtman demonstrated the anaerobic cleavage of DMSP by *Clostridium propionicum* (Wagner and Stadtman 1962). Recently, a *Desulfovibrio acrylicus* strain was isolated in our laboratory; this organism reduces sulfate and acrylate and is able to cleave DMSP very rapidly. It did not ferment the acrylate produced from the cleavage reaction, but used it as an electron acceptor (van der Maarel et al. 1996c).

Demethylation of DMSP. The initial demethylation of DMSP results in MTPA (Fig. 1). The MTPA can be demethylated further to mercaptopropionate, or the MTPA can be converted to methanethiol and further to dimethyldisulfide. Demethylating bacteria have been isolated from both aerobic (Taylor and Gilchrist 1991; Visscher and Taylor 1994; Diaz and Taylor 1996) and anaerobic (van der Maarel et al 1993, 1996b) environments.

Examples of aerobic organisms able to demethylate DMSP are strain DG-C1, strain MM-P, and *Alteromonas macleodii*. These bacteria produced methanethiol from DMSP (Taylor and Gilchrist 1991; Diaz and Taylor 1996; Ledyard et al. 1993). Strain BIS-6 demethylated DMSP stoichiometrically to mercaptopropionate (Visscher and Taylor 1994). Completely new was the observation from Gonzalez et al. (1999) that five marine bacterial strains were not only able to cleave the DMSP, but also possessed the demethylation pathway, with methanethiol as sulfur-containing end product. Kiene et al. (1999) showed that these bacteria, and also the DMSP-cleaving organisms isolated in that study, used DMSP and methanethiol as a source of sulfur for methionine and protein-sulfur.

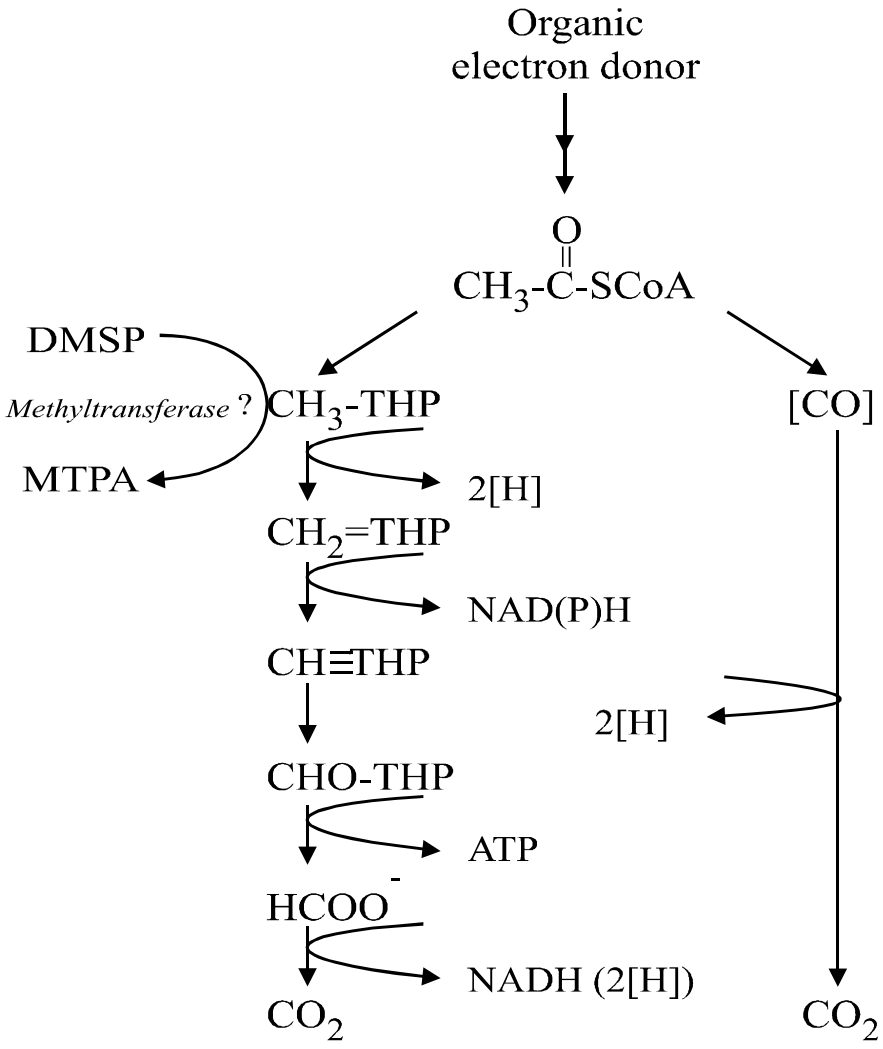

Fig. 3 Oxidative acetylCoA/CO dehydrogenase pathway used by sulfate-reducing bacteria such as *Desulfobacterium* and *Desulfotomaculum* (THP = tetrahydropterin, see Widdel and Hansen 1992)

Anaerobic demethylation of DMSP by a pure bacterial culture was demonstrated by van der Maarel et al. (1993). The marine sulfate-reducing bacterium *Desulfobacterium* strain PM4 oxidized one methyl group of DMSP and coupled this to the reduction of sulfate. The product of this demethylation reaction, MTPA, was not used by this strain. Several other marine sulfate-reducing bacteria belonging to the genus *Desulfobacterium* (or very similar to it) are able to demethylate DMSP stoichiometrically to MTPA, and in this way grow on the basis of the oxidation of the methyl group (van der Maarel et al. 1996b). Most likely, these bacteria channel the methyl group into the oxidative acetyl-CoA/CO dehydrogenase pathway, which is normally involved in the oxidation of acetyl-CoA (Fig. 3; van der Maarel et al. 1996b).

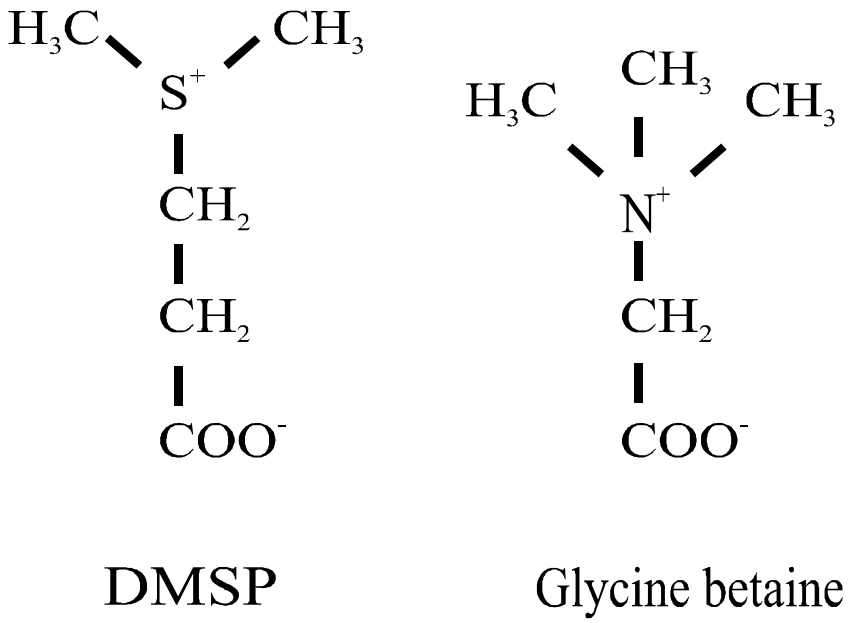

Fig. 4 Comparison of dimethylsulfoniopropionate and glycine betaine

Besides the already mentioned *Desulfobacterium* strain PM4, also *Db. niacini*, *Db. vacuolatum* and strain WN did not only demethylate DMSP, but also the N-containing structural analog of DMSP, glycine betaine (Fig. 4), which is a widely used compatible solute (Yancey et al. 1982). However, *Db. autotrophicum* grows on glycine betaine, but is unable to grow on DMSP (van der Maarel et al. 1996b). Acetogenic bacteria are obligate anaerobes that synthesize acetyl-CoA from C_1-compounds both for conservation of energy and for growth (Drake 1994). Generally, acetate is the main end product, but also longer fatty acids, like butyrate, can be produced (Fig. 5; more detailed information about acetogenic bacteria is described in the part dealing with the methyltransferases in acetogenic bacteria). Several acetogenic bacteria are able to grow on glycine betaine (see, for example, Heijthuijsen and Hansen 1990), but the ability of acetogens to demethylate DMSP was not known when this thesis project started. Kiene and Taylor (1988b) suggested that such acetogens might be involved in the demethylation of DMSP in anoxic sediments. Data from van der Maarel et al. (1996b) showed that the marine DMSP-demethylating sulfate-reducing bacteria, probably play an important role in this conversion in anoxic sediments due to their low K_m values for DMSP degradation and their high abundance. Whether acetogens play a major role in DMSP demethylation in marine, anoxic sediments is not known, but appears not very likely. Several acetogens, originating mainly from non-marine environments, are also able to demethylate DMSP to MTPA, but growth was not observed on this substrate and K_m values for DMSP degradation were rather high (van der Maarel et al. 1996b; chapter 3 in this thesis).

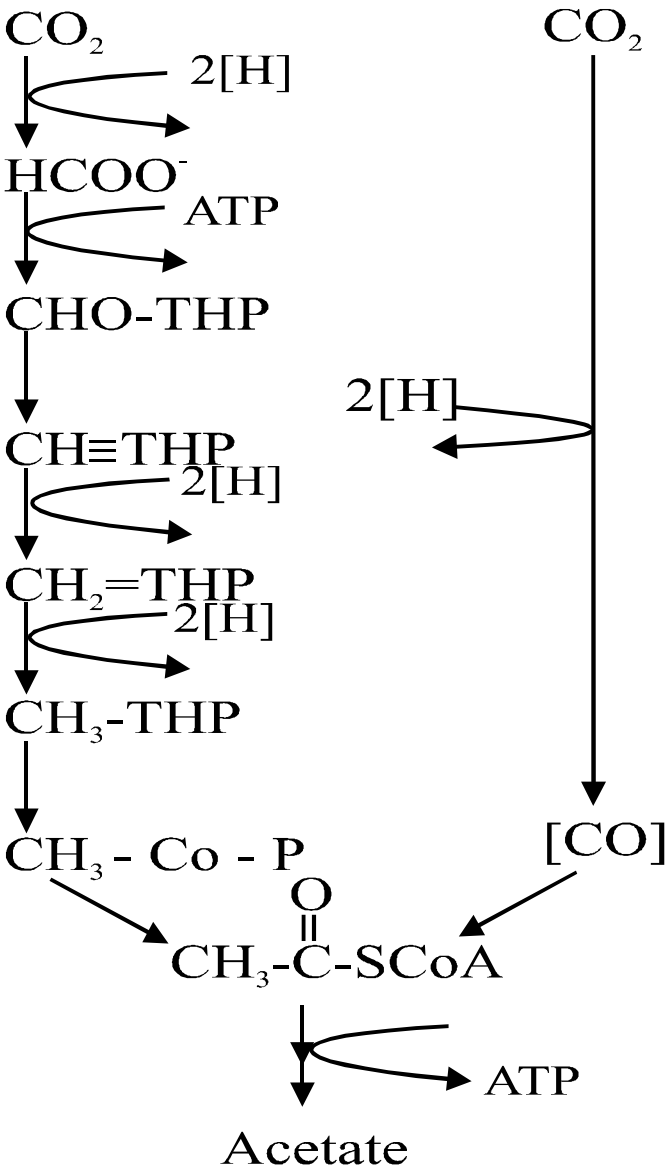

Fig. 5 Acetate synthesis from CO_2 by homoacetogenic bacteria (Acetyl CoA/ CO dehydrogenase pathway; THP = tetrahydropterin, CH_3-Co-P = methylated corrinoid/Fe-S protein); see Diekert and Wohlfarth 1994

Biochemical aspects of DMSP demethylation reactions

Until a few years ago, virtually nothing was known about the biochemistry of DMSP demethylation. This is somewhat surprising, because of the large amounts of DMSP produced in the marine environment. As shown above, the DMSP degraded by bacteria proceeds not only via an initial cleavage reaction, but is also possible via a demethylation pathway (Fig. 1). Isolation of bacteria growing aerobically on MTPA (and DMSP) was very easy (Taylor and Gilchrist 1991), and the authors concluded that the demethylation of DMSP to MTPA might be a significant process in the marine environment. The quantitative importance of the demethylating bacteria of the total DMSP degraders in the Caribbean Sea was demonstrated by Visscher et al. 1992). Estimated dimethylsulfide emissions from the oceans to the atmosphere are approximately 50 million tons S/year (Barnard et al. 1982, Malin 1996). Since not all DMSP is cleaved to dimethylsulfide and dimethylsulfide consumption seems to be a

very important factor (Kiene and Bates 1990), this implies that the flux of DMSP via the demethylation pathway must be large. DMSP lyases have been purified from a number of bacterial strains (de Souza and Yoch, 1995b; van der Maarel et al. 1996a). In contrast reports describing purified bacterial enzymes that catalyze the demethylation of DMSP are not available.

Possible DMSP demethylation reactions. Theoretically, aerobic or anaerobic demethylation of DMSP can be catalyzed by several enzymes.
(A) In aerobes a DMSP-oxidase may be involved, which results in the following reaction:
DMSP + O_2 + tetrahydrofolate $\rightarrow$ MTPA + H_2O_2 + methylenetetrahydrofolate

An example of an enzyme catalyzing such a type of reaction is sarcosine oxidase. The enzyme from *Corynebacterium* sp. P-1 catalyzes the oxidative demethylation of sarcosine which yields glycine, hydrogen peroxide and methylenetetrahydrofolate (Chlumsky et al. 1995). In the absence of tetrahydrofolate, formaldehyde is produced.
(B) A second possibility in aerobes is an oxygenase:
DMSP + O_2 + electron $donor_{reduced}$ $\rightarrow$ MTPA + H_2O + electron $donor_{oxidized}$ + HCHO (formaldehyde)

An example of an enzyme catalyzing such a type of reaction is dimethylsulfide monooxygenase. This enzyme catalyzes the oxidation of dimethylsulfide to methanethiol in *Hyphomicrobium* S. Most probably, formaldehyde is produced and assimilated by the serine pathway (de Bont et al. 1981).
(C) A third possibility is a DMSP dehydrogenase:
DMSP + electron $acceptor_{oxidized}$ + H_2O $\rightarrow$ MTPA + electron $acceptor_{reduced}$ + HCHO

The flavoprotein dimethylglycine dehydrogenase (EC 1.5.99.2) catalyzes such a type of reaction, where demethylation of dimethylglycine results in sarcosine formation (Frisell and MacKenzie 1962).
(D) A fourth possibility is a methyltransferase:
DMSP + methyl acceptor $\rightarrow$ MTPA + methylated methyl acceptor

Methyl acceptors that might play a role in this demethylation reaction are, for example, tetrahydrofolate (THF, also called tetrahydropteroylglutamate) and related compounds, such as tetrahydropterin (THP, similar to THF with 2 or more glutamate molecules), or homocysteine. During the thesis project we made considerable progress in understanding DMSP demethylation in certain anaerobes. Oxygen-dependent reactions are impossible in such organisms. We detected a DMSP-demethylating enzyme which belongs to the methyltransferases. Therefore, in the last part of this chapter we discuss methyltransferase reactions with THF or homocysteine as a methyl acceptor, and methyl transfer in methanogenic archaea and acetogenic bacteria.

Methyltransferases with tetrahydrofolate or homocysteine as methyl acceptor. THF is a methyl acceptor in trimethylsulfonium-THF methyltransferase in a Pseudomonas strain (Wagner et al. 1967). This enzyme catalyzes the demethylation of trimethylsulfonium chloride to dimethylsulfide, where THF acts as methyl acceptor.
Trimethylsulfonium + THF $\rightarrow$ dimethylsulfide + methyl-THF + H^+

This enzyme does not use DMSP or *S*-adenosylmethionine as methyl donor, and also important to mention for the remainder of this chapter, no vitamin B_{12} derivative is associated with the enzyme.

An example of an enzyme where homocysteine acts as a methyl acceptor is methionine synthase (EC 2.1.1.14). This reaction is important in the synthesis of methionine. Methionine synthase catalyzes the transfer of the methyl group from methyl-THF to homocysteine, which yields THF and methionine (see, for a recent review, Ludwig and Matthews 1997).

Methyl-THF + homocysteine → THF + methionine

Certain methionine synthases are corrinoid-dependent and others are corrinoid-independent (Stupperich 1993). Corrinoids are compounds containing cobalt and four reduced pyrrole rings. The best known example of this group is vitamin B_{12}. Over the years several different corrinoids have been isolated from methanogenic archaea and acetogenic bacteria (Stupperich et al. 1990).

Another example where homocysteine acts as a methyl acceptor is in the reaction catalyzed by the corrinoid independent betaine:homocysteine methyltransferase (EC 2.1.1.5):

Glycine betaine + homocysteine → dimethylglycine + methionine

This protein is found not only in bacteria (White et al. 1973; Smith et al. 1988), but also in eukarya (Garrow 1996). More than 40 years ago, it was reported that a thetin:homocysteine transmethylase from horse liver used besides dimethylacetothetin (modern name: dimethylsulfonioacetate) also glycine betaine and DMSP (Durell et al. 1957). Later it was confirmed by other researchers, that DMSP could serve as methyl donor for these enzymes. Physiological and biochemical evidence shows that in *Sinorhizobium meliloti* dimethylsulfonioacetate, the acetate analog of DMSP, is demethylated via the glycine betaine demethylating system, most likely a glycine betaine:homocysteine methyltransferase (Smith et al. 1988). DMSP, however, is not demethylated by *Sinorhizobium meliloti* and is used only as an osmoprotectant (Pichereau et al. 1998). Similarly, in extracts of *Pseudomonas denitrificans* glycine betaine and dimethylsulfonioacetate can function as methyl donors for homocysteine methylation whereas DMSP cannot (White et al. 1973).

Methyltransferases in methanogenic archaea. A great deal of research has been done to elucidate the mechanism of the methyl transfer reactions involved in the degradation of several methylated substrates in methanogenic archaea. Methanogenic archaea are involved in the conversion of compounds such as methanol (Daas et al. 1996; Sauer et al. 1997), methylamines (Ferguson and Krzycki 1997; Asakawa et al. 1998; Wassenaar et al. 1998) and methylthiols (Tallant and Krzycki 1997) to methane. Corrinoid proteins play an important role in these methyl transfer reactions to coenzyme M. The conversion of methanol to methane in *Methanosarcina barkeri* proceeds via methyl-coenzyme M. Two methyltransferases play an important role (van der Meijden et al 1983). First, the corrinoid of methyltransferase 1 (methanol: 5-hydroxybenzimidazolylcobamide methyltransferase) is methylated by methanol. Next, the methyl group is transferred to coenzyme M by methyltransferase 2 (methylcobamide:coenzyme M methyltransferase). Several reviews deal with the methyl transfer reactions in methanogens (see, for example, Ferry 1992; Keltjens and Vogels 1993; Blaut 1994).

Methyltransferases in acetogenic bacteria. Methyltransferases play an important role in the degradation of C_1-substrates by acetogenic bacteria. These obligately anaerobic bacteria, synthesizing acetyl-CoA for conservation of energy and growth, usually form acetate as main end product (Fig. 5). The methyl group of acetate is formed from CO_2, via formate, formyl-THP, methenyl-THP, methylene-THP, and methyl-THP. The formation of the carboxyl group is catalyzed by the key enzyme in this pathway, CO dehydrogenase. CO is synthesized from CO_2 by CO dehydrogenase which also catalyzes the formation of acetyl-CoA from the methyl group and CO (Fig. 5). Acetyl-CoA is then converted either to acetate in catabolism or used for biosynthesis (see for a review, Diekert and Wolfarth 1994). Homoacetogenic bacteria can utilize different methylated compounds. Examples of such substrates are methanol, methoxylated aromatic compounds such as vanillate or syringate (Diekert and Wohlfarth 1994), and glycine betaine (see, for example, Heijthuijsen and Hansen 1990). Methyltransferases are important enzymes in the degradation of these substrates. A number of experiments with cell extracts of acetogenic bacteria showed that THF served as a methyl acceptor in demethylation reactions (Table 1). It was demonstrated for *Acetobacterium woodii*, grown on phenyl methylethers, that THF and ATP were necessary for the oxygen sensitive *O*-demethylation (Table 1; Berman and Frazer 1992). Activity measured in extracts of syringate-grown *Clostridium thermoaceticum* was found to be relatively oxygen insensitive and reductive activation, by, for example, titanium(III)-nitrilotriacetic acid, was not required. Propyl iodide is known to inhibit proteins containing a corrinoid, but inhibition of the *O*-demethylase by propyl iodide was not observed (Kasmi et al. 1994). The involvement of corrinoids in these reactions can be demonstrated by the light-reversible inhibition by propyl iodide (Brot and Weissbach 1965). In cell fractions of Sporomusa ovata, grown on methanol or 3,4-dimethoxybenzoate, activities were very low (2.0-3.3 $nmol.min^{-1}.mg^{-1}$ protein) and oxygen insensitive (Stupperich and Konle 1993). For measuring the *O*-demethylating activity of *Holophaga foetida*, a new coupled photometric assay was developed (Kreft and Schink 1994; Kreft and Schink 1997). In this assay the NADPH-consuming phloroglucinol reductase reaction was coupled to the phloroglucinol-yielding demethylation of 3,5-dihydroxyanisole. In this way it is possible to study the kinetics of the indicator enzyme phloroglucinol reductase, because phloroglucinol starts to accumulate until it is removed by phloroglucinol reductase at the rate of its production. THF methylation in this organism is probably catalyzed by a multi component enzyme system (Kreft and Schink 1994).

For *Acetobacterium dehalogenans* (formerly known as strain MC; Kaufman et al. 1998) a comparable coupled enzyme assay was developed to measure methyl chloride demethylation and *O*-demethylation (Meβmer et al. 1996). This assay is based on quantification of methyl-THF by a set of three indicator enzymes. First reaction is the methylation of THF by the *O*-demethylase or methyl chloride dehalogenase:

1. $CH_3\text{-}X + THF \rightarrow CH_3\text{-}THF + XH$
   (*O*-demethylase or methyl chloride dehalogenase)

Subsequentely, the methyl-THF is converted to methylene-THF by an NAD^+-dependent methylene-THF reductase:

2. $CH_3\text{-}THF + NAD^+ \rightarrow CH_2{=}THF + NADH + H^+$
   (methylene-THF reductase)

Table 1 Comparison of methyl transfer reactions in cell extracts of the sulfate-reducing bacterium strain WN and acetogenic bacteria

Organism	Substrate	Propyliodide Inhibition	ATP required	Oxygen sensitive	Activity (mU[a] /mg protein)	Substrate specificity	Reference
Strain WN	DMSP	Yes	No	Yes	560	ND	Chapter 4
Eubacterium limosum str PM31	DMSP	ND[b]	No	ND	10.4	ND	Chapter 3
Acetobacterium woodii	PME[c]	Yes	Yes	Yes	14.2	ND	Berman & Frazer (1992)
Clostridium thermoaceticum	Syringate	No	±	No	15	Broad	Kasmi et al. (1994)
Sporomusa ovata	Methanol	Yes	Yes	No	2.0	ND	Stupperich & Konle (1993)
	3,4-DMB[d]	Yes	Yes	No	3.3	ND	
Holophaga foetida	PME	Yes	Yes	ND	81-105	Broad	Kreft & Schink (1994, 1997)
Acetobacterium dehalogenans	Methyl chloride	No	Yes	ND	20	ND	Meβmer et al. (1993, 1996)

[a]: one Unit is defined as µmol/min; [b] : Not Determined; [c]: Phenylmethylethers; [d]: dimethoxybenzoate

The methylene-THF is converted to methenyl-THF by the $NADP^+$-dependent methylene-THF dehydrogenase:

3. CH_2=THF + $NADP^+ \rightarrow$ CH≡THF^+ + NADPH
   (methylene-THF dehydrogenase)

Lactate dehydrogenase was required to drive the thermodynamically unfavorable reaction with methylene-THF reductase:

4. $Pyruvate^- + NADH + H^+ \rightarrow lactate^- + NAD^+$
   (lactate dehydrogenase)

In this way it is possible to measure the formation of NADPH and methenyl-THF photometrically. The advantage of this method is, besides the possibility to do kinetic experiments, that it can be used for all THF-dependent reactions. Using this assay, the group of Diekert succeeded in purifying the ether-cleaving enzyme system, *O*-demethylase, from *A. dehalogenans* (Kaufman et al. 1997). This *O*-demethylase consisted of four different components that all were required for catalysis of the transfer of the methyl group from phenyl methyl ethers to THF. Component B (methyltransferase I) mediated the conversion of the substrate to the reduced corrinoid protein component A. Component D functions as the methylcorrinoid:THF methyltransferase (methyltransferase II). Component C probably functions as an activating protein, reversing oxidation of the protein-bound cobalamin in the presence of ATP and reducing equivalents supplied by the enzymatic oxidation of hydrogen (Kaufman et al. 1998).

Aim and outline of this thesis

The first aim of this thesis project was to identify microorganisms that have the ability to demethylate the algal osmolyte DMSP to the flavor precursors MTPA or mercaptopropionate. This was done by isolating new strains and by screening of culture collections for potential candidates. The second goal was to study the biochemistry of the demethylation reaction of DMSP to MTPA. At the start of this project, a marine sulfate-reducing bacterium, *Desulfobacterium* strain PM4, was known to demethylate DMSP to MTPA (van der Maarel et al. 1993). Other closely related marine sulfate reducers also catalyzed this demethylation reaction (van der Maarel et al. 1996b). This demonstrated the possibility to use microorganisms for the production of MTPA from DMSP in a natural way.

Chapter 2 focuses on aerobic DMSP demethylation. A new isolate belonging to the α-Proteobacteria was found to be able to demethylate DMSP to MTPA. The MTPA was secreted, but subsequently degraded further with a transient accumulation of methanethiol and with dimethyldisulfide as sulfur-containing end product. The closest relative of the isolate, *Ruegeria atlantica*, was also able to demethylate DMSP.

Chapter 3 describes the ability of several acetogenic bacteria to demethylate DMSP stoichiometrically to MTPA. The conversion did not result in any significant increase in biomass. An N-containing structural analog of DMSP, glycine betaine, did support good growth of these organisms.

The sulfate-reducing bacteria that are able to demethylate DMSP (*Db. niacini*, *Db. vacuolatum*, strain PM4 and strain WN) all use the oxidative acetyl-CoA/CO dehydrogenase pathway. It was hypothesized that these bacteria channel the methyl group to the methyl branch of this pathway (Fig. 3; van der Maarel et al. 1996b). Indeed, when cell extracts of DMSP-grown sulfate-reducing bacteria were incubated under anaerobic conditions with THF as a methyl acceptor, MTPA and methyltetrahydrofolate formation was detected (chapter 4). These bacteria also demethylated glycine betaine to dimethylglycine, but cell extracts of DMSP- or glycine betaine grown bacteria did not catalyze the demethylation of glycine betaine with THF as a methyl acceptor.

The demethylation reaction was studied in more detail in chapter 5. In this chapter the purification and characterization of an enzyme specifically catalyzing the demethylation of DMSP (DMSP:tetrahydrofolate methyltransferase) of one of the sulfate-reducing bacteria (strain WN) is described.

Thus far, no anaerobic microorganisms have been isolated that are able to demethylate DMSP completely to mercaptopropionate. Furthermore, the anaerobic demethylation of MTPA to mercaptopropionate by pure cultures of microorganisms was never reported. Chapter 6 shows that under certain conditions the demethylation of MTPA to mercaptopropionate can be demonstrated in sediment suspensions. Organisms responsible for this reaction were methanogenic archaea. With pure cultures of marine *Methanosarcina* strains we showed that MTPA was a good and novel substrate for these archaea.

Finally, the results described in these chapters are discussed and summarized (chapter 7).

2

Production of 3-methylthiopropionate from dimethylsulfoniopropionate by *Ruegeria frisia*, sp. nov.

Michael Jansen · Marc J.E.C. van der Maarel · Irma van der Veen · Theo A. Hansen

The algal osmolyte DMSP is a potential substrate for the production of certain natural flavor compounds or flavor precursors. A newly isolated, aerobic, rod-shaped bacterium, strain WNA2, was found to convert DMSP to substoichiometric concentrations of MTPA. The isolate was obtained from enrichments inoculated with a 10^6-fold diluted slurry of intertidal mud and water from the Wadden Sea. MTPA was degraded further with a transient accumulation of methanethiol and with dimethyldisulfide as the sulfur-containing end-product. Growth of strain WNA2 on glycine betaine, which can be considered as an N-containing structural analogue of DMSP, was strongly inhibited by dimethyldisulfide. The sequence of its 16S rRNA encoding gene identified strain WNA2 as a member of the α-subclass of the Proteobacteria with *Ruegeria atlantica* as its closest relative. A phenotypic analysis of strain WNA2, and DNA/DNA hybridization studies with the type strain of this species, allowed assignment of strain WNA2 to a new species, *Ruegeria frisia* sp. nov.

Key words: Dimethylsulfoniopropionate – methylthiopropionate – flavor compounds – dimethyldisulfide – *Ruegeria*

Introduction

Microbial conversions can be used for the production of natural flavors. Examples are the synthesis of vanillin from eugenol by bacteria (Washisu et al. 1993) or from ferulate by fungi (Lesage-Meesen et al. 1999) and the production of γ-decalactone from ricinoleic acid by *Saccharomyces cerevisiae* (Boog et al. 1998).

Certain esters of MTPA and of mercaptopropionate are natural fruity flavors (Fenaroli 1995; Hansen and van der Maarel 1998). MTPA itself is associated with a cheesy flavor (Berger et al. 1999). Because of their role as flavors or flavor precursors there is interest in the biological production of natural MTPA and mercaptopropionate (e.g. Hansen and van der Maarel 1998; Hansen et al. 1998). A potentially useful precursor for the synthesis of MTPA and mercaptopropionate is DMSP. This compound is present in high concentrations in certain marine algae (Reed 1983; Keller 1988) and some plants (Hanson and Gage 1996).

Anaerobic demethylation of DMSP has been shown to be carried out by several sulfate-reducing bacteria belonging to the δ-Proteobacteria and to support growth; they include *Desulfobacterium* strain PM4, *Db. niacini*, *Db. vacuolatum* and strain WN (van der Maarel et al. 1996 b). Such bacteria stoichiometrically convert DMSP to MTPA. We recently demonstrated demethylation of DMSP to MTPA by acetogenic bacteria such as *Eubacterium limosum*, but this conversion did not support growth (Jansen and Hansen, submitted). Both the sulfate-reducing bacteria and the acetogens require anoxic conditions for growth and are rather difficult to handle. The goal of the present work was to obtain aerobic bacteria that can produce high concentrations of MTPA from DMSP. The isolate we studied in most detail turned out to be a new species of the genus *Ruegeria*.

Materials and Methods

Organisms and cultivation. Serial tenfold dilutions (in medium) of surface sediment or seawater collected at the intertidal area of the Wadden Sea near Westernieland (The Netherlands) were used as 1-ml inoculum for enrichment cultures. The enrichments were incubated statically at room temperature. The medium with DMSP (0.6 mM) was made according to Visscher and Taylor (1994). Alternatively, a Tris-buffered medium with the following composition per l was used: 30 g NaCl, 1.0 g $MgCl_2 \cdot 6H_2O$, 4.0 g Na_2SO_4, 0.15 $CaCl_2 \cdot 2H_2O$, 0.7 g KCl, 0.5 g NH_4Cl, 0.2 g $NaHCO_3$, 6.0 g Tris, 1 ml trace elements solution SL7 (Laanbroek and Pfennig 1981), 1 ml vitamins solution (Widdel 1980), 2 ml KH_2PO_4 (1.0 M), and 0.5 l sterile seawater (pH 7.8). Pure cultures were obtained by repeated plating on Tris-buffered media solidified with 1.5% (wt/vol) Noble Agar (Difco Laboratories, Detroit, MI, USA). Pure cultures were grown in the Tris-buffered medium (pH 7.8) with 0.02% yeast extract (without the sterile seawater) in erlenmeyer flasks on a gyratory shaker (100-150 rpm) at 30^0C. The temperature optimum for growth was determined with glycine betaine as a substrate (10 mM); the temperature range was tested on marine agar plates (Difco). The pH range was determined with glycine betaine as a substrate (10 mM); the salinity range was determined with glucose (0.1%) without yeast extract. Potential growth substrates were tested in shaken erlenmeyer flasks at concentrations of 0.1% (unless otherwise indicated); growth was corrected for the contribution of the yeast extract in the medium. Oxidase, catalase, denitrification and nitrate reduction were determined according to Smibert and Krieg (1994). *Ruegeria atlantica* DSM 5823^T [formerly *Agrobacterium atlanticum*; reclassified by Uchino et al. (1998)] was grown on marine agar (Difco) or in Tris-buffered medium as described above.

Cell suspension experiments. Cultures were grown on DMSP (10 mM), glycine betaine (10 mM) or DMSP (10 mM) plus glycine betaine (10 mM), harvested by centrifugation (15 min at 8,000 x *g* at 4^0C), and washed at least twice in medium without substrate. Cells were resuspended to a final protein concentration of 1.0 mg/ml, and 10 ml of the suspension was transferred to 120-ml vials. Vials were closed with teflon-coated stoppers (Supelco, Bellefonte, PA, USA) to avoid loss of the sulfur compounds in the stoppers (Kiene and Capone 1988). Production of methanethiol and dimethyldisulfide by the cell suspensions was measured by head space analysis.

Mole % G + C. The G+C content of the DNA was determined by HPLC based on the method of Mesbah et al. (1989) by the DSMZ (Braunschweig, Germany).

DNA hybridization. DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion et al (1977). DNA-DNA hybridization was carried out by the DSMZ as described by De Ley et al. (1970), with the modification described by Huss et al. (1983) and Escara and Hutton (1980) using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992).

Denaturing gradient gel electrophoresis. Denaturing gradient gel electrophoresis (DGGE) was performed as described by Muyzer et al. (1998) using a Hoefer SE600 series vertical electrophoresis unit connected to a circulating water bath. A urea-formamide gradient from 40% to 70% was used. The gel was run at 55^oC and a

constant voltage of 50V for 18h. PCR-DGGE fragments were generated from amplified 16S rDNA (see below) by using the forward primer S-D-Bact-341-a-S-17 with a 5' GC-rich extension and the reversed primer S-*-Univ-909-b-A-17 under the PCR conditions as described by Muyzer et al. (1998).

16S rRNA gene sequence determination and phylogenetic analysis. DNA of strain WNA2 was extracted as described previously (Van der Maarel et al. 1996b). The 16S rRNA gene was amplified using the S-D-Bact-0008-a-S-19 and S-*-Bact-1392-a-A-15 primers and *Taq* DNA polymerase (Pharmacia Amersham Biotech, Uppsala, Sweden) on a Progene thermal cycler (Techne, Cambridge, UK) according to the method of Van der Maarel et al. (1996b). After purification of the PCR product with the Wizard PCR DNA purification system (Promega, Madison, WIS, USA), the complete 16S rDNA sequence was determined using the BigDye terminator cycle sequencing kit of Perkin Elmer (Foster City, CA, USA) according to the manufacturer's manual. The following primers were used for the cycle sequencing reaction:

1. S-*-Bact-0050-a-S-19 (5'-AACACATGCAAGTCGAACG);
2. S-*-Univ-0519-a-S-18;
3. S-*-Univ-0519-a-A-18;
4. S-*-Univ-0909a-S-20 (5'-AAACTCAAATGAATTGACGG-3');
5. S-*-Univ-0909-a-A-20 (5'-CCGTCAATTCATTTGAGTTT);
6. S-*-Univ-1392 -a-A-15 [notation of the primers according to Alm et al. (1996)].

Cycle sequencing products were analyzed on an ABI 310 automated DNA analyser (Perkin Elmer). Sequences were aligned using the Dedicated Comparative Sequence Editor programme of De Rijk and de Wachter (1993) and corrected by hand. Subsequently, a distance matrix based on the algorithm of Jukes and Cantor (1969) and the neighbor-joining method was calculated. A phylogenetic dendrogram was constructed with the aid of the TREECON software package (van de Peer and de Wachter 1993).

Nucleotide sequence accession numbers. The 16S rRNA gene sequence determined in this study has been deposited in Genbank under accession number AF124521. The accession numbers of the sequences used in the phylogenetic analyses are: *Roseobacter gallaeciensis* (Y13244), *Ruegeria algicola* (X78315), *Ruegeria atlantica* (D88526), *Ruegeria gelatinovorans* (D88523), *Roseovarius tolerans* (Y11551), *Roseobacter denitrificans* (M96746), *Roseobacter litoralis* (X78312), *Antarctobacter heliothermus* (Y11552), *Silicibacter lacuscaerulensis* (U77644), *Sulfitobacter pontiacus* (Y13155), *Sulfitobacter mediterraneus* (Y17387), *Sulfitobacter brevis* (Y16425), *Sagittula stellata* (U58356), *Staleya guttiformis* (Y16427), *Octadecabacter arcticus* (U73725), '*Marinosulfonomonas methylotropha*' (U62894), DMSP-degrading strain LFR (L15345), *Paracoccus versutus* (D32244), *Rhodovulum sulfidophilum* (D16423), *Rhodobacter veldkampii* (D13477).

Scanning electron microscopy. Bacteria, grown on glycine betaine (10 mM) in medium without yeast extract, were fixed with 6% glutaraldehyde and by 1% osmium tetroxide. The sample was dehydrated with a graded series of ethanol followed by critical-point drying. Samples were examined using a JEOL 6300 scanning electron microscope.

Analytical methods. DMSP was determined as dimethylsulfide or acrylate after

overnight treatment with 1 M NaOH (White 1982). Acrylate, MTPA and mercaptopropionate concentrations were analyzed by HPLC using a C18 column (Jansen and Hansen 1998). Mercaptopropionate was measured after reduction of the sample with tributylphosphine (Humphrey and Potter 1965). Methanethiol, dimethylsulfide and dimethyldisulfide were measured in the headspace of vials by gas chromatography as described by Jonkers et al. (1998); for dimethyldisulfide analyses the oven temperature was 170^{o}C instead of 120^{o}C. Cell protein was determined according to Lowry et al. (1951) after treatment of the cell pellet with 1 M NaOH at 100^{0}C for 10 min. The optical density of cultures was measured in a 1 cm cuvette in a Starrcol colorimeter (Hoorn, The Netherlands) at 660 nm.

Chemicals. DMSP was synthesized from acrylic acid and dimethylsulfide (Chambers et al. 1987) or obtained from CASS (University of Groningen, Groningen, The Netherlands). MTPA was obtained by alkaline hydrolysis of its methylester (Aldrich, Steinheim, Germany). Tributylphosphine was purchased from Acros Organics (Geel, Belgium), and dimethyldisulfide and methanethiol from Fluka Chemika (Buchs, Switzerland).

Results

Isolation of DMSP-demethylating strains. Strains were isolated from dilution series of surface sediment or seawater of the Wadden Sea near Westernieland (The Netherlands), using media with 0.6 mM DMSP as sole source of carbon and energy. After 10 days of incubation, the highest dilution with visible growth (10^6 dilution) was streaked onto solid media with 0.6 mM DMSP. Approximately 40 colonies were tested for production of MTPA or 3-mercaptopropionate in liquid media containing 10 mM DMSP. Two isolates were obtained that produced more than 0.1 mM MTPA after 10 days of incubation; strain WNA1 was isolated from a carbonate-buffered medium and strain WNA2 from a Tris-buffered medium. Colonies were transparent and yellowish to colorless. Both isolates were short, irregular (approx. 0.4-0.7 by 2.2-4.5 µm), gram-negative rods; a few cells were motile by means of a polar flagellum. Furthermore, occasionally a star-shaped aggregation of cells could be observed (Figure 1). Because both isolates had similar morphological and physiological characteristics and partial 16S rDNA fragments of both strain WNA1 and WNA2 migrated to the same positions when using denaturing gradient gel electrophoresis (data not shown), more detailed experiments were done only with strain WNA2.

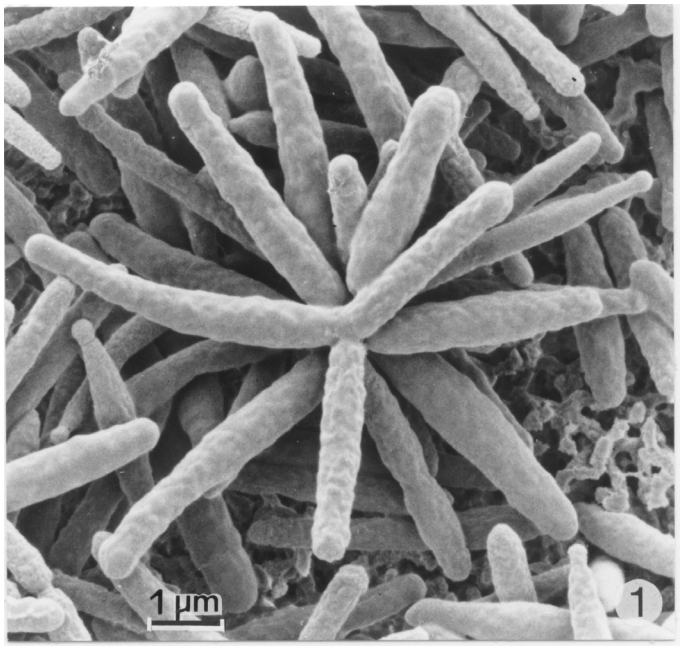

Fig. 1 Scanning electron micrograph of strain WNA2 showing rosette formation (bar, 1 µm)

Strain WNA2 grew between 4 and 37^{0}C (28-30^{0}C optimum). Optimal growth occurred at pH 8.0 (range: 7.0-9.5). The optimal salinity for growth on glycine betaine was approximately 3.0%. No growth was observed with NaCl concentrations below 0.1 M or above 2.1 M in media with glucose. The organism grew on DMSP, glycine betaine, dimethylglycine, sarcosine, glycine, choline, acetate, propionate, acrylate, butyrate, pyruvate, glycerol, mannitol, sorbitol, L-serine, L-alanine, L-histidine, L-glutamate, L-aspartate, L-arginine, L-methylcysteine, glucose, fructose, galactose, mannose, xylose, cellobiose, succinate, L-malate, citrate, and *p*-hydroxybenzoate. No growth was observed on the following compounds: MTPA (10 mM), 3-mercaptopropionate (10 mM), dimethylsulfide (1 mM), methanethiol (1 mM), dimethyldisulfide (1 mM), formate (10 mM), L-lactate (10 mM), gluconate, L-methionine, L-methylmethionine, methanol (10 mM), ethanol (10 mM), sucrose, lactose, trehalose, maltose, arabinose, dimethylamine (10 mM), monomethylamine (10 mM) and trimethylamine (10 mM). Starch and gelatine were not utilized. Strain WNA2 was catalase and oxidase-positive. In mineral media with glucose it required biotin as a growth factor. Nitrite formation from nitrate, gas formation from nitrate and anaerobic growth on fructose were not observed. The G+C content of the DNA was 60.4 mol%.

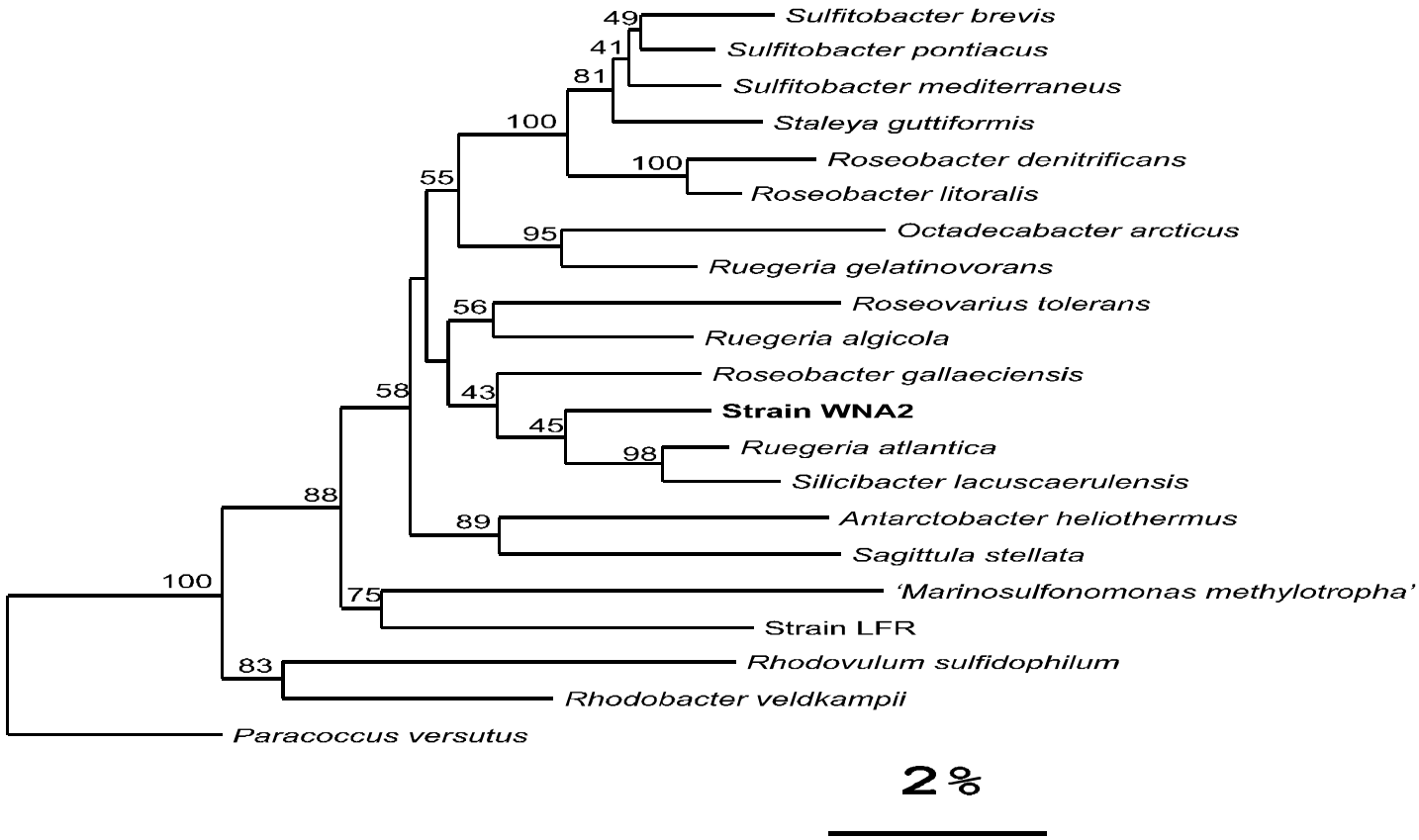

Fig. 2 Dendogram showing the relatedness between the 16S rRNA gene sequences of strain WNA2 and a number α-subclass Proteobacteria. The scale bar corresponds to two nucleotide substitutions per 100 sequence positions. The dendrogram was constructed using the DCSE alignment program and the TREECON for Windows software package. The distance was estimated using the two parameter model of Jukes and Cantor (1969) and the tree was constructed by the neighbor joining method as implemented in the TREECON software package. The numbers indicate absolute bootstrap values per 100; only bootstrap values above 25 are shown.

A phylogenetic analysis based on the 16S rRNA gene sequences revealed that strain WNA2 is a member of the α-subclass of the Proteobacteria. It clusters in the group of the marine α-Proteobacteria; these bacteria are also designated as α-3-Proteobacteria or the *Roseobacter* group (Uchino et al. 1998; González et al. 1999). *Ruegeria atlantica* (97.2% similarity) and *Silicibacter lacuscaerulensis* (96.7% similarity) are the closest relatives of strain WNA2 (Figure 2). DNA-DNA hybridization of strain WNA2 with *R. atlantica* showed a value of 20.9 % which indicates that strain WNA2 does not belong to *R. atlantica*.

Growth of strain WNA2 on DMSP and product formation. When strain WNA2 was grown in a medium with 19 mM DMSP and 0.02% yeast extract, DMSP was converted slowly to MTPA (Figure 3). Unlike growth on betaine (t_d 8 h) growth on DMSP was not exponential; during growth on DMSP a transient accumulation of MTPA was observed. A DMSP:MTPA stoichiometry of 1:1 was never observed.

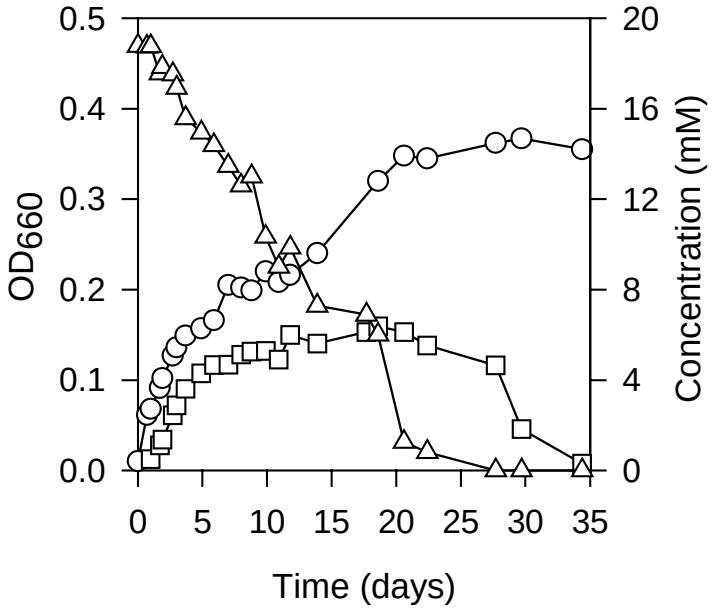

Fig. 3 Growth of strain WNA2 on 19 mM DMSP (Δ), (□) MTPA concentration. (○) OD_{660}.

The degradation product(s) of MTPA were measured using closed vials containing washed cell suspensions of strain WNA2 grown on DMSP (10 mM), glycine betaine (10 mM), or glycine betaine plus DMSP (10 mM). DMSP (100 µM) was added to these cell suspensions (incubated at 30^0C) and they were analyzed for methanethiol and dimethyldisulfide formation. After 1 h, 18-20 µM MTPA could be detected in the cell suspensions with DMSP. Several hours later a low concentration of methanethiol was detected (<5 µmol methanethiol/l liquid medium), and dimethyldisulfide formation started. In total 53 ± 6 µmol dimethyldisulfide/l liquid medium (average of eight incubations) was found in these suspensions after 5 days; methanethiol and MTPA could no longer be detected. This showed that all DMSP was converted to dimethyldisulfide. Dimethyldisulfide was probably formed due to oxidation of two methanethiol molecules, as indicated by control experiments were methanethiol was added to Tris-buffered medium and the dimethyldisulfide production rate was measured. Cell suspension experiments with MTPA (100 µM) showed similar results as for cell suspensions supplemented with DMSP, with dimethyldisulfide as sulfur-containing end-product (data not shown). Mercaptopropionate or dimethylsulfide were never detected as intermediate or end-product in DMSP-grown cultures or cell suspensions of strain WNA2.

When a well-aerated stationary phase fermenter culture of strain WNA2 after growth on 50 mM glycine betaine (OD_{660} 2.5; pH kept constant at 7.8) was supplemented with 80 mM DMSP, 30.5 mM DMSP was utilized during 72 h of incubation with the concomittant production of 19.5 mM MTPA. After 225 h this concentration of MTPA was still present, and the DMSP concentration had hardly decreased further (Figure 4).

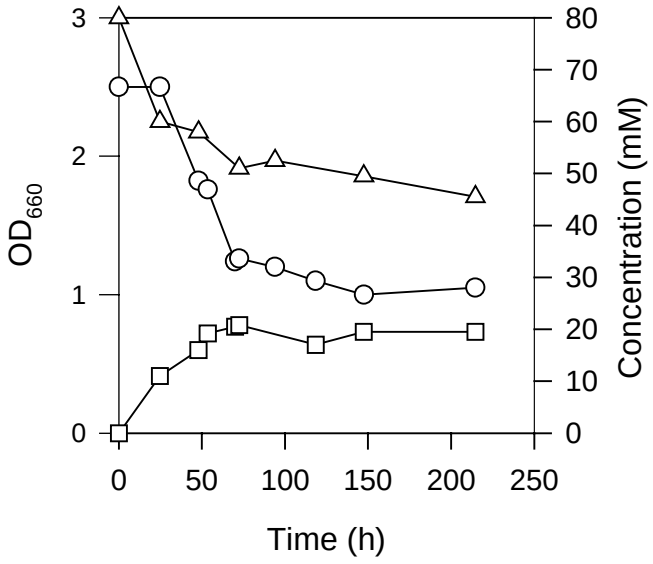

Fig. 4 MTPA production (□) from DMSP (Δ) by strain WNA2 pregrown on 50 mM glycine betaine. (○) OD_{660}.

MTPA addition to batch cultures of strain WNA2 resulted in reduced growth rates and less dense growth. When 20 mM MTPA was added at t_0 to cultures growing on 10 mM glycine betaine, the maximal optical density reached was approximately 25% lower than in the control without MTPA. No growth on glycine betaine was observed in the presence of 40 mM MTPA. Dimethyldisulfide strongly inhibited growth of strain WNA2 on glycine betaine even at far lower concentrations than MTPA. These experiments were performed with 10-ml cultures growing on 10 mM glycine betaine in 120-ml vials closed with teflon-coated stoppers, and with dimethyldisulfide added at t_0 in various concentrations: 3.75, 1.5 and 0.75 mmol dimethyldisulfide/litre added in the liquid phase (dimethyldisulfide was measured in the gas phase). A concentration of 0.75 mmol dimethyldisulfide/litre in the liquid phase already resulted in a 50% lower final optical density of growth on glycine betaine, compared to a control without dimethyldisulfide. Virtually no growth was observed when 3.75 mmol dimethyldisulfide/litre was added. When a similar experiment was done with DMSP as a substrate, no clear difference in final optical densities was observed between the control without dimethyldisulfide and with various concentrations dimethyldisulfide (0.75-3.75mmol/litre).

DMSP degradation by *Ruegeria atlantica*. In view of the high similarity of *Ruegeria atlantica* with strain WNA2 (Figure 2), we tested the type strain of this species for growth on DMSP and MTPA. After a lag phase of 6 days, cultures of *R. atlantica* degraded 15 mM DMSP in 6-8 days, with a transient accumulation of up to 4 mM MTPA. The cultures reached an optical density (600 nm) of 0.4. Similar optical densities were found when *R. atlantica* was grown on 15 mM MTPA; in contrast,

strain WNA2 does not grow on MTPA (see above). In cultures of *R. atlantica* grown in closed vials, supplemented with DMSP or MTPA (10 mM), methanethiol and dimethyldisulfide were detected. As was found for strain WNA2, *R. atlantica* grew more rapidly in media with glycine betaine as a substrate than with DMSP.

Discussion

The use of aerobic bacteria such as strain WNA2 or *R. atlantica* for the production of MTPA from DMSP is possible but major disadvantages are the substoichiometric conversion and the rather low MTPA concentrations obtained (approximately 20 mM). The production of MTPA from DMSP by sulfate-reducing bacteria was stoichiometric (van der Maarel et al. 1996b); the same was true for the bioconversion of DMSP by acetogenic bacteria such as *Eubacterium limosum* PM31 (Jansen and Hansen, submitted). Unlike strain WNA2, *R. atlantica* can grow on MTPA and form colonies on plates with MTPA; one would expect that if a mutant of *R. atlantica* impaired in the degradation of MTPA can be obtained that such a mutant would carry out a stoichiometric conversion of DMSP to MTPA.

Aerobic marine bacteria that degrade DMSP via an initial demethylation have been known since 1991 (Taylor and Gilchrist 1991; Visscher and Taylor 1994; Diaz and Taylor 1996). The phylogenetic position of the isolates studied by these authors is not clear. Recently, González et al. (1999) reported production of methanethiol from DMSP by several marine bacterial strains; this indicates demethylation of the substrate followed by production of methanethiol from MTPA. All of these isolates belonged to the α-Proteobacteria but differed from strain WNA2. It is not known whether any of the DMSP-metabolizing strains studied earlier excretes substantial amounts of MTPA during the degradation of DMSP. In this study we demonstrated that the type strain of *R. atlantica*, a close relative of our strain WNA2, also produces appreciable concentrations of MTPA.

On the basis of the DNA/DNA hybridization studies with *R. atlantica* and the considerable difference in G+C mol % of the DNA with this species (G+C mol % of *R. atlantica* is 55; Rüger and Höfle 1992; G+C mol % of strain WNA2 is 60.4%) we conclude that strain WNA2 can not be affiliated with *R. atlantica*. Phenotypic differences include the absence of nitrate reduction to nitrite or gas and the lack of utilization of trehalose, maltose and sucrose by strain WNA2. We assign WNA2 to a new species for which we propose the name *Ruegeria frisia*, sp. nov.

Description of the type species Ruegeria frisia, sp. nov.

Fri.si.a, M.L. fem. adj. frisia, Frisian, pertaining to the Northern regions of the Netherlands and Germany.

Gram-negative somewhat irregular rods, 0.4-0.7 x 2.2-4.5 µm. Usually immotile, but sometimes motile by means of polar flagellum. May form rosettes. Temperature range from 4 to 37 ^{o}C (optimum at 28-30 ^{o}C); pH range for growth 7.0 to 9.5 (optimum at pH 8.0). No growth below 0.1 M NaCl or above 2.1 M NaCl in minimal media with glucose; optimal salinity 3 %. Aerobic heterotrophic growth. Catalase and oxidase-positive. No fermentative growth on glucose or fructose; no reduction of nitrate. No pigment produced. Grows in mineral media supplemented with a carbon-

and energy source and biotin. Starch and gelatine not hydrolyzed. Utilizes glucose, fructose, mannose, galactose, xylose, cellobiose, mannitol, sorbitol, glycerol, acetate, propionate, butyrate, succinate, malate, acrylate, citrate, pyruvate, glycine, alanine, serine, aspartate, glutamate, histidine, arginine, betaine, dimethylglycine, sarcosine, and *p*-hydroxybenzoate. Does not utilize methanol, ethanol, methylamine, dimethylamine, trimethylamine, lactose, maltose, sucrose, arabinose, gluconate, lactate, and methionine. Excretes methylthiopropionate during growth on dimethylsulfoniopropionate (in the presence of yeast extract). The DNA base composition is 60.4 mol % G+C.

The type strain WNA2 was isolated from an intertidal area (Dutch Wadden Sea); it will be deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Acknowledgements

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3

Non-growth associated demethylation of dimethylsulfoniopropionate by (homo)-acetogenic bacteria

Michael Jansen · Theo A. Hansen

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The demethylation of the algal osmolyte DMSP to MTPA by (homo)-acetogenic bacteria was studied. Five *Eubacterium limosum* strains (including the type strain), *Sporomusa ovata* DSM 2662^T, *Sporomusa sphaeroides* DSM 2875^T, and *Acetobacterium woodii* DSM 1030^T were shown to demethylate DMSP stoichiometrically to MTPA. The (homo)-acetogenic fermentation based on this demethylation did not result in any significant increase in biomass. The analogous demethylation of glycine betaine to dimethylglycine does support growth of acetogens. In batch cultures of *E. limosum* strain PM31 DMSP and glycine betaine were demethylated simultaneously. In mixed substrates experiments with fructose/DMSP or methanol/DMSP, DMSP was used rapidly but only after exhaustion of the fructose or the methanol. In steady-state fructose-limited chemostat cultures (at a dilution rate of 0.03 h^{-1}) with DMSP as a second reservoir substrate, DMSP was biotransformed to MTPA but this did not result in higher biomass values than in cultures without DMSP. Cells from such cultures demethylated DMSP at rates of approximately 50 nmol min^{-1} mg^{-1} protein, both after growth in the presence of DMSP and after growth in its absence. In cell extracts of glycine betaine-grown strain PM31, DMSP demethylation activities of 20-25 nmol min^{-1} mg^{-1} protein were detected with THF as methyl acceptor; activities with glycine betaine were approximately tenfold lower. A speculative explanation for the demethylation of DMSP without an obvious benefit for the organism is that the DMSP-demethylating activity is catalyzed by the glycine betaine-demethylating enzyme and that a transport-related factor, in particular a higher energy demand for DMSP transport across the cytoplasmic membrane than for glycine betaine transport, may reduce the overall ATP yield of the fermentation to virtually zero.

Introduction

Certain marine sulfate-reducing bacteria belonging to the *Desulfobacterium* - *Desulfobacter* cluster of the delta-Proteobacteria and possessing the oxidative CO dehydrogenase pathway for acetyl-coenzyme A oxidation use the algal osmolyte DMSP [$(CH_3)_2$-S^+-CH_2-CH_2-COO^-] for growth and convert it to MTPA [CH_3-S-CH_2-CH_2-COO^-, van der Maarel et al. 1996b]. These bacteria use a specific DMSP:THF methyltransferase for the demethylation reaction (Jansen and Hansen 1998; Jansen and Hansen 2000). Glycine betaine [$(CH_3)_3$-N^+-CH_2-COO^-], an N-containing structural analog of DMSP, was demethylated to dimethylglycine [$(CH_3)_2$-N-CH_2-COO^-] by these bacteria, but in cell extracts no activity was detected with THF as methyl acceptor and glycine betaine as a substrate. Both glycine betaine and DMSP are important osmolytes (Blunden and Gordon 1986; Csonka and Hanson 1991; Galinski 1995). DMSP is produced by many marine algae and some plants where it is synthesized from methionine (Gage et al. 1997; Rhodes and Hanson 1993). The occurrence of high DMSP concentrations in certain types of biological material, and the possibility to convert DMSP to other sulfur-containing compounds, including MTPA, by using bacterial cultures, makes DMSP of potential interest for the natural flavor industry (Hansen and van der Maarel 1998, Hansen et al. 1998).

(Homo)-acetogenic bacteria synthesize acetyl-coenzyme A from C_1-compounds with involvement of the reductive CO dehydrogenase pathway, which is the reverse of the route used by the sulfate-reducing bacteria for acetyl-coenzyme A oxidation. The acetyl-coenzyme A can be converted to acetate or to acetate and longer acids such as butyrate (Heijthijsen and Hansen 1990). See for a discussion of the term acetogenic bacteria or acetogens ref. Drake (1994). Since the discovery in 1981 that the acetogenic bacterium *Eubacterium limosum* can demethylate glycine betaine to dimethylglycine, with acetate and butyrate as fermentation products (Müller et al. 1981), many other acetogenic bacteria have been shown to grow by demethylation of betaine (Heijthuijsen and Hansen 1990). A possible involvement of organisms such as *E. limosum* in DMSP demethylation in anoxic sediments was already suggested by Kiene and Taylor (1988b), but without direct experimental evidence. Recently, in our laboratory a slow demethylation of DMSP to MTPA was demonstrated in experiments with an *E. limosum*-like strain isolated from intertidal mud; growth of this strain was very poor (van der Maarel et al. 1996b). In this paper we show that under certain conditions the biotransformation of DMSP to MTPA can be carried out at appreciable rates by this strain and by a number of other acetogenic bacteria; this process, however, does not support growth in contrast with the analogous demethylation of glycine betaine.

Materials and methods

Microorganisms, media and cultivation. Bacterial strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) unless otherwise indicated. *Eubacterium limosum* strain PM31 (isolated by J.H.F.G. Heijthuijsen in our laboratory), *Eubacterium limosum* DSM 20543^T, *Eubacterium limosum* DSM 20517, *Eubacterium limosum* DSM 2594 (strain 11A), '*Butyribacterium methylotrophicum*' strain Marburg (obtained from J.G. Zeikus), and *Sporomusa sphaeroides* DSM 2875 were grown in 120-ml vials containing 50 ml medium with the following composition per l: 1.0 g NaCl, 1.0 $MgSO_4.7H_2O$, 0.5 g NH_4Cl, 0.3 g KCl, 0.1 g $CaCl_2.2H_2O$, 1.0 g yeast extract (Difco, Detroit, Mi.), 0.5 mg resazurin, 0.1 μM Na_2SeO_3, 0.1 μM Na_2WO_4, and 1 ml trace elements solution (Laanbroek and Pfennig 1981). After autoclaving the basal medium was supplemented with 1 ml of a vitamins solution (Widdel 1980), 2 ml of a phosphate buffer (KH_2PO_4, 1.58 M; K_2HPO_4, 0.93 M), 50 ml 1 M sodium bicarbonate, 4 ml 0.5 M sodium sulfide and substrate as indicated. The vials were gassed with an oxygen-free mixture of N_2/CO_2 (80/20 % v/v). Incubation temperature was 37^{0}C, except for *Sporomusa sphaeroides*. *Acetobacterium woodii* DSM 1030 and *Sporomusa ovata* DSM 2662 were cultured at 30^{0}C in medium 135 and 311, respectively, as described in Deutsche Sammlung von Microorganismen und Zellkulturen, Catalogue of Strains (1993).

DMSP and glycine betaine demethylation by cell suspensions and chemostat cultures. Strain PM31 was grown in fructose-limited chemostats with a working volume of 730 ml. The following conditions were employed: dilution rate 0.03 h^{-1}, reservoir medium (for composition see above) with 3 mM fructose, pH 7.2 (kept constant by automatic titration with 2 N NaOH), temperature 37^{o}C, gas phase above reservoir and culture N_2/CO_2 (80/20 % v/v). The medium reservoir was slowly stirred

to avoid a possible loss of precipitated trace elements. At steady state (after at least five volume changes) the medium feed was stopped and DMSP from an anoxic 1 M stock solution was added or cells were removed anoxically from the culture vessel and 50 ml were transferred into 120-ml vials in an anaerobic glove box equipped with a palladium catalyst (R020; BASF, Ludwigshafen, Germany) under an atmosphere of N_2:H_2 (approximately 95:5%, v/v). The vials were gassed with N_2/CO_2 (80/20% v/v) and used in experiments with protein synthesis inhibitors. Cell suspension experiments with batch-grown strain PM31 were done with anoxically harvested cells that had been grown on various concentrations of glycine betaine or glycine betaine/ DMSP. These cells were washed once or twice with complete sulfide-reduced medium without yeast extract or with anoxic 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol.

Cell extract preparation and enzyme measurements. Extracts of cells (3 - 10 mg protein/ml), grown on medium with 15 mM DMSP, 15 mM glycine betaine or 15 mM glycine betaine + 15 mM DMSP, were prepared under anoxic conditions as described by Hensgens et al. (1993) with the following minor modifications: the cells were washed and suspended in 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol and passed three times through a French pressure cell. The enzyme assays were performed in an anaerobic glove box; the assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.2), 2 mM dithiothreitol, 2.5 mM titanium(III)-10 mM nitrilotriacetic acid, 3 mM THF, 0.2 mM cyanocobalamin, 2 mM ATP, 8 mM $MgCl_2$, and cell extract in a total volume of 1 ml. After 10 min of incubation at 37^0C, the reaction was started by the addition of 10 mM substrate. Reactions were stopped with 60 mM HCl and after approximately 15 min the reaction mixture was centrifuged (5 min; 2000 x *g*) in the glove box. The supernatants were transferred to airtight vials and the THF and methyl-THF concentrations were measured by high-performance liquid chromatography (HPLC).

rDNA sequencing and sequence comparison. DNA of strain PM31 and '*Butyribacterium methylotrophicum*' for 16S rRNA gene sequence analysis was extracted and amplified as described previously (van der Maarel et al. 1996b). The PCR product was purified using the Wizard PCR purification system (Promega, Madison, Wi.) and subsequently sequenced on an ABI 310 automated sequencer (Perkin Elmer, Norwalk, Conn.) using the dye-terminator cycle sequencing method of Perkin Elmer in combination with custom primers based on the conserved regions of the 16S rRNA gene. Reaction conditions for the cycle sequencing reaction were according to the manufacturer's manual. The similarity of the sequences was determined by alignment of 1460 nucleotides using the DCSE program of De Rijk and De Wachter (1993) and subsequently calculating the number of common nucleotides. The 16S rDNA sequence of *E. limosum* DSM 20543^T was obtained from GenBank.

Analytical procedures. DMSP was determined as acrylate after conversion to dimethylsulfide and acrylate by overnight treatment with 1 M NaOH (White 1982). Acrylate, MTPA and mercaptopropionate were analyzed by HPLC (Jansen and Hansen 1998). Mercaptopropionate was measured after reduction of the sample with up to 20 mM tributylphosphine. Betaine and dimethylglycine were measured by HPLC according to Heijthuijsen and Hansen (1989), with acetonitrile/ water (80%:20% v/v)

as a mobile phase instead of acetonitrile with a 10 mM sodium phosphate buffer (pH 7.5). THF and methyl-THF were measured by HPLC with UV detection at 280 nm as described by Stupperich and Konle (1993). Homocysteine and methionine were assayed by HPLC after derivative formation with *ortho*-phthalaldehyde (Euverink et al. 1995). Fructose was measured by HPLC using a Polyspher OA HY column (Merck, Darmstadt, Germany) and refractometric detection; the flow rate of the mobile phase (0.01 N H_2SO_4) was 0.6 ml/min. The detection limit for fructose was 20 µM. Protein in cell extracts was determined according to Bradford (1976) using the BioRad reagent with bovine serum albumin as a standard. Protein content of whole cells was measured after treatment with 1 M NaOH at 100^{o}C for 10 min according to Lowry et al. (1951). The optical densities of cultures were measured in a 1 cm cuvette in a Starrcol colorimeter (Hoorn, The Netherlands) at 660 nm. An OD_{660} value of 1.0 corresponds to 0.26 mg/ml protein. Cell carbon was determined as described in (Heijthuijsen and Hansen 1989). Dimethylsulfide and methanethiol were assayed by GC as described previously (van der Maarel et al. 1993; van der Maarel et al. 1995).

Chemicals. DMSP was synthesized from acrylic acid and DMS (Chambers et al. 1987) or obtained from CASS (Groningen, The Netherlands). MTPA was obtained by alkaline hydrolysis of its methylester (Aldrich, Steinheim, Germany). 5,6,7,8-Tetrahydrofolic acid was obtained from Sigma (St. Louis, Mo.) or Schircks Laboratories (Jona, Switzerland); 5-methyl-5,6,7,8-tetrahydrofolic acid was from Merck (Darmstadt, Germany). Cyanocobalamin was purchased from Sigma (St. Louis, Mo.). Titanium(III)-nitrilotriacetic acid stock solutions were prepared according to Moench and Zeikus (1983).

Nucleotide sequence accession numbers. The 16S rDNA sequences of '*Butyribacterium methylotrophicum*' and *Eubacterium limosum* strain PM31 were deposited with GenBank under accession numbers AF 064241 and AF 064242, respectively.

Results

Conversion of DMSP by pure cultures of acetogenic bacteria. Recently, we reported that the acetogenic strain PM31, which had been isolated from intertidal mud and been tentatively identified as *Eubacterium limosum*, was able to demethylate DMSP to MTPA, but growth was very poor and the conversion was slow (van der Maarel et al. 1996b). Here, we describe in more detail the demethylation of DMSP by this bacterium. When 5% of a culture (grown on 15 mM DMSP/15 mM betaine) was inoculated into medium with 15 mM DMSP and 0.1% yeast extract, 5.6 mM MTPA was produced from 6.0 mM DMSP in 163 h of incubation (Fig. 1A and 1B). Other products in this culture were 1.7 mM acetate and 0.7 mM butyrate. Other possible sulfur-containing end-products such as dimethylsulfide, methanethiol and mercaptopropionate were not detected. The increase in optical density in these cultures (OD_{660} 0.12) was not higher than in cultures without DMSP and therefore most likely due to utilization of components from the yeast extract. This increase took place during the first 40 hours; in this period only 1.4 mM MTPA had been formed, again indicating that growth could not have been supported by DMSP demethylation.

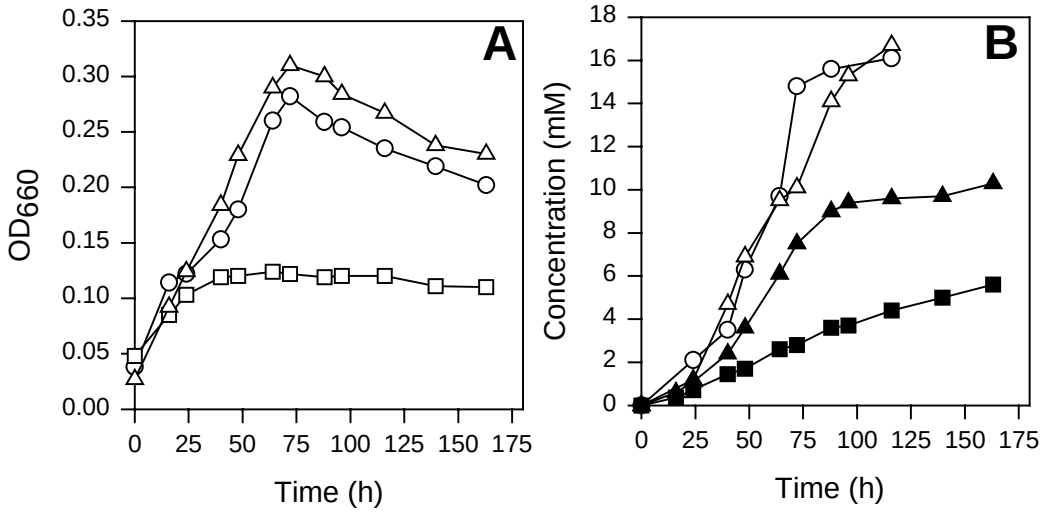

Fig. 1 Growth (A) of strain PM31 on 15 mM glycine betaine (-○-), 15 mM DMSP (-□-) and 15 mM DMSP + 15 mM glycine betaine (-Δ-). In (B) the product concentrations are shown; dimethylglycine during growth on 15 mM glycine betaine (-○-), or 15 mM DMSP + 15 mM glycine betaine (-Δ-), and MTPA during growth on 15 mM DMSP (-■-) and 15 mM DMSP + 15 mM glycine betaine (-▲-).

Incubations with 15 mM glycine betaine resulted in a maximum optical density of 0.28 (Fig. 1A), a value considerably above the control with only yeast extract; products of the growth on glycine betaine were 16.1 mM dimethylglycine, 6.6 mM acetate and 0.8 mM butyrate. The growth yield on glycine betaine in this experiment was 3.0 g dry weight cells/ mol glycine betaine. In earlier work (van der Maarel et al. 1996b) DMSP had been tested for its use as a growth substrate by acetogens; since it now appeared that DMSP utilization was not associated with growth we reinvestigated a possible biotranformation of DMSP to MTPA by other acetogens. Besides strain PM31, several other acetogenic bacteria were indeed found to be able to demethylate DMSP; *E. limosum* DSM 20543^{T}, *E. limosum* DSM 20517, *E. limosum* DSM 2594, *Sporomusa ovata* DSM 2662, *Sporomusa sphaeroides* DSM 2875, *Acetobacterium woodii* DSM 1030, and '*Butyribacterium methylotrophicum*' produced MTPA from DMSP, at rates that are comparable with the DMSP demethylation rates in cultures of strain PM31. Also cultures of these strains showed no significant increase in optical density when DMSP was added to the medium. This is in agreement with the observation of van der Maarel et al. (1996b), that these pure cultures were unable to grow on DMSP. Importantly, with all of these bacteria the demethylation of glycine betaine to dimethylglycine did support growth.

Characteristics and phylogenetic position of strain PM31. Strain PM31 was chosen as the model organism for the detailed studies described below because it originates from an environment where DMSP is known to occur. Strain PM31 is strictly anaerobic, salt-tolerant, nonmotile, nonsporeforming, Gram-positive and rod-

shaped (0.6 to 0.9 μm by 2.0 to 3.0 μm); it was isolated from an enrichment culture inoculated with anoxic intertidal sediment of the Wadden Sea (The Netherlands) with 10 mM vanillate as a substrate (19). It showed good growth in freshwater medium at 37^{O}C on several substrates including methanol, H_2/CO_2 (80%/20% v/v), glucose, fructose, various methoxylated aromatic compounds, and glycine betaine. Acetate and butyrate were the main fermentation products.

The 16S rRNA genes of strain PM31 and '*Butyribacterium methylotrophicum*' were found to differ in only one nucleotide position (99.9% similarity). Based on their 16S rRNA gene sequences strain PM31 and '*Butyribacterium methylotrophicum*' are very closely related to the type strain of *E. limosum* (similarities of 99.5% and 99.4%, respectively). These data and its phenotypic properties support assignment of strain PM31 to *E. limosum*. It is also evident that '*Butyribacterium methylotrophicum*' is in fact a *E. limosum* strain, which is in agreement with the phenotypic similarity. Its major difference with *E. limosum*, namely its ability to produce spores, has been questioned by Cato et al. (4).

Sequential and simultaneous utilization of substrates by batch cultures of *E. limosum* strain PM31. Demethylation of DMSP and glycine betaine in cultures of strain PM31 occurred simultaneously (Fig. 1B). In cultures inoculated with 5% of a glycine betaine (15 mM)/DMSP (15 mM) pregrown culture, the change in dimethylglycine concentration was similar to those of cultures supplemented with only glycine betaine; the production of MTPA was slower. In such cultures glycine betaine and DMSP were demethylated stoichiometrically to dimethylglycine and MTPA, respectively. Under these conditions demethylation of DMSP was faster than in cultures supplemented with only DMSP (Fig. 1B). In batch fermenter cultures (pH kept constant at 7.2) with media containing 15 mM fructose and 30 mM DMSP, strain PM31 rapidly demethylated DMSP after a growth-supporting utilization of the fructose (Fig. 2); during the linear decrease in the DMSP concentration between 40 and 60 h the demethylation rate was 50 nmol min^{-1} mg^{-1} protein. Similarly, in experiments with methanol and DMSP as substrates, DMSP was only demethylated after virtual exhaustion of the methanol. Also in these experiments no or negligible growth was observed when DMSP was demethylated. In cultures of strain PM31 with fructose and glycine betaine, first fructose was used and subsequently the glycine betaine was demethylated. All other tested acetogenic bacteria (see material and methods) also showed faster DMSP demethylation in media containing a true growth substrate and DMSP.

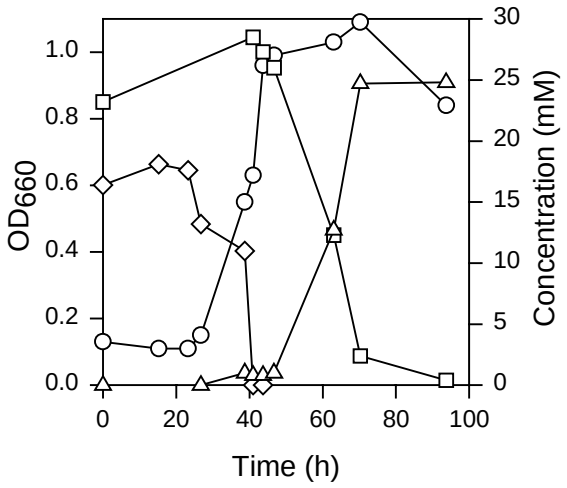

Fig. 2 Sequential utilization of fructose and DMSP by strain PM31. Symbols: -○- OD_{660}, -□- DMSP, -∆- MTPA, -◊- fructose.

DMSP and glycine betaine demethylation by cells of *Eubacterium limosum* strain PM31. Cells obtained from glycine betaine/DMSP/yeast extract or DMSP/yeast extract grown batch cultures, washed in medium without yeast extract, did not demethylate DMSP or, after a lag phase of many hours, at rates lower than 5 nmol min^{-1} mg^{-1} protein despite the use of anaerobic techniques throughout the manipulations. To obtain active cells, experiments were carried out with fructose-limited chemostat cultures, where at steady state at a dilution rate of 0.03 h^{-1} the medium flow was stopped and DMSP was added; at steady state the fructose concentration was below the detection level (lower than 20 µM). DMSP demethylation occurred at a rate of approximately 50 nmol min^{-1} mg^{-1} protein and started immediately after addition of DMSP (Fig. 3). Over the period during which the DMSP was demethylated there was a small OD_{660} decrease, again showing that DMSP did not support growth. The apparent K_m value for DMSP was approximately 2 mM as calculated from the substrate depletion curve. When DMSP and glycine betaine, both at 10 mM, were added to a fructose-limited chemostat culture, both compounds were demethylated at the same time at similar rates (data not shown).

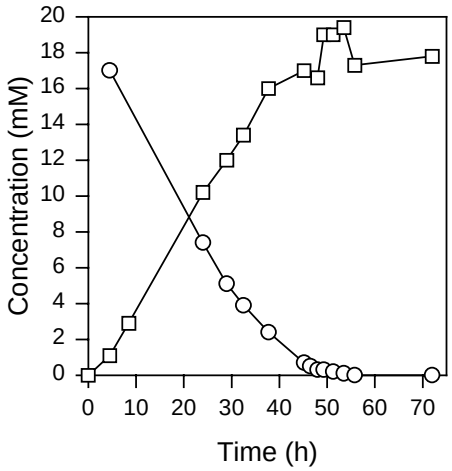

Fig. 3 DMSP demethylation by cells (protein concentration 0.16 mg/ml) obtained from fructose-limited chemostat cultures of strain PM31. Symbols: -○- DMSP, -□- MTPA.

Steady state cultures at a dilution rate of 0.03 h^{-1} with 3.0 mM fructose and 9.0 mM DMSP in the medium reservoir contained 0.9 mM DMSP and 8.0 mM MTPA. The OD values of fructose-limited cultures with DMSP in the reservoir were almost the same as without DMSP (0.47 *vs.* 0.50). When 10 mM DMSP was added to such a culture immediately after stopping the medium flow, approximately the same DMSP demethylation rate was found as with cultures grown in the absence of DMSP (53 nmol min^{-1} mg^{-1} protein). These results show that the DMSP demethylation system does not require induction by DMSP. This was confirmed by using tetracycline as an inhibitor of de novo protein synthesis; rifampicin (4 µg/ml) and chloramphenicol (25 - 100 µg/ml) could not be used (data not shown). Tetracycline (20 µg/ml) blocked the growth of strain PM31 on fructose, and strongly reduced the rate of fructose degradation. With cells growing in batch culture in a medium with initial concentrations of 3 mM fructose and 20 mM DMSP and already producing MTPA (after exhaustion of the fructose), addition of 20 µg/ml tetracycline did not affect the demethylation of DMSP. When, after growth on fructose (in the absence of DMSP), DMSP and tetracycline were added simultaneously, strain PM31 was still able to demethylate DMSP. With cells obtained from fructose-limited chemostat cultures similar results were obtained.

Yeast extract is known to contain 1-3% (w/w) glycine betaine (Galinski 1995); 1 g/l yeast extract in the media therefore may lead to an initial glycine betaine concentration of 0.27 mM; such a concentration might be sufficient for the induction of the glycine betaine demethylation system (if it is inducible) and, because of the structural similarity of DMSP and glycine betaine, of a specific DMSP demethylating enzyme, if such an enzyme exists. We therefore tried to culture strain PM31 in fructose-limited chemostat cultures without yeast extract in the reservoir medium. After 9.4 volume changes there was considerably more wall growth and a lower OD (0.3) than under culture conditions

with yeast extract present, but the residual fructose concentration remained below the detection limit. These cells were able to demethylate DMSP in the presence of 10 µg/ml tetracycline, albeit very slowly (approximately 1 nmol min^{-1} mg^{-1} protein; the control without tetracycline demethylated DMSP at 7.5 nmol. min^{-1} mg^{-1} protein).

Effects of MTPA and dimethylglycine on the growth of *Eubacterium limosum* strain PM31. Dimethylglycine and MTPA both have an inhibitory effect on the growth on betaine of strain PM31 (Fig. 4). This effect was small at initial dimethylglycine or MTPA concentrations of 5 mM but became pronounced at initial concentrations of 10 mM or higher. The effect of MTPA was not stronger than that of dimethylglycine. The effect on growth with 3 mM fructose appeared to be smaller than on growth with betaine but during growth on betaine an increasing concentration of dimethylglycine is produced. Up to 10 mM dimethylglycine or MTPA hardly affected the growth on fructose but 20 mM dimethylglycine or MTPA strongly reduced the growth rate and the final optical density of the culture was approximately 25 % lower than in the control. These data exclude a far stronger inhibitory effect of MTPA than that of dimethylglycine on the growth as an explanation for the inability of strain PM31 to utilize DMSP as a growth-supporting substrate.

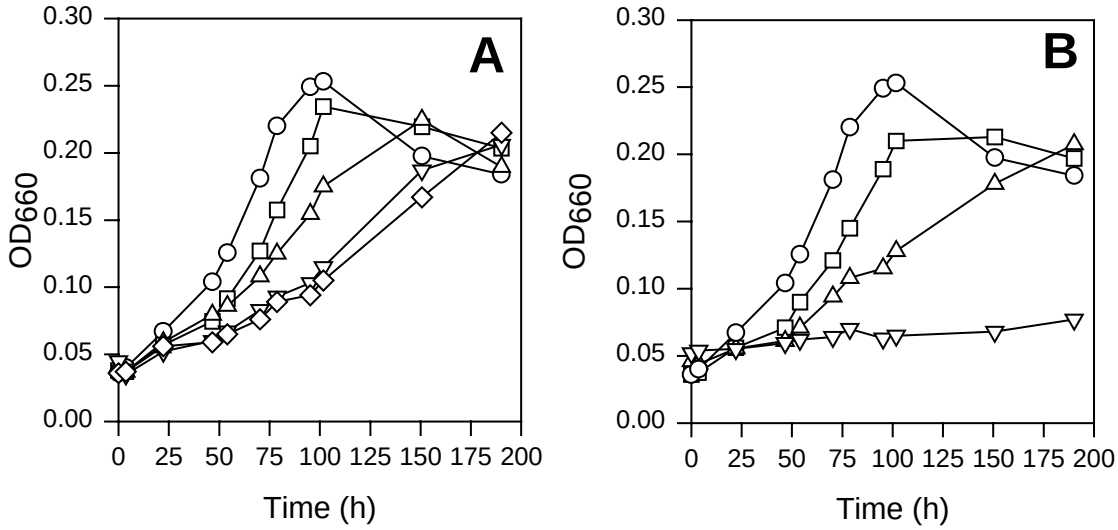

Fig. 4 Effect of MTPA (A) and dimethylglycine (B) on growth of strain PM31 on glycine betaine (15 mM). Symbols (A): -○- control (minus addition), addition at t=0 of 5 (-□-), 10 (-Δ-), 15 (-∇-) and 20 (-◊-) mM MTPA; (B) -○- control (minus addition), addition at t=0 of 5 (-□-), 10 (-Δ-) and 20 (-∇-) mM dimethylglycine.

DMSP demethylating activities in cell extracts of *Eubacterium limosum* strain PM31. DMSP:THF methyltransferase was identified as the key enzyme in DMSP demethylation in sulfate-reducing bacteria. Using a modified assay system we also detected DMSP:THF methyltransferase activity in the acetogenic strain PM31 but the activities were lower than in the sulfate reducers (Table 1). DMSP-THF methyltransferase activities of approximately 20 - 25 nmol min^{-1} mg^{-1} protein were detected with cell extracts of glycine betaine- or glycine betaine/DMSP-grown strain PM31. Activities with glycine betaine as substrate were significantly lower, even in cells grown on glycine betaine (Table 1). No activity was detected without titanium-nitrilotriacetic acid. ATP and Mg^{2+} were not obligatory for activity, but stimulated the DMSP demethylating activities in cell extracts of strain PM31. When cyanocobalamin was omitted from the assay mixture, DMSP demethylating activities were approximately 10-30 % lower. Hydroxocobalamin did not have a stimulatory effect on the DMSP demethylation activities. In extracts of cells that had been grown on fructose or on fructose and DMSP (harvested when they were demethylating DMSP) no or negligible DMSP:THF methyltransferase (and no activity with betaine) could be detected.

Table 1 DMSP and glycine betaine demethylation with THF as methyl acceptor in cell extracts of strain PM31[a]

Substrate(s)[b]	DMSP-demethylating activity (nmol min^{-1} mg^{-1} protein)	Betaine-demethylating activity (nmol min^{-1} mg^{-1} protein)
DMSP	10.4 ± 1.5	1.0 ± 0.4
Glycine betaine	24.2 ± 3.0	2.2 ± 0.3
Glycine betaine + DMSP	20.9 ± 0.8	2.6 ± 0.2

[a] Results are means of three experiments ± standard deviation, except for DMSP-demethylating activities with DMSP (mean of five experiments) and glycine betaine as a growth substrate (mean of eight experiments).
[b] All media contained 0.1% yeast extract.

A DMSP or glycine betaine demethylating activity with homocysteine as a methyl acceptor (glycine betaine:homocysteine methyltransferase) was not found; in titanium-nitrilotriacetic acid reduced assays with cell extracts of glycine betaine-grown strain PM31 and with glycine betaine or DMSP as a substrate, we did not detect a decrease in homocysteine concentration or an increase in methionine concentration, not even after incubations for two hours.

Discussion

This study shows that demethylation of DMSP to MTPA under anoxic conditions can not only be performed by certain sulfate-reducing bacteria of the *Desulfobacter* - *Desulfobacterium* cluster of the delta-Proteobacteria (van der Maarel et al. 1996b), but also by several acetogenic bacteria. These acetogenic bacteria are also able to demethylate the N-containing analog of DMSP, glycine betaine, but unlike DMSP,

glycine betaine was a growth substrate whereas DMSP was only biotransformed without supporting growth. In view of this finding and the poor K_m value for DMSP (mM range) of *E. limosum* strain PM31 compared to the value for the sulfate-reducing bacteria (µmolar range) the observed demethylation of DMSP by such acetogens is probably of no or very limited ecological significance. The demethylation of DMSP to MTPA at a rate of 50 nmol min^{-1} mg^{-1} protein, as shown with strain PM31, is of clear biotechnological relevance, however (Hansen and van der Maarel 1998). Biologically produced MTPA can be used in the production of natural flavors.

Since the demethylation of DMSP is of no obvious benefit for the organism, the process is most probably catalyzed by an enzyme which is normally involved in demethylation of a structurally related true substrate such as glycine betaine. In analogy with the biochemistry of the metabolism of other methylated substrates in acetogens (methanol, methoxylated aromatics; see Ref. Kaufmann et al. 1997; Kaufmann et al. 1998; Stupperich and Konle 1993) one would expect the presence of a DMSP- and a glycine betaine demethylating enzyme which would feed the methyl group into the methyl branch of the Wood-Ljungdahl (reductive CO dehydrogenase) pathway for acetyl-CoA synthesis. Recently, the *O*-demethylase from *Acetobacterium dehalogenans* was purified and shown to consist of four components that were all required for the efficient catalysis of the methyl transfer from phenyl methyl ethers to THF (Kaufmann et al. 1997; Kaufmann et al. 1998). Purification of the DMSP- and glycine betaine-demethylating system(s) will be necessary to reveal whether these systems are indeed identical and of a similar complexity as the *O*-demethylating system. The low activities of a glycine betaine methyltransferase we detected are only a first indication for the nature of the methyltransferase reaction. The media used contained yeast extract, because the strain grew poorly in its absence; therefore glycine betaine was always present in low concentrations in the media. Under these conditions DMSP demethylation by cells grown under fructose limitation does not require de novo protein synthesis. Furthermore, glycine betaine and DMSP were demethylated simultaneously. The low activities of the methyltransferase reaction with glycine betaine did not allow a detailed kinetic analysis of the effect of DMSP on the activity. There are some examples in the literature that show that DMSP can indeed be a substrate (and sometimes with higher activities!) for a glycine betaine utilizing enzyme but this is not a general rule. Mammalian betaine:homocysteine methyltransferase is known to be active towards DMSP (Garrow 1996). In *Sinorhizobium meliloti* dimethylsulfonioacetate, the acetate analog of DMSP, is demethylated via the glycine betaine demethylating system, which in this organism is thought to be a glycine betaine:homocysteine methyltransferase (Smith et al. 1988), but DMSP is not demethylated by *S. meliloti* and is used only as an osmoprotectant (Pichereau et al. 1998). Similarly, in extracts of *Pseudomonas denitrificans* glycine betaine and dimethylsulfonioacetate can function as methyl donors for homocysteine methylation whereas DMSP cannot (White et al. 1973). In the sulfate-reducing bacterium strain WN demethylation of DMSP is catalyzed by a DMSP:THF methyltransferase which is not active towards glycine betaine (Jansen and Hansen 1998; Jansen and Hansen 2000).

The utilization of both DMSP and glycine betaine is completely inhibited as long

as fructose is present in low mM concentrations but not when fructose is the limiting substrate in chemostat cultures; we do not know what mechanism underlies this phenomenon in our strain. In experiments with a different strain of *E. limosum*, Genthner and Bryant (1987) observed a similar repression of the utilization of methanol, hydrogen and isoleucine by 2 mM glucose leading to their utilization after glucose depletion, and a clear lag phase

Why these bacteria grow on glycine betaine and show marginal or no growth on DMSP can only be speculated about. At the moment there are no reasons to believe that the intracellular conversion of DMSP and carbon dioxide to MTPA, acetate and butyrate yields less ATP than the analogous fermentation of glycine betaine and carbon dioxide. Our value of the molar growth yield on glycine betaine was considerably lower (approximately 3.0 g dry weight cells/ mol) than the value (9 g/mol) reported by Müller et al. (1981) for another strain of *E. limosum* but the order of magnitude in both cases shows that less than one ATP per betaine is produced which can be used for biosynthesis (cf. Badziong and Thauer 1978). Three factors may contribute to differences in the amount of ATP available for biosynthesis as a result of betaine and DMSP fermentation: differences in the energetic costs of betaine and DMSP uptake, differences related to product (dimethylglycine and MTPA) export, and maintenance energy effects. The transport of DMSP across the membrane may be energetically more expensive than the transport of glycine betaine. How DMSP and glycine betaine are transported in *E. limosum* strain PM31 is not known, but both in *Escherichia coli* and *Bacillus subtilis* different mechanisms for glycine betaine transport exist (Kappes et al. 1996; Lucht and Bremer 1994). In *E. coli* glycine betaine can be transported across the membrane by the constitutive, low affinity, proton-motive-force-driven system ProP, and the inducible, high affinity, ATP-consuming system ProU (Lucht and Bremer 1994; Mimmack et al. 1989). Differences in the energetic costs of glycine betaine and DMSP transport in *E. limosum* may strongly affect the growth yield. If DMSP is transported mainly by an ATP-consuming system and glycine betaine mainly in symport with one proton, a considerable difference in molar growth yield may be expected. Interestingly, recent work has shown that in *B. subtilis* DMSP is not taken up by the proton-motive-driven secondary betaine transporter OpuD; DMSP is taken up only by the ABC transporters OpuA and OpuC (Nau-Wagner et al. 1999). Differences in the mechanism of export of dimethylglycine and MTPA from the cells may also play a role. Because of the stronger structural similarity of butyrate and MTPA the energy-consuming export system described for butyrate in *Eubacterium limosum* might also be involved in MTPA export and not in dimethylglycine export (Lebloas et al. 1996). Maintenance energy is known to affect the molar growth yields at low specific growth rates; the rates of DMSP and glycine betaine utilization did not differ so much that maintenenance energy effects alone can easily explain the lack of growth on DMSP.

Acknowledgements

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4

Tetrahydrofolate serves as a methyl acceptor in the demethylation of dimethylsulfoniopropionate in cell extracts of sulfate-reducing bacteria

Michael Jansen · Theo A. Hansen

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THF was shown to function as a methyl acceptor in the anaerobic demethylation of DMSP to MTPA in cell extracts of the sulfate-reducing bacterium strain WN. DMSP-dependent activities were 0.56 µmol methylTHF min^{-1} (mg protein)$^{-1}$ and were higher than required to explain the growth rate of strain WN on DMSP. The reaction did not require ATP or reductive activation by titanium(III)-nitrilotriacetic acid. Preincubation of the extract under air significantly decreased the activity (35% loss in 3 h). Three other DMSP-demethylating sulfate reducers, *Desulfobacterium niacini, Desulfobacterium vacuolatum*, and *Desulfobacterium* strain PM4, had DMSP:THF methyltransferase activities of 0.16, 0.05, and 0.24 µmol min^{-1} (mg protein)$^{-1}$, respectively. No methyltransferase activity to THF was found with betaine as a substrate, not even in extracts of betaine-grown cells of these sulfate reducers. DMSP demethylation in cell extracts of strain WN was completely inhibited by 0.5 mM propyl iodide; in the light, the inhibition was far less strong, indicating involvement of a corrinoid-dependent methyltransferase.

Key words: Dimethylsulfoniopropionate - Methylthiopropionate - Sulfate-reducing bacteria - *Desulfobacterium* - Tetrahydrofolate - Methyltransferase

Introduction

Demethylation has been shown to be one of the initial steps in the breakdown of the algal osmolyte DMSP [$(CH_3)_2$-S^+-CH_2-CH_2-COO^-] by several bacteria, including both aerobes (Taylor and Gilchrist 1991; Diaz and Taylor 1996; Visscher and Taylor 1994) and anaerobes. Among the anaerobes, several sulfate-reducing bacteria are able to demethylate DMSP to MTPA (CH_3-S-CH_2-CH_2-COO^-) and to grow on the basis of the oxidation of the methyl group with the concomitant reduction of sulfate to hydrogen sulfide (van der Maarel et al. 1996b). Most likely, these bacteria channel the methyl group to the oxidative acetyl-CoA/CO dehydrogenase pathway, which is normally involved in the oxidation of acetyl-CoA (van der Maarel et al. 1996b). Tetrahydropterins play a key role in the acetyl-CoA/CO dehydrogenase pathway. *Desulfobacterium autotrophicum* uses tetrahydropteroyltetraglutamate as a one-carbon carrier (Länge et al. 1989), but extracts of another bacterium with this pathway, *Desulfotomaculum acetoxidans*, have been shown to have activity of the enzymes of the methyl branch of this pathway with THF, a tetrahydropterin with only one glutamate (Spormann and Thauer 1988). The mechanism of DMSP demethylation in sulfate-reducing bacteria and in aerobic demethylating bacteria remains to be elucidated. Here, we report on a novel type of methyl transfer in cell extracts of DMSP-demethylating sulfate reducers in which THF is used as a methyl acceptor for the demethylation of DMSP.

Materials and methods

Organisms, cultivation, and cell extract preparation. For growth of strain WN (van der Maarel et al. 1996b) and *Desulfobacterium autotrophicum* strain HRM2

(DSM 3382), medium 383 as described in the Deutsche Sammlung von Mikroorgananismen und Zellkulturen Catalogue of Strains (1993) was used with yeast extract (0.025%) and 10 mM substrate (betaine, DMSP, or ethanol) as indicated. *Desulfobacterium niacini* strain Nav1 (DSM 2650), *Desulfobacterium vacuolatum* strain IbRM (DSM 3385), and *Desulfobacterium* strain PM4 were grown in mineral medium according to Heijthuijsen and Hansen (1989) with yeast extract (0.025%), 20 mM Na_2SO_4, and 10 mM substrate (DMSP or betaine) as indicated. Extracts of cells (5-15 mg protein ml^{-1}) were prepared under anoxic conditions as described by Hensgens et al. (1993) with the following minor modifications: the cells were washed twice in 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol, 21 g NaCl/l, and 3.0 g $MgCl_2 \cdot 6H_2O$/l and were suspended in buffer without NaCl and $MgCl_2 \cdot 6H_2O$.

Enzyme assays. The methyltransferase assays were performed in an anaerobic glove box equipped with a palladium catalyst (R020; BASF, Ludwigshafen, Germany) under an atmosphere of N_2:H_2 (approximately 95:5 v/v) and at $28^{O}C$. No difference in activities was observed with an atmosphere of 100% N_2. The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM dithiotreitol, 2.5 mM titanium(III)-10 mM nitrilotriacetic acid, 1 mM THF, 5 mM DMSP or betaine, and cell extract in a total volume of 1.0 ml. Reactions were stopped with 60 mM HCl and were centrifuged in the glove box. The supernatants were transferred to air-tight vials; THF and methyl-THF concentrations were measured by HPLC. Activities are expressed as µmol methyl-THF produced min^{-1}.

Analytical procedures. DMSP concentrations were determined as acrylate after conversion to dimethylsulfide and acrylate by overnight treatment with 1 M NaOH (White 1982). Acrylate and MTPA concentrations were analyzed by HPLC using a C18 column (Econosil C18-5µ- 250 x 4.6 mm, Alltech, Breda, The Netherlands) with 7.5% acetonitrile in 1% (w/v) phosphoric acid as the mobile phase at a flow rate of 1 ml/min. The eluate was monitored at 205 nm. THF and methyl-THF were separated by HPLC according to Stupperich and Konle (1993); the eluate was monitored at 280 nm. Protein was measured according to Bradford (1976) using the BioRad reagent with bovine serum albumin as a standard. Cell carbon was determined according to Heijthuijsen and Hansen (1989).

Chemicals. DMSP was synthesized from acrylic acid and dimethylsulfide (Chambers et al. 1987). MTPA was obtained by alkaline hydrolysis of its methylester (Aldrich, Steinheim, Germany). 5,6,7,8-Tetrahydrofolic acid was obtained from Sigma (St. Louis, Mo., USA) and Schircks Laboratories (Jona, Switzerland); 5-methyl-5,6,7,8-tetrahydrofolic acid was from Merck (Darmstadt, Germany); and propyl iodide was from Fluka Chemika (Buchs, Switzerland). Titanium(III)-nitrilotriacetic acid stock solutions were prepared according to Moench and Zeikus (1983).

Results and discussion

Recently, three photometric continuous assay systems for methyltransferase reactions have been used for studies of demethylation reactions in acetogenic bacteria (Meßmer et al. 1996; Kreft and Schink 1996, 1997). For the measurement of DMSP:THF

methyltransferase, we developed a discontinuous assay. Despite the fact that our method is rather time-consuming, it is easy to use. With this method we detected high activities of a DMSP:THF methyltransferase in extracts of DMSP-grown cells of strain WN [in the presence of titanium(III)-nitrilotriacetic acid, approximately 0.56 μmol min^{-1} (mg protein)$^{-1}$]. The demethylation of DMSP is the first observation of a methyltransferase using THF in sulfate-reducing bacteria. The activity in strain WN is higher than the demethylating activity with THF found in most acetogenic bacteria that convert C_1-compounds such as methanol, methyl chloride, or methoxylated aromatic compounds. Recently, high activities of 3,5-dihydroxyanisole methyl transfer to THF [approximately 0.1 μmol min^{-1} (mg protein)$^{-1}$] have been observed in extracts of *Holophaga foetida* (Kreft and Schink 1997) but, for example, activities with methanol in cell fractions of *Sporomusa ovata* were only 2.0 nmol min^{-1} (mg protein)$^{-1}$ (Stupperich and Konle 1993), and in cell extracts of the homoacetogenic strain MC methylchloride was demethylated at a rate of 20 nmol min^{-1} (mg protein)$^{-1}$ (Meβmer et al. 1993). The specific activity measured with THF in strain WN is more than enough to explain the growth rate of this organism on DMSP; a DMSP demethylation rate of 0.23 μmol min^{-1} (mg protein)$^{-1}$ can be calculated for cultures growing on DMSP (with a growth yield of 2.9 g cell carbon (mol substrate)$^{-1}$ and a specific growth rate of 0.04 h^{-1} as determined earlier by van der Maarel et al. 1996b).

From DMSP, equal concentrations of MTPA and methyl-THF were formed. With an excess of DMSP (initial concentration, 5 mM), from 0.9 mM THF approximately 0.5 mM methyl-THF was formed even after prolonged incubation (Fig. 1), most likely because only of one the enantiomers of the (*R*,*S*)-THF was used. Reduction of the assay mixture by titanium(III)-nitrilotriacetic acid was not necessary in order to obtain these high activities; activities without titanium(III)-nitrilotriacetic acid were sometimes 10-20% lower, or a lag phase was observed. ATP (1.0 mM) was not stimulatory in this reaction, whereas a positive effect has been reported for several other demethylation reactions in acetogenic bacteria (e.g. Berman and Frazer 1992; Stupperich and Konle 1993). Heat treatment (30 min at 70^{O}C) of the cell extract destroyed all activity. Activity increased proportionally with the protein concentration (range tested: 0.03-0.6 mg ml^{-1}), implying that the reaction was catalyzed by a single enzyme. This is not true for certain other demethylation reactions found in anaerobic bacteria (Kreft and Schink 1993). Incubation of the extract of strain WN (at 0^{O}C) under air without stirring (1 ml in a 10-ml vial) led to a 35% loss of activity within 3 h, which suggests that the enzyme is at least slightly oxygen-labile. After 17 h of incubation, 25% of the activity was still present. Under anoxic conditions, the activity remained the same over the period of a day.

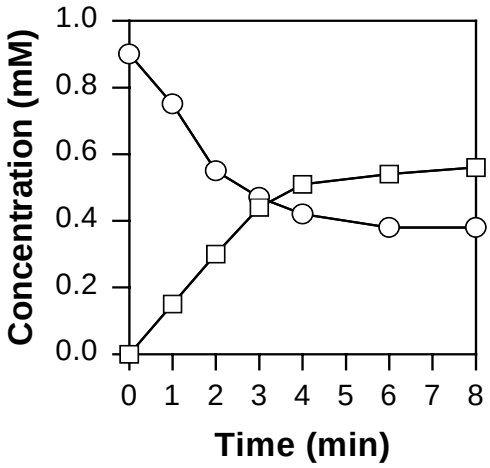

Fig. 1 Demethylation of DMSP by cell extracts of DMSP-grown strain WN: ○ consumption of THF and □ production of methyl-THF. Protein concentration, 0.29 mg ml^{-1}

In cell extracts of three other DMSP-demethylating sulfate reducers, *Desulfobacterium* strain PM4, *Desulfobacterium niacini*, and *Desulfobacterium vacuolatum*, DMSP-demethylating activities were detected using the same assay system (Table 1). ATP did not have a stimulatory effect on the activity in extracts of these organisms.

Table 1 Demethylation of DMSP in cell extracts of sulfate-reducing bacteria with THF as the methyl acceptor. Organisms were grown on DMSP except for *Desulfobacterium autotrophicum*, which was grown on betaine

Organism	DMSP-demethylating activity [µmol min^{-1} (mg protein)$^{-1}$]
Strain WN	0.56
Desulfobacterium strain PM4	0.24
Desulfobacterium niacini	0.16
Desulfobacterium vacuolatum	0.05
Desulfobacterium autotrophicum	0.00

Betaine [$(CH_3)_3$-N^+-CH_2-COO^-] structurally resembles DMSP and can be demethylated to dimethylglycine [$(CH_3)_2$-N-CH_2-COOH] by the four tested strains; interestingly, some examples are known in which a sulfonium analog can substitute for betaine, such as in the betaine-homocysteine methyltransferase reaction (see, for example, Garrow 1996 and White et al. 1973). Cell extracts of betaine-grown cells did not catalyze the demethylation of betaine or DMSP with THF as a methyl acceptor; addition of ATP did not lead to any activity with betaine. Also, cell extracts of these bacteria grown on DMSP did not demethylate betaine with THF. Cell extracts of betaine-grown *Db. autotrophicum*, an organism not able to demethylate DMSP (van

der Maarel et al. 1996b), did not have any activity with THF when betaine or DMSP was the substrate (Table 1). These results indicate that probably another compound serves as the methyl acceptor for demethylation of betaine or that a very different assay system is required. In this respect, it is important to note that the physiological one-carbon carrier detected in cells of *Db. autotrophicum* was not THF but a pterin containing 4 mol L-glutamate per mol pterin (Länge et al. 1989); with this cosubstrate, the specific formyltetrahydropterin synthetase activity was 170-fold higher than with THF. We do not know what type of pterins is the major cosubstrate in the DMSP-demethylating strains; therefore, some of our data may be underestimates of the actual specific activities if the proper pterin were used in the assay. With cell extracts of ethanol-grown strain WN, no activity of DMSP:THF methyltransferase was observed. Supplementation of the medium with 5 mM MTPA when strain WN was grown on ethanol or betaine also did not result in any activity. MTPA was tested because in some reactions, the product can induce the enzyme responsible for the formation of this product (see, for example, van der Maarel et al. 1996a). The results indicated that these bacteria have an enzyme system that is induced only by DMSP.

Most corrinoid-dependent methyltransferases are known to be inactivated by propyl iodide (see, for example, Brot and Weissbach 1965; Kengen et al. 1988). Incubation of the cell extract of strain WN with 0.5 mM propyl iodide (added from a 25 mM solution in ethanol; the ethanol caused an inhibition of only approximately 10% of the reaction) for 10 min in the dark inhibited the reaction completely, but when after 10 min the reaction mixture was incubated in the light, the activity was partly restored. The activity of the control experiment without titanium(III)-nitrilotriacetic acid incubated in the light (150-W incandescent light bulb at 20 cm) was 0.54 μmol min^{-1} $(mg\ protein)^{-1}$; incubation with 0.5 mM propyl iodide in the light (after 10 min of dark incubation) resulted in activities of 0.33 μmol min^{-1} $(mg\ protein)^{-1}$. This effect of light on the inhibition of the DMSP-demethylating reaction suggests the involvement of a corrinoid-dependent enzyme although reductive activation was not required. Corrinoids may play a role in the transfer of the methyl group of C_1-compounds in sulfate reducers, as is the case in some acetogenic bacteria (see, for example, Kreft and Schink 1993; Stupperich and Konle 1993). A purification and characterization of the enzyme responsible for the demethylation of DMSP in strain WN will reveal whether a corrinoid is involved in this enzyme activity.

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5

DMSP:tetrahydrofolate methyltransferase from the marine sulfate-reducing bacterium strain WN

Michael Jansen · Theo A. Hansen

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DMSP, an important compatible solute of many marine algae, can be metabolised by bacteria via cleavage to dimethylsulfide and acrylate or via an initial demethylation. This is the first report on the purification of an enzyme that specifically catalyzes the demethylation of DMSP. The enzyme was isolated from the sulfate-reducing bacterium strain WN, which grows on DMSP and demethylates it to MTPA. DMSP:THF methyltransferase from strain WN was purified 76-fold [to a specific activity of 40.5 µmol.min^{-1}(mg protein)$^{-1}$]. SDS polyacrylamide gel electrophoresis showed two bands of approximately 10 and 35 kDa; in particular the 35 kDa polypeptide became significantly enriched during the purification. Storage of the purified fraction at -20 °C under nitrogen resulted in a 99 % loss of activity in two days. The activity could be partially restored by addition of 200 µM cyanocobalamin, hydroxocobalamin or coenzyme B_{12}. ATP did not have any positive effect on activity. Reduction of the assay mixture by titanium(III)nitrilotriacetic acid slightly stimulated the activity. Gel filtration chromatography revealed a native molecular mass between 45 and 60 kDa for the DMSP:THF methyltransferase. The enzyme was most active at 35 °C and pH 7.8. Glycine betaine, which can be considered an N-containing structural analog of DMSP, did not serve as a methyl donor for DMSP:THF methyltransferase. Various sulfur-containing DMSP-analogs were tested but only methylethylsulfoniopropionate served as methyl donor. None of these compounds inhibited methyl transfer from DMSP to THF. Strain WN did not grow on any of the sulfur-containing DMSP-analogs.

Keywords: Dimethylsulfoniopropionate - Methyltransferase - Sulfate-reducing bacteria

Introduction

During the past ten years considerable progress has been made in the understanding of the bacterial metabolism of DMSP [$(CH_3)_2$-S^+-CH_2-CH_2-COO^-]. DMSP is an important compatible solute in many marine algae and some plants. Its metabolism can be initiated by a cleavage reaction yielding dimethylsulfide and acrylate or by a demethylation. DMSP lyases have been purified from a number of bacterial strains (de Souza and Yoch 1995a; van der Maarel et al. 1996a) but data on purified enzymes that specifically catalyze the demethylation of DMSP are not available. The product of the first demethylation reaction is MTPA (CH_3-S-CH_2-CH_2-COO^-); this compound is also of interest as a flavor precursor (Hansen and van der Maarel 1998). Both aerobic (e.g. González et al. 1999; Kiene et al. 2000) and anaerobic bacteria that can demethylate DMSP are known. Certain representatives of the *Desulfobacterium*/*Desulfobacter* cluster of the the delta-Proteobacteria have been identified as important DMSP-demethylating bacteria in marine sediments (van der Maarel et al. 1996b). These anaerobic, sulfate-reducing bacteria, including our strain WN, stoichiometrically convert DMSP to MTPA which is not degraded further. No data are available on the

mechanism of DMSP demethylation in aerobic bacteria. In this domain, however, considerable progress has been made in work with anaerobic bacteria from marine sediments. Cell extracts of DMSP-grown sulfate reducers were shown to possess rather high activities of an oxygen-labile enzyme (or enzyme system) that catalyzes methyl transfer from DMSP to THF, yielding MTPA and methyl-THF (Jansen and Hansen 1998). Methyl-THF is an intermediate in the oxidative acetyl CoA/CO dehydrogenase pathway (see, for example, Widdel and Hansen 1992); oxidation of the methyl group to CO_2 is thought to proceed via this pathway.

The objective of the work described here was the purification and characterization of the methyltransferase involved in the demethylation of DMSP in the sulfate-reducing bacterium strain WN. This is the first report on the purification of an enzyme that specifically catalyzes the demethylation of DMSP.

Material and methods

Organism, cultivation, cell extract preparation, and enzyme assay. Strain WN from our laboratory collection (Van der Maarel et al 1996b) was grown in 23-l glass vessels which, under a gas phase of N_2/CO_2 (80/20% v/v), contained 20 l medium 383 (without $CaCl_2$) as described in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (1993) Catalogue of Strains, supplemented with yeast extract (0.025%) and 7 mM DMSP. The vessels were inoculated (5% v/v) with cultures grown in similar media. After 3-4 days of incubation at 28 ^{0}C, cells were harvested by centrifugation (8000g at 4^0C) and washed twice in 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol, 21 g NaCl/l, and 3.0 g $MgCl_2 \cdot 6H_2O$/l. Cells were suspended in 10 mM potassium phosphate buffer without NaCl and $MgCl_2$. Cell extracts (10-30 mg protein/ml) were prepared using a French Pressure cell under anoxic conditions as described by Hensgens et al. (1993). The methyltransferase assays were performed according to Jansen and Hansen (1998). One unit of activity is the amount of enzyme that forms 1 µmol of CH_3-tetrahydrofolate per min.

Purification of the DMSP:THF methyltransferase. Throughout the purification an anaerobic chamber, equipped with a palladium catalyst (R020; BASF, Ludwigshafen, Germany) and containing an atmosphere of N_2:H_2 (approximately 95:5, v/v), was used to prevent rapid loss of activity which occurred with cell extracts under aerobic conditions (Jansen and Hansen 1998). Cell extract (50 ml) was loaded onto a Q-Sepharose XL (Pharmacia) column (16 by 2.6 cm; cooled to 4^0C) and equilibrated with 10 mM potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol at a flow rate of 3.0 ml/min. The column was eluted (3.0 ml/min) with a linear gradient of 0-0.5 M KCl (500 ml). Activity eluted between 250-350 mM KCl, and fractions with 250-310 mM KCl (56 ml) were pooled. The Q-Sepharose pool was subjected to 40% ammonium sulfate precipitation by slowly adding ammonium sulfate crystals under gentle stirring at 4 ^{0}C. After 45 min the suspension was anaerobically centrifuged (48 000*g*; 30 min at 4^0C) and the supernatant was applied to a Phenyl Sepharose CL-4B (Pharmacia) column (20 by 1.6 cm; 4^0C) equilibrated with 1 M ammonium sulfate. The column was eluted with a linear gradient of 1 to 0.4 M ammonium sulfate (100 ml) at a

flow rate of 1.5 ml/min and then with buffer lacking ammonium sulfate. Activity eluted from 75 mM to 0 mM ammonium sulfate and was pooled. This pool (7.5 ml) was applied to a hydroxyapatite (BioRad) column (10 by 1.6 cm; 4^0C; flow rate 1.0 ml/min). The column was eluted with a linear gradient (100ml; 10-500 mM potassium phosphate pH 7.2 containing 2 mM dithiothreitol). Activity eluted between 150-200 mM potassium phosphate.

Gel filtration chromatography. A Sephacryl S-300 HR (Pharmacia) column (1.6 by 30 cm) was used in an anaerobic chamber. Cell extract or Phenyl Sepharose CL-4B pool (1 ml; 4.9 and 0.39 mg protein, respectively) was loaded on the column (flow rate 1.0 ml/min) equilibrated with 10 mM potassium phosphate buffer (pH 7.2) plus 2 mM dithiothreitol. Molecular mass standards (BioRad) included thyroglobulin (670.0 kDa), gamma globulin (158.0 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa); they were run under the same conditions.

Analytical procedures. Tetrahydrofolate and methyltetrahydrofolate were separated by HPLC according to Stupperich and Konle (1993); the eluate was monitored at 280 nm. DMSP concentrations were determined as acrylate after conversion to dimethylsulfide and acrylate by overnight treatment with 1 M NaOH (White 1982). Acrylate and MTPA were analyzed by HPLC using a C18 column (Jansen and Hansen 1998). Protein was measured according to Bradford (1976) using the BioRad reagent, with bovine serum albumin as a standard. Denaturing polyacrylamide gel electrophoresis (12.5% acrylamide) was done according to Laemmli (1970) with 0.1% sodium dodecyl sulfate using the mini-Protean II electrophoresis system of BioRad. Molecular mass markers (BioRad) were myosin (200.0 kDa), β-galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). Gels were stained with 0.1 % Coomassie brilliant blue G-250.

Chemicals. DMSP was synthesized from acrylic acid and dimethylsulfide (Chambers et al 1987) or obtained from CASS (Groningen, The Netherlands). Dimethylsulfonioacetate, dimethylsulfoniobutanoate, dimethylsulfoniopentanoate, and methylethylsulfoniopropionate were synthesized by CASS (Groningen, The Netherlands). MTPA was obtained by alkaline hydrolysis of its methylester (Aldrich, Steinheim, Germany). 5,6,7,8-Tetrahydrofolic acid was from Sigma (St. Louis, USA) and Schircks Laboratories (Jona, Switserland), 5-methyl-5,6,7,8-tetrahydrofolic acid from Merck (Darmstadt, Germany). Trimethylsulfonium iodide and hydroxocobalamin were from Fluka (Buchs, Switzerland), cyanocobalamin, and coenzyme B_{12} from Sigma. Titanium(III)-nitrilotriacetic acid stock solutions were prepared after Moench and Zeikus (1983).

Results and discussion

Purification and stability of DMSP:THF methyltransferase. Purification of DMSP:tetrahydrofolate methyltransferase turned out to be rather difficult. In view of the oxygen lability of the activity in cell extracts (Jansen and Hansen 1998), all steps were carried out under anoxic conditions. Using a purification scheme involving anion-

exchange chromatography, ammonium sulfate precipitation, hydrophobic interaction chromatography, and hydroxyapatite chromatography, a 76-fold purification with a yield of 1% was obtained (Table 1). In one of the best purifications a 234-fold increase of the specific activity [142.9 μmol.min^{-1}(mg protein)$^{-1}$] at a 1% yield was achieved, but the activity was completely lost during overnight incubation under nitrogen at -20 ^{o}C. SDS polyacrylamide gel electrophoresis showed that one band with a molecular mass of approximately 35 kDa significantly increased in density during purification (Fig. 1). A band of 10 kDa was visible on SDS polyacrylamide gel electrophoresis in the hydroxyapatite pool. Minor contaminations, between 5-10% of the 10 μg total protein loaded onto the gel, were observed on SDS-polyacrylamide gel (12.5%). When the hydroxyapatite pool was loaded onto a 7.5% native polyacrylamide gel only one band was visible.

Although activity in cell extracts was stable for several weeks when stored at –20 ^{0}C, activity in partially purified samples was very unstable. Activity of the hydroxyapatite pool obtained in the experiment of Table 1 decreased in two days from 40.5 to 0.4 μmol.min^{-1}.(mg protein)$^{-1}$, when stored at -20 ^{0}C under nitrogen. This activity could be partially restored by addition of 200 μM of the B_{12}-derivatives cyanocobalamin, hydroxocobalamin, and coenzyme B_{12} to the assay mixture [activities increased to 2.7, 2.8, and 3.4 μmol.min^{-1}.(mg protein)$^{-1}$]. Titanium(III)nitrilotriacetic acid reduction of the assay mixture was not absolutely required; without titanium(III)nitrilotriacetic acid activities were approximately 10-20% lower. ATP (2 mM) plus $MgCl_2$ (8 mM) did not have any positive effect on activity when added to the assay mixture at any time. When cell extract (91 μg protein) with DMSP:THF methyltransferase activity was added to the assay mixture with the purified enzyme (preincubated for 5 min) no higher activities were obtained. Addition of cell extract to an inactive hydroxyapatite pool did not result in measurable activities. This indicates that the loss of activity is not caused by the lack of ATP or another compound which could be present in the cell extract. Different observations were made in studies of the methyl transfer from phenyl methyl ethers to THF in the acetogenic bacterium *Acetobacterium dehalogenans*. Upon purification of this *O*-demethylase, it turned out to be a multi-component system; already in the first chromatographic step (Q-Sepharose column) four distinct protein components were separated. All four proteins were required for the efficient catalysis of the methyl transfer from the substrate to tetrahydrofolate; additional requirements were ATP and low-potential reducing equivalents supplied by, for example, titanium(III) citrate (Kaufmann et al. 1997, 1998). Similarly, experiments with the tetramethylammonium:coenzyme M methyltransferase system from *Methanococcoides* sp. revealed a multi-component system with a corrinoid protein involved; in this case the presence of titanium(III) citrate and ATP was necessary (Asakawa at al. 1998).

Table 1 Partial purification of DMSP:THF methyltransferase from DMSP-grown strain WN

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Cell extract[a]	810	430	0.53	100	1.0
Q-Sepharose XL	71.9	260	3.62	61	6.8
40% AS[b]	50.8	224	4.4	52	8.3
Phenyl-Sepharose CL-4B	3.3	74	22.4	17	42.2
Hydroxyapatite	0.09	4	40.5	1	76.3

[a] From 40 l culture of DMSP-grown strain WN.
[b] Supernatant of 40% ammonium sulfate precipitation.

Properties of partially purified DMSP:THF methyltransferase. The optimum conditions for DMSP:THF methyltransferase activity were 35 ^{o}C and pH 7.8. At 5 ^{o}C the enzyme had only 20% of the activity; above 45 ^{o}C inactivation was considerable. The reaction had a broad pH optimum with still 85% of the activity present at pH 5.5 and 8.5. DMSP:THF methyltransferase is a highly specific enzyme; activity was observed only with DMSP and methylethylsulfoniopropionate (Table 2). Glycine betaine did not serve as methyl donor (Table 2), and did not inhibit the reaction with DMSP when the purified enzyme was preincubated for 5 min with glycine betaine (concentrations up to 100 mM tested). In contrast, in the case of mammalian glycine betaine:homocysteine methyltransferase a sulfonium analog such as DMSP could substitute for glycine betaine as methyl donor (Garrow, 1996). None of the DMSP-analogues (7.5 mM) listed in Table 2 inhibited the reaction with DMSP (preincubation with the enzyme for 15 min). Except for glycine betaine, strain WN was not able to utilize these compounds for growth (tested with 10 mM substrate, 0.025% yeast extract and a 5% inoculum of a DMSP-grown culture). Preincubation of the enzyme for 3 min with Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, or Zn^{2+} (2 mM each) resulted in relative activities of 89%, 80%, 85%, 87%, 100%, 76%, and 9%, respectively.

Table 2 Activity of DMSP:THF methyltransferase with other substrates

Substrate (7.5 mM)	Specific activity (U/mg protein)
DMSP	38.4
Trimethylsulfonium chloride	0.0- 0.8
Dimethylsulfonioacetate	0.0
Dimethylsulfoniopentanoate	2.8
Dimethylsulfoniobutanoate	3.2
Methylethylsulfoniopropionate	20.8
Glycine betaine	0.0

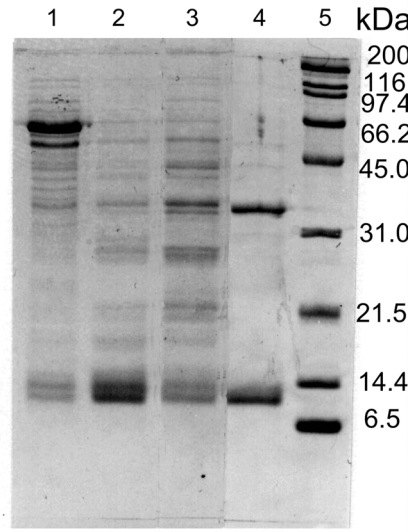

Fig. 1 SDS-polyacrylamide gel showing the four steps in the purification of DMSP:THF methyltransferase from strain WN. Lane 1, cell extract (9.0 µg); lane 2, Q-sepharose-pool (7.7 µg); lane 3, Phenyl-Sepharose CL-4B-pool (11.0 µg); lane 4, Hydroxyapatite-pool (11.0 µg); lane 5, molecular mass markers (BioRad): myosin (200.0 kDa), ß-galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). The gel was stained with 0.1% Coomassie brilliant blue R-250.

With cell extract of DMSP-grown strain WN we showed the inhibition (and reactivation with light) of DMSP:THF methyltransferase by propyl iodide (Jansen and Hansen 1998), which is indicative of involvement of a corrinoid as cofactor. Corrinoid proteins have been shown to play a role in methyl transfer reactions during the anaerobic degradation of various other *O*-, *S*-, or *N*-methylated compounds such as methanol, methoxylated aromatics, methylated sulfides, and methylated amines by Bacteria (Stupperich and Konle 1993; Kaufmann et al. 1997, 1998) and Archaea (Daas et al. 1996; Ferguson and Krzycki 1997; Sauer et al. 1997; Tallant and Krzycki 1997; Asakawa et al. 1998; Wassenaar et al. 1998). A corrinoid protein which is not involved in anaerobic degradation is the cobalamin-dependent methionine synthase which catalyzes the transfer of the methyl group from methyl-THF to homocysteine (see, for example, Frasca et al. 1988). Methionine synthesis from glycine betaine and homocysteine also involves the transfer of a methyl group but this reaction is not catalysed by a corrinoid protein (see, for example, Garrow 1996). Similarly, the protein that catalyzed the methyl transfer of trimethylsulfonium chloride to THF in an aerobic bacterium did not contain a corrinoid (Wagner et al. 1967).

With respect to the presence of a corrinoid in the DMSP:THF methyltransferase it can only be said at the moment that the strongest evidence in favour of such a presence is the effect of propyl iodide and light on the activity in cell extracts (see above) and the reactivation by cobalamins of inactivated enzyme after storage as discussed above. A reddish-brown color, indicative for a corrinoid as a cofactor, was never observed in the purified preparation. This lack of color is not unexpected because of the low protein concentration used in this experiment. More and more concentrated purified

protein will be required to obtain information about the presence of corrinoids. Since the purification of Table 1 with a yield of 90 µg of purified protein was based on 40 l of DMSP-grown culture, clearly very large culture volumes will be required to obtain milligram quantities of purified enzyme even if the maximal optical density of the cultures is considerably improved.

The native molecular mass of DMSP:THF methyltransferase was between 45 and 60 kDa, as judged by gel filtration chromatography. Almost all activity was lost when 1 ml of the Phenyl Sepharose CL-4B pool (0.39 mg protein) was loaded onto a Sephacryl S-300 HR column. The yield was only 1% and this remaining activity was completely lost within a few hours. The activity could not be restored by addition of 200 µM cyanocobalamin, hydroxocobalamin, or coenzyme B_{12} to the assay mixture. Attempts to restore activity using combinations of several fractions were not successful. Activity also decreased significantly when 1 ml of a cell extract of DMSP-grown strain WN was loaded onto the gel filtration column; only approximately 10% of the total activity was detected in the eluate. Again, addition of 200 µM cyanocobalamin, hydroxocobalamin, or coenzyme B_{12} to the assay mixture did not result in higher activities. The estimated molecular mass of the DMSP:THF methyltransferase calculated from the experiment with cell extracts was between 30 and 70 kDa. When PD-10 or HiTrap desalting columns were used, the activity decreased only 10-20%. It is difficult to draw conclusions about the subunit composition of the native DMSP:THF methyltransferase from these experiments in combination with the results of Fig. 1. Furthermore, what causes the inactivation when using gel filtration chromatography is not clear. Whether an activation mechanism exists for the DMSP:THF methyltransferase of strain WN remains to be established. Three different activation mechanisms have been reported for the methanol methyltransferase in the methanogen *Methanosarcina barkeri* (Daas 1996); two of these require or are stimulated by ATP (for example, van de Wijngaard et al. 1991; Daas et al. 1993). The other activation mechanism is ATP-independent, like the activation of the corrinoid/Fe-S protein involved in methyl transfer from acetylcoenzyme A to tetrahydrosarcinapterin (Grahame 1991, 1993).

This is the first report on a highly purified enzyme preparation that specifically catalyzes the demethylation of DMSP. Future efforts will be directed towards obtaining larger quantities of the purified protein to allow a more detailed characterisation.

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6

Methanogenic conversion of 3-*S*-methylmercaptopropionate* to 3-mercaptopropionate

Marc J.E.C. van der Maarel · Michael Jansen · Theo A. Hansen

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*3-*S*-Methylmercaptopropionate (MMPA) is the same compound as methylthiopropionate (MTPA) mentioned in the other chapters

Anaerobic metabolism of DMSP, an osmolyte of marine algae, in anoxic intertidal sediments involves either cleavage to dimethylsulfide or demethylation to methylmercaptopropionate (MMPA) and subsequently to mercaptopropionate. The methanogenic archaea *Methanosarcina* sp. strain MTP4 (DSM 6636), *Ms. acetivorans* DSM 2834, and *Ms. (Methanolobus) siciliae* DSM 3028 were found to use MMPA as a growth substrate and to convert it stoichiometrically to mercaptopropionate. Approximately 0.75 mol methane was formed per mol MMPA degraded; methanethiol was not detected as an intermediate. Eight other methanogenic strains did not carry out this conversion. We also studied the conversion of MMPA in anoxic marine sediment slurries. Addition of MMPA (500 μM) resulted in the production of methanethiol which was subsequently converted to methane (417 μM). In the presence of the antibiotics ampicillin, vancomycin and kanamycin (20 μg/ml each), 275 μM methane was formed from 380 μM MMPA; no MT was formed during these incubations. Only methanethiol was formed from MMPA when 2-bromoethanesulfonate (25 mM) was added to a sediment suspension. These results indicate that in natural environments MMPA could be directly or indirectly a substrate for methanogenic archaea.

Introduction

DMSP, an osmolyte found in many marine algae and certain plants (Reed 1983; Keller 1988; Gröne and Kirst 1991), is the major source of dimethylsulfide in the marine environment. In the atmosphere dimethylsulfide contributes through its oxidation products methanesulfonic acid and sulfuric acid to cloud formation and acid precipitation (Charlson et al. 1987). DMSP and dimethylsulfide are biologically converted in oxic as well as anoxic environments (Kiene et al. 1986; Kiene 1988; Kiene and Capone 1988; Kiene and Taylor 1988a and b; Taylor and Gilchrist 1991). The production of DMSP by benthic algae and the settling of dead algae cause the presence of DMSP in marine sediments. Relatively high concentrations of DMSP (from approx. 1 up to 70-110 μmol/l sediment) have been measured in the surface sediment of a salt marsh and other types of intertidal sediments (Kiene 1988; Visscher et al. 1994). In estuarine carbonate sediments DMSP is degraded by cleavage to dimethylsulfide and acrylate or by a demethylation to MMPA and subsequently to mercaptopropionate (Kiene and Taylor 1988a and b). MMPA is also degraded to methanethiol and presumably acrylate (Kiene and Taylor 1988a); acrylic acid and hydrogen sulfide can slowly react to form mercaptopropionate in a chemical process (Variavamurthy and Mopper 1987). Kiene and Taylor (1988a) suggested that a *Eubacterium limosum*-like organism might be responsible for the sequential demethylation of DMSP to mercaptopropionate. Thus far, no anaerobic microorganisms have been isolated that are able to demethylate DMSP all the way to mercaptopropionate. Anoxic most probable number incubations (in the presence of 5 mM NO_3^-) of mud from the upper 5 mm of a cyanobacterial mat indicated the presence of considerable populations of mercaptopropionate-producing microorganisms (Visscher et al. 1994), but the characteristics of the organisms are unknown. Recently, it was shown that the marine sulfate-reducing bacterium *Desulfobacterium* strain PM4 demethylates DMSP to MMPA (van der Maarel et al. 1993). This organism is not able

to demethylate MMPA to mercaptopropionate. In this communication we present data that show that some methanogenic archaea are able to demethylate MMPA to mercaptopropionate.

Materials and methods

Sediment sampling, preparation, and incubation. Anoxic intertidal sediment was collected from the Wadden Sea near Westernieland, The Netherlands. The sediment consisted of a black sulfide-rich layer covered by a 0- to 2 cm-thick oxic sandy layer. Sediment cores were taken using perspex cores (10-cm length; 2.5-cm diameter). After sampling the cores were sealed with butylrubber stoppers. The samples were transported in a N_2-flushed anaerobic jar at ambient temperature and suspensions were made in an anaerobic glove box (equipped with catalyst R0-20 of BASF Aktiengesellschaft, Ludwigshafen, Germany) within a few hours. Fresh sediments contained 40-60 µM DMSP when measured as dimethylsulfide by headspace analysis after alkalinization of the sample with NaOH (final concentration, 5 M).

The sediment was suspended in degassed seawater (approximately 4 ml/g of sediment [wet weight]). The suspension was thoroughly mixed with a blender for one min and 40-ml aliquots were poured into 70-ml bottles, while the sediment was kept in suspension. The bottles were sealed with a screw cap containing a butyl rubber stopper through the central hole; a Viton disc which is impermeable to volatile sulfur compounds was placed beneath the rubber stopper (Dr. J. Gerritse, personal communication). The headspace was flushed with oxygen-free N_2 for 2 min. Then, if required, the inhibitor 2-bromoethanesulfonic acid (final concentration 25 mM) or the antibiotics ampicillin, vancomycin, and kanamycin (20 µg/ml final concentration each) were added from aqueous stocks and the suspension was incubated at 25^{o}C overnight to remove remaining oxygen; then the incubations were started by addition of the substrate.

Microorganisms and growth conditions. An enrichment culture of MMPA-degrading microorganisms was obtained by inoculation of anoxic sediment (2.5-ml suspension) in bicarbonate-buffered (50 mM) mineral medium (Heijthuijsen and Hansen 1989) with sulfate (20 mM), yeast extract (50 mg/l), and MMPA (10 to 20 mM). Sulfate was omitted after several transfers into fresh medium. Incubations were done in 120-ml bottles filled with 50 ml medium at 28^{o}C.

Methanosarcina sp. strain MTP4 (DSM 6636) was grown in 120-ml bottles filled with 50 ml medium according to Finster et al. (1992) under an atmosphere of N_2-CO_2 (80/20) at 30 ^{o}C; inoculation (5%) was from late-log phase cultures. Strain MTP4 was isolated with methanethiol as a substrate by Finster et al. (1992) from sediment of salt marsh near Bordeaux, France. Growth was followed by measuring the optical density at 430 nm. The following strains were also used: *Ms. acetivorans* MS (DSM 2834) precultured on methanol (10 mM); *Ms.mazeii* C16 (DSM 3318; also known as '*Ms. frisia'* C16) precultured on methanol (25 mM); *Ms. siciliae* T4/M (DSM 3028; this strain was formerly designated *Methanolobus siciliae*; cf. Ni et al. 1994) precultured on methanol (25 mM); *Methanobacterium* sp. strain C8 (DSM 3821) precultured on H_2-CO_2 (80/20); *Methanococcoides methylutens* TMA-10 (DSM 2657) precultured on

trimethylamine (25 mM); *Methanohalophilus zhilinaeae* WeN5 (DSM 4017) precultured on trimethylamine (10 mM); *Methanospirillum hungateii* JF1 (DSM 864) precultured on H_2-CO_2 (80/20) and acetate (2.5 mM); *Ms. barkeri* Fusaro precultured on acetate (5 mM), and *Ms. barkeri* MS precultured on methanol (10 mM). *Ms. barkeri* strains Fusaro and MS were kindly provided by J.T. Keltjens, University of Nijmegen, The Netherlands. All of these strains were cultivated in the media as described in the Deutsche Sammlung von Mikroorganismen und Zellkulturen catalog of strains (1993, Braunschweig, Germany). *Methanococcoides* sp. strain PM2 (Culture collection of the Department of Microbiology, University of Groningen), precultured on methanol (10 mM), was cultivated in the medium of Heijthuijsen and Hansen (1989b). The strains were grown at 37°C except for the *Methanococcoides* strains (30°C), *Methanohalophilus zhilinaeae* (45°C) and *Methanobacterium* sp. Strain C8 (30°C).

Analyses. Headspace analyses of methane and methanethiol were performed by a slightly modified method of Visscher and van Gemerden (1991), in which a Supelpak S instead of a Porapak column was used. The concentration of methanethiol in the liquid phase was calculated using a distribution coefficient of 7.9 (Kiene and Capone 1988). In pure culture studies methane was measured by gas chromatography on a Porapak Q column with thermal conductivity detection (Heijthuijsen and Hansen 1989).

MMPA and mercaptopropionate were measured after esterification of 0.5 ml of sample (after centrifugation to remove cells or sediment) with methanol in sulfuric acid (50%) by the gas chromatographic method of Laanbroek et al. (1982) for analysis of lactate. Succinate was used as an internal standard. MMPA and mercaptopropionate were also analyzed by HPLC as described previously (van der Maarel et al. 1993).

Chemicals. MMPA was made by alkaline hydrolysis of its methyl ester (Aldrich, Steinheim, Germany) as described by Wackett et al. (1987). The identity and purity of the product was checked by ^{1}H-NMR; the MMPA content was estimated by organic carbon analysis. Mercaptopropionate was obtained from Aldrich (Steinheim, Germany).

Results

Enrichment culture. After inoculation of mineral medium containing MMPA (20 mM) and yeast extract (50 mg/l) with anoxic marine sediment, MMPA was converted to methane, with methanethiol as an intermediate. After several transfers into fresh medium MMPA was still converted to methane, also when sulfate was omitted from the medium. The enrichment culture that was obtained in this way produced 14 mM methane from 20 mM MMPA. No methanethiol or acrylate could be detected. Epifluorescence microscopy showed large numbers of irregular coccoid cells which had a characteristic fluorescence at 420 nm. After treatment with the antibiotics ampicillin, vancomycin, and kanamycin (20 µg/ml final concentration each), which all act against bacteria but not against archaea, MMPA was still converted to methane and mercaptopropionate. When 2-bromoethanesulfonic acid (25 mM), a specific inhibitor of methanogenesis (Sparling and Daniels 1987), was added, no MMPA was converted to mercaptopropionate and methane. These observations made us speculate that methanogenic archaea present in the enrichment culture might have directly converted

MMPA to mercaptopropionate and methane. Because of the morphological similarity of the methanogens present in the enrichment culture to coccoid *Methanosarcina* strains and the ability of *Methanosarcina* sp. strain MTP4 to metabolize methanethiol (Finster et al. 1992), a possible intermediate of MMPA degradation, we tested strain MTP4 for the ability to convert MMPA to mercaptopropionate and methane.

Conversion of MMPA by pure cultures of methanogenic archaea. *Methanosarcina* sp. strain MTP4 was found to be able to grow with MMPA as a substrate. A lag phase of approximately 7 days was observed when a methanol-grown culture was transferred to medium containing MMPA as a substrate. Transfer of a MMPA-grown culture to fresh medium with methanol or MMPA as a substrate gave no significant lag phase. During growth, MMPA was converted to mercaptopropionate and methane (Fig. 1A). The specific growth rate was 0.033 h^{-1} (t_d=21 h), based on the exponential production of methane between 51 and 119 hours. In a separate experiment, the conversion stoichiometry was determined; from 13.5 mM MMPA 13.5 mM mercaptopropionate and 10.2 µmol methane per ml medium were formed. The conversion corresponds to the following equation: 4 MMPA + 2 H_2O $\rightarrow$ 4 mercaptopropionate + CO_2 + 3 CH_4. The identity of the organic compound formed after growth of strain MTP4 on MMPA was established to be mercaptopropionate by ^{3}H-nuclear magnetic resonance and cochromatography (HPLC and gas chromatography) with authentic mercaptopropionate as a reference (data not shown).

Ten other methanogenic strains were tested for the ability to grow on MMPA. Only *Ms. acetivorans* DSM 2834 (Fig. 1B) and *Ms. siciliae* DSM 3028 (data not shown) were found to be able to grow on MMPA; the latter strain grew more slowly than strain MTP4 and *Ms. acetivorans*. *Ms. mazeii* DSM 3318, *Methanobacterium* sp. strain C8 DSM 3821, *Methanococcoides methylutens* DSM 2657, *Methanohalophilus zhilinaeae* DSM 4017, *Methanospirillum hungateii* DSM 864, *Ms. barkeri* strain Fusaro, *Ms. barkeri* strain MS, and *Methanococcoides* sp. strain PM2 did not grow with MMPA as a single substrate and did not convert MMPA during growth on their regular substrate (see Materials and Methods). The MMPA-utilizing methanogenic strains were unable to convert DMSP.

Conversion of MMPA in sediment suspensions. Methanethiol was formed within one day after addition of MMPA (500 µM) to a sediment suspension (Fig. 2A). The maximum concentration of methanethiol was approximately 200 µM; methanethiol started to decrease after two days. No methanethiol could be detected after 5 days. Methane formation started in the same period and reached its maximum after 5 days (417 µM). No methanethiol was formed in the presence of the antibiotics ampicillin, vancomycin, and kanamycin (Fig. 2B). Methane formation in these incubations was much slower than in the incubations without additions. The concentration of MMPA decreased at a rate similar to the increase in the concentration of methane. Methanethiol but not methane was formed when 2-bromoethanesulfonic acid (25 mM) was added to the suspension.

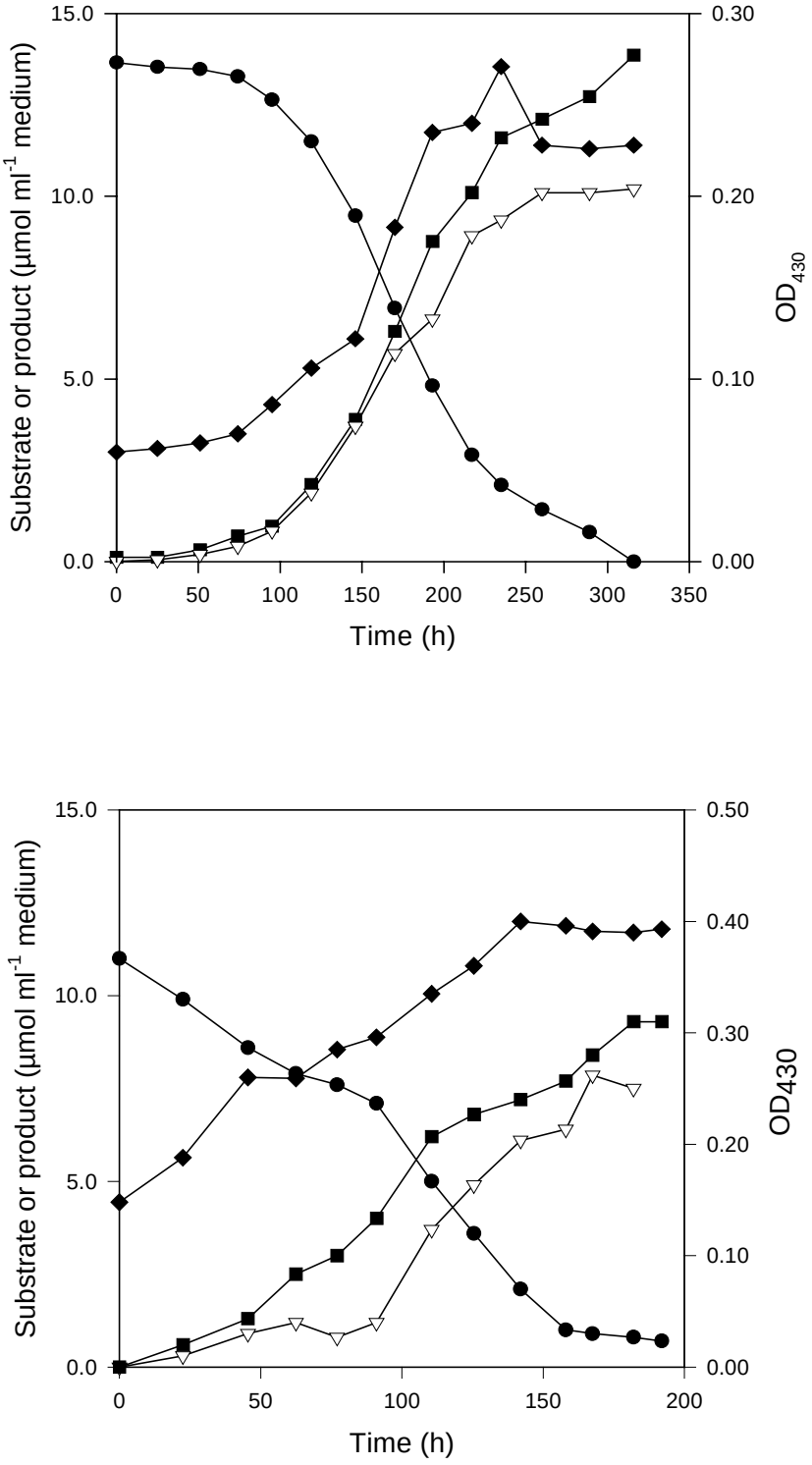

Fig. 1. (A) Growth of *Methanosarcina* sp. strain MTP4 on MMPA (13.5 mM). (B) Growth of *Ms. acetivorans* DSM 2834 on MMPA (10 mM). Symbols: ♦, optical density at 430 nm (OD_{430}); ∇, methane; ●, MMPA; ■, mercaptopropionate. Cultures were grown in 120-ml crimp-seal bottles with 50 ml medium under an atmosphere of N_2-CO_2 (80/20 [vol/vol]). The methane line indicates the total amounts present in both the gas and liquid.

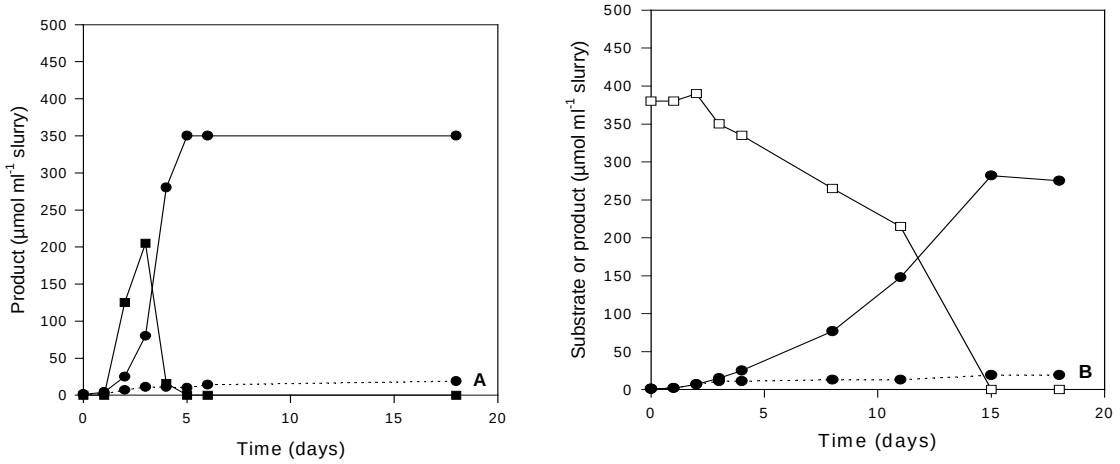

Fig. 3 (A) Accumulation of methanethiol (■) and methane (●) in a sediment suspension after the addition of MMPA (500 µM). (B) Accumulation of methane (●) in a sediment suspension after the addition of MMPA (□) and antibiotics; no methanethiol was detected in the presence of antibiotics. Dotted lines are controls without MMPA addition. Sediment slurries (40 ml) were incubated in 70-ml crimp-seal bottles under a nitrogen atmosphere. Methane and methanethiol are the total amounts present in both the gas and liquid.

Discussion

This is the first report in which it is shown that a pure culture of a methanogenic archaeon can utilize MMPA as a substrate for growth. It adds to the limited number of compounds that are known as methanogenic substrates or electron donors for methanogenesis: H_2-CO_2, formate, CO, methanol, acetate, tri-, di-, and monomethylamine, dimethylsulfide, methanethiol, 1-propanol, 2-propanol, ethanol, 1-butanol, 2-butanol, 1,3-butanediol, cyclopentanol, and pyruvate (O'Brien et al. 1984; Oremland et al. 1989; Whitman et al. 1992; Finster et al. 1992; Bock et al. 1994; Rajagopal and LeGall 1994). The most important methanogenic substrates usually are H_2-CO_2 and acetate but in marine environments methylated compounds such as trimethylamine and dimethylsulfide are thought to predominate (Whitman et al. 1992). *Methanosarcina* strain MTP4 utilizes MMPA as a typical C_1-substrate and demethylates it to mercaptopropionate. *Methanosarcina* strain MTP4 was isolated from a salt marsh with methanethiol as the enrichment substrate (Finster et al. 1992); it can also grow on dimethylsulfide. Dimethylsulfide and methanethiol can be formed from methoxylated aromatics (Finster et al. 1990), but in the marine environment DMSP is most probably the major source of dimethylsulfide. DMSP is also a precursor of MMPA as suggested by sediment slurry experiments (Kiene and Taylor 1988a and b) and shown in pure culture studies with *Desulfobacterium* PM4 (van der Maarel et al. 1993). Thus, strain MTP4 originates from an environment where both dimethylsulfide and MMPA are present. Similarly, *Ms. acetivorans* DSM 2834 was isolated from marine sediment and is now known to metabolize both dimethylsulfide (Ni et al. 1994) and MMPA (this study). *Ms. siciliae* DSM 3028 was shown to be closely related to *Ms. acetivorans* (Ni et al. 1994).

The biochemical mechanism of MMPA demethylation by methanogens is still obscure. Wackett et al. (1987) showed that in crude cell extracts of H_2-CO_2-grown *Methanobacterium thermoautotrophicum* ΔH MMPA, which is a structural analogue of methyl-*S*-coenzyme M, can serve as a substrate for the methyl-*S*-coenzyme M

reductase, an enzyme involved in the last step of methanogenesis (Ferry 1992). It is therefore possible that strain MTP4 is able to take up MMPA and use the methyl-S-coenzyme M reductase to convert MMPA. However, out of the eleven methanogenic strains tested only *Methanosarcina* sp. strain MTP4, *Ms. acetivorans* DSM 2834 and *Ms. siciliae* DSM 3028 were able to convert MMPA. It is therefore not very likely that MMPA utilization by strain MTP4, strain DSM 2834, and strain DSM 3028 is due to a general lack of specificity of the methyl-S-coenzyme M reductase. Alternatively, a MMPA:coenzyme M methyl transferase system might be used in the conversion of MMPA. Specific methyltransferases are known to be involved in the metabolism of methanol and methylamines, respectively (Ferry 1992).

The methanogenic conversion of MMPA to mercaptopropionate has also been found to occur in slurries prepared from anoxic marine sediments, but only when antibiotics were added. Under normal conditions, MMPA was readily converted to methanethiol and presumably acrylate. Methanethiol is further converted to methane by methanogenic archaea. These results suggest that in situ MMPA can serve as a substrate for methanogenic archaea but the major pathway for conversion might be demethiolation; it should be kept in mind, however, that at the low natural concentrations of MMPA the ratio between demethylation and demethiolation may be very different. Kiene and coworkers (Kiene and Taylor 1988a; Kiene et al. 1990) concluded that demethylation is a major transformation pathway for MMPA in intertidal sediments. They suggested that *Eubacterium limosum*-like bacteria might be responsible for the sequential demethylation of DMSP. Thus far, acetogenic bacteria that can demethylate DMSP have not been isolated. The combined activities of DMSP-demethylating, sulfate-reducing bacteria (van der Maarel et al. 1993) and MMPA-demethylating methanogenic archaea may also be responsible for the observed conversion of DMSP to mercaptopropionate.

Cleavage of DMSP results in the formation of dimethylsulfide. Part of the dimethylsulfide escapes to the atmosphere, where it is oxidized to sulfuric acid and methanesulfonic acid (Charlson et al. 1987). These compounds may act as cloud condensation nuclei, and thus dimethylsulfide may exert a negative effect on global warming. Anaerobic metabolism of dimethylsulfide results in the formation of methane (Kiene et al. 1986; Finster et al. 1992), which can act as a greenhouse gas (Hogan et al. 1991), although part of the dimethylsulfide might be oxidized to CO_2 by sulfate-reducing bacteria (Kiene et al. 1986). Demethylation as well as demethiolation of MMPA can also result in the direct or indirect formation of methane. Part of the methane that is formed in the anoxic sediment can be oxidized in the upper oxic layer by methane-oxidizing bacteria but methane fluxes from salt marshes into the atmosphere have been found to exist (Senior et al. 1982). Therefore, we conclude that anaerobic demethylation of DMSP results in the production of a positive effector (methane) of global warming, whereas the cleavage into dimethylsulfide and acrylate leads to both a positive (methane) and a negative (dimethylsulfide) effector.

Acknowledgements

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7

Concluding remarks/ Summary/ Samenvatting

Michael Jansen

Concluding remarks

In this thesis the microbial demethylation of dimethylsulfoniopropionate (DMSP) was investigated. This involved characterization of the organisms catalyzing the demethylation reactions (aerobic α-Proteobacteria, acetogenic bacteria and methanogenic Archaea). At the start of this project, a few aerobic bacteria and certain marine sulfate-reducing bacteria able to demethylate DMSP were known. In the work described here we show that acetogenic bacteria can demethylate DMSP to methylthiopropionate (MTPA) and that methanogenic archaea are able to demethylate MTPA to mercaptopropionate. Also the DMSP:tetrahydrofolate methyltransferase enzyme responsible for the demethylation reaction of DMSP to MTPA in a marine sulfate-reducing bacterium was characterized.

The importance of marine α-Proteobacteria, like strain WNA2 (chapter 2), in the degradation of DMSP was shown by Gonzalez and coworkers. The presence of α-Proteobacteria is abundant in seawater samples off the coast of the southeastern United States (Gonzalez and Moran 1997). Whether other aerobic DMSP-demethylating strains such as DG-C1 (Taylor and Gilchrist 1991), BIS-6 (Visscher and Taylor 1994), and MM-P (Diaz and Taylor 1996) belong to the α-Proteobacteria remains to be established. Most probably, DMSP demethylation is not restricted to this group. The marine bacterium *Alteromonas macleodii*, a member of the γ-subclass of Proteobacteria (Gauthier et al. 1995), was able to produce methanethiol from DMSP (Ledyard et al. 1993), but whether this proceeds via an initial demethylation to MTPA or via the cleavage pathway to dimethylsulfide is not known.

The biochemistry of the DMSP demethylation reaction requires further studies. In future, efforts should be directed towards obtaining larger quantities of the purified protein from the sulfate reducer strain WN, to allow a more detailed characterization. Several cobalt containing methyltransferases were isolated over the years from methanogenic archaea and acetogenic bacteria; once a preparation of more purified enzyme has been obtained it will be possible to see whether cobalt is present in DMSP:tetrahydrofolate methyltransferase in strain WN. Furthermore, the gene sequence of the DMSP:tetrahydrofolate methyltransferase is of interest. Comparison of the gene sequence of the enzyme with other methyltransferases will show if it is closely related to any other type of methyltransferase.

Almost nothing is known about the demethylation of DMSP in aerobic bacteria. The strain isolated in chapter 2, strain WNA2, was unable to use tetrahydrofolate as a methylacceptor in the demethylation of DMSP. Other enzymes that might catalyze the demethylation of DMSP are described in chapter 1. A DMSP-oxidase, oxygenase, dehydrogenase or a methyltransferase with an acceptor other than tetrahydrofolate might catalyze the demethylation of DMSP, but future studies are necessary to see what enzyme is responsible for this reaction.

Whether the enzyme (or enzyme system) responsible for the demethylation of DMSP in the acetogenic bacteria described in chapter 3 is a methyltransferase is not known at this moment, but appears very likely. In analogy with the biochemistry of the metabolism of other methylated substrates in acetogens (methanol, methoxylated aromatics; see Kaufman et al. 1997, 1998; Stupperich and Konle 1993), one would expect the presence of a DMSP- and a glycine betaine demethylating enzyme which

would feed the methyl group into the methyl branch of the Wood-Ljungdahl (reductive CO dehydrogenase) pathway for acetyl-CoA synthesis. Purification of the *O*-demethylase from *Acetobacterium dehalogenans* showed that this demethylase consists of four components that were all required for the efficient catalysis of the methyl transfer from phenyl methyl ethers to tetrahydrofolate (Kaufman et al. 1997, 1998). Purification of the DMSP- and betaine-demethylating system(s) will be necessary to reveal whether these systems are indeed identical and of a similar complexity as the *O*-demethylating system. There are some examples in the literature that show that DMSP can indeed be a substrate (and sometimes with higher activities!) for a betaine utilizing enzyme, but this is not a general rule. Mammalian betaine:homocysteine methyltransferase is known to be active towards DMSP (Garrow 1996). In extracts of *Pseudomonas denitrificans* betaine and dimethylsulfonioacetate can function as methyl donors for homocysteine methylation whereas DMSP cannot (White et al. 1973).

The biochemical mechanism of MTPA demethylation was still unknown when this project started; in chapter 6 it is speculated that an MTPA:coenzyme M methyltransferase might be catalyzing this reaction in methanogens. After completion of the work described in chapter 6, it was shown by Tallant and Krzycki (1997) that a methylthiol:coenzyme M methyltransferase was responsible for the demethylation of MTPA. This 480-kDa corrinoid protein also catalyzed the demethylation of dimethylsulfide to methanethiol. Whether this methyltransferase is present in all the methanogens growing on MTPA is not known at this moment.

Summary

As discussed in chapter 1, there is an increased interest in the production of certain natural sulfur-containing flavor compounds or flavor precursors. Production of natural flavors is becoming increasingly important, because consumers tend to prefer natural compounds for health reasons. With the aid of extraction techniques it is possible to obtain flavors directly from plant material, but these methods are time consuming and expensive, because the most interesting flavors are present in only very low concentrations. A more recent method to produce flavors is based on a biotechnological approach where natural precursors, isolated mainly from plant material, can be converted to the desired flavor in a bioreactor with the aid of enzymes and/or microorganisms. The algal osmolyte dimethylsulfoniopropionate (DMSP) is a potential substrate for the production of naturally occurring sulfur-containing flavors. Demethylation of DMSP results in the formation of methylthiopropionate (MTPA). MTPA can be demethylated further to mercaptopropionate. Ethyl- or methyl esters of these compounds can be used as flavors. At the start of the thesis project basically two types of DMSP-demethylating microorganisms were known: marine aerobic microorganisms and marine sulfate-reducing bacteria. These organisms, or other still unknown strains, could be of interest for the production of the sulfur-containing flavors. The organisms used in this study were isolated from environments where the presence of DMSP is very likely; also potential candidates obtained from culture collections were screened for their ability to demethylate DMSP to MTPA/mercaptopropionate or MTPA to mercaptopropionate. Furthermore, detailed studies were carried out to gain more insight in the biochemical aspects of the demethylation of DMSP.

Aerobic demethylation of DMSP. An aerobic rod-shaped bacterium, strain WNA2, was obtained from enrichments with DMSP inoculated with a 10^6-fold diluted slurry of intertidal mud and water from the Wadden Sea (chapter 2). The enrichment cultures were screened for the presence of MTPA or mercaptopropionate in the supernatant. Strain WNA2 was found to convert DMSP to substoichiometric concentrations of MTPA, which was excreted in the culture medium. The MTPA was degraded further with a transient accumulation of methanethiol and with dimethyldisulfide as the sulfur-containing end-product. The sequence of its 16S rRNA encoding gene identified strain WNA2 as a member of the α-subclass of the Proteobacteria with *Ruegeria* and *Roseobacter* species as closest relatives. Strain WNA2 is a new species designated *Ruegeria frisia*.

Anaerobic demethylation of DMSP. Stoichiometric demethylation of DMSP to MTPA is possible with the aid of certain marine sulfate-reducing bacteria (van der Maarel et al. 1996b). Preliminary evidence that demethylation of DMSP to MTPA can be catalyzed by an acetogen was obtained in our laboratory (van der Maarel et al. 1996b). Therefore, the anaerobic demethylation of DMSP by acetogenic bacteria was studied in more detail (chapter 3). Acetogenic bacteria are obligate anaerobes that synthesize acetyl-CoA from C_1-compounds for both conservation of energy and growth. Furthermore, several acetogens show good growth on an N-containing structural analog of DMSP, glycine betaine. Eight acetogens, mostly originating from non-marine environments were shown to demethylate DMSP stoichiometrically to

MTPA. The acetogenic fermentation based on this demethylation did not result in any significant increase in biomass. Demethylation of glycine betaine to dimethylglycine does support growth of the tested acetogens. In batch cultures of *Eubacterium limosum* strain PM31 DMSP and glycine betaine were demethylated simultaneously. In mixed substrates experiments with fructose/DMSP or methanol/DMSP, DMSP was used rapidly but only after exhaustion of the fructose or the methanol. In steady-state fructose-limited chemostat cultures (at a dilution rate of 0.03 h^{-1}) with DMSP as a second reservoir substrate, DMSP was biotransformed to MTPA but this did not result in higher biomass values than in cultures without DMSP. Cells from fructose-limited chemostat cultures demethylated DMSP at rates of approximately 50 nmol min^{-1} mg^{-1} protein, both after growth in the presence of DMSP and after growth in its absence. In cell extracts of glycine betaine-grown strain PM31, DMSP demethylation activities of 20-25 nmol min^{-1} mg^{-1} protein were detected with tetrahydrofolate as methyl acceptor; activities with glycine betaine were approximately tenfold lower.

Biochemical aspects of DMSP demethylation. Until a few years ago there was a complete lack of knowledge concerning the biochemistry of bacterial demethylation of DMSP. In chapters 4 and 5 the results of the first successful study into the nature of the DMSP-demethylating reaction in a bacterium, namely the sulfate-reducing bacterium strain WN, are described. In cell extracts of strain WN grown on DMSP, tetrahydrofolate functioned as a methyl acceptor in the anaerobic demethylation of DMSP to MTPA. DMSP-dependent activities were 0.5-0.6 µmol methyltetrahydrofolate produced min^{-1} mg^{-1} protein; preincubation of the extract under air significantly decreased the activity (35% activity loss in 3 h). The reaction did not require ATP or reductive activation by titanium(III)-nitrilotriacetic acid. Three other DMSP-demethylating sulfate reducers, *Desulfobacterium niacini*, *Db. vacuolatum*, and *Db.* strain PM4, had DMSP:tetrahydrofolate methyltransferase activities of 0.16, 0.05, and 0.24 µmol min^{-1} mg^{-1} protein, respectively. No methyltransferase activity to tetrahydrofolate was found with glycine betaine as a substrate, not even in extracts of glycine betaine-grown cells of these sulfate reducers. DMSP demethylation in cell extracts of strain WN was completely inhibited by 0.5 mM propyl iodide; in the light, the inhibition was far less strong, indicating involvement of a corrinoid-dependent methyltransferase. The data obtained indicated that a purification of DMSP:tetrahydrofolate methyltransferase might be feasible.

Chapter 5 describes the purification of an enzyme that specifically catalyzes the demethylation of DMSP. The enzyme was isolated from the marine sulfate-reducing bacterium strain WN. DMSP:tetrahydrofolate methyltransferase from strain WN was purified 76-fold [to a specific activity of 40.5 µmol.min^{-1}mg $protein^{-1}$]. SDS polyacrylamide gel electrophoresis showed two bands of approximately 10 and 35 kDa; in particular the 35 kDa polypeptide became significantly enriched during the purification. The methyltransferase activity was extremely unstable; storage of the purified fraction at -20 ^{o}C under nitrogen resulted in a 99 % loss of activity in two days. The activity could be partially restored by addition of 200 µM cyanocobalamin, hydroxocobalamin or coenzyme B_{12}. ATP did not have any positive effect on activity. Reduction of the assay mixture by titanium(III)-nitrilotriacetic acid slightly stimulated the activity. Gel filtration chromatography revealed a native molecular mass between 45 and 60 kDa for the DMSP:tetrahydrofolate methyltransferase. The optimum

conditions for the enzyme activity were 35 ^{o}C and pH 7.8. Glycine betaine did not serve as a methyl donor for DMSP:tetrahydrofolate methyltransferase. Various other sulfur-containing DMSP-analogs were tested but only methylethylsulfoniopropionate served as a methyl donor. None of these compounds inhibited the methyl transfer from DMSP to tetrahydrofolate. Strain WN did not grow on any of the sulfur-containing DMSP-analogs tested.

Demethylation of MTPA to mercaptopropionate by Archaea. Demethylation of MTPA to mercaptopropionate has been demonstrated to occur in aerobic microorganisms; whether anaerobic microorganisms are able to catalyze this reaction was not known. The methanogenic archaea *Methanosarcina* sp. strain MTP4, *Ms. acetivorans* and *Ms. siciliae* were found to use MTPA as a growth substrate and to convert it stoichiometrically to mercaptopropionate (chapter 6). Approximately 0.75 mol methane was formed per mol MTPA degraded; methanethiol was not detected as an intermediate. Eight other methanogenic strains did not carry out this conversion. Addition of MTPA (500 µM) to anoxic marine sediment slurries resulted in the production of methanethiol which was subsequently converted to methane (417 µM). In the presence of the antibiotics ampicillin, vancomycin and kanamycin (20 µg/ml each), methane was also formed from MTPA, but methanethiol could not be detected during these incubations. When 2-bromoethanesulfonate (25 mM), a specific methanogenic inihibitor, was added to a sediment suspension with MTPA, methanethiol formation was observed. These results indicate that in natural environments MTPA could directly or indirectly be a substrate for methanogenic archaea.

Samenvatting

Microbiële demethylering van dimethylsulfoniopropionaat en methylthiopropionaat

In toenemende mate bestaat er interesse om verschillende natuurlijke zwavelbevattende smaakstoffen of smaakstofprecursors te produceren. De productie van natuurlijke smaakstoffen wordt steeds belangrijker nu de consument van tegenwoordig producten verlangt met natuurlijke smaakstoffen, omdat deze gezonder zouden zijn. Met behulp van extractietechnieken is het mogelijk om de smaakstoffen direct uit plantenmateriaal te verkrijgen. Een nadeel van deze techniek is dat deze veel tijd kost en duur is, omdat de smaakstoffen vaak in een zeer lage concentratie voorkomen.

Een andere, meer recentelijk gebruikte methode is gebaseerd op een biotechnologische aanpak, waarbij natuurlijke precursors, voornamelijk geïsoleerd uit plantenmateriaal, in een bioreactor omgezet kunnen worden in de benodigde smaakstof. Dit kan worden bereikt met behulp van enzymen en/of micro-organismen. In veel mariene algen komt dimethylsulfoniopropionaat (DMSP) voor. Deze verbinding zou als substraat kunnen dienen voor de natuurlijke productie van zwavelbevattende verbindingen. Als DMSP wordt gedemethyleerd resulteert dat in de vorming van methylthiopropionaat (MTPA). MTPA op zijn beurt kan ook gedemethyleerd worden tot mercaptopropionaat. Ethyl- of methyl esters van deze verbindingen kunnen worden gebruikt als smaakstoffen. In het begin van dit project waren twee typen DMSP-demethylerende bacteriën bekend: mariene, aërobe bacteriën en een aantal mariene, sulfaatreducerende bacteriën. Deze organismen, of nog onbekende micro-organismen, zouden interessant kunnen zijn om zwavelbevattende smaakstoffen te produceren. De organismen die in deze studie zijn gebruikt zijn geïsoleerd uit milieus waar het zeer waarschijnlijk is dat DMSP aanwezig is; ook zijn geselecteerde organismen uit cultuurcollecties getest of deze in staat zouden zijn om DMSP of MTPA te demethyleren. Verder is er onderzoek gedaan naar de biochemische aspecten van de demethylering van DMSP.

Aërobe demethylering van DMSP. Een aëroob staafvormig isolaat, stam WNA2, is verkregen uit verrijkingscultures beënt met sedimentslurries uit de Waddenzee en DMSP als substraat (hoofdstuk 2). De supernatanten van de verrijkingscultures werden doorgemeten op de mogelijke vorming van MTPA of mercaptopropionaat. Stam WNA2 demethyleert DMSP naar substoichiometrische concentraties MTPA. De MTPA werd uitgescheiden in het kweekmedium en werd vervolgens verder afgebroken met methaanthiol als tussenproduct en dimethyldisulfide als zwavelbevattend eindproduct. De sequentie van het 16S rRNA gen van stam WNA2 liet zien dat het organisme behoort tot de α-subklasse van de Proteobacteriën met *Ruegeria* en *Roseobacter* soorten als meest nauwverwante organismen.

Anaërobe demethylering van DMSP. Stoichiometrische demethylering van DMSP naar MTPA is mogelijk met behulp van sommige mariene sulfaatreducerende bacteriën. Het eerste bewijs dat de demethylering van DMSP naar MTPA gekatalyseerd kan worden door een acetogeen is verkregen in ons laboratorium. In dit proefschrift is deze anaërobe demethylering van DMSP door acetogene bacteriën in

meer detail bestudeerd (hoofdstuk 3). Acetogene bacteriën zijn anaërobe micro-organismen die acetyl-CoA uit C_1-verbindingen synthetiseren voor conservering van energie en groei. Verder zijn een aantal van deze organismen in staat om goed te groeien op een stikstofbevattende, structureel analoge verbinding van DMSP, glycine betaine. Acht acetogenen, voornamelijk geïsoleerd uit niet-mariene milieus, bleken in staat te zijn om DMSP stoichiometrisch om te zetten naar MTPA. De fermentatie van DMSP resulteerde niet in een significante toename van de biomassa. Demethylering van glycine betaine naar dimethylglycine daarentegen, liet wel een belangrijke toename in biomassa zien. In batch cultures van *Eubacterium limosum* stam PM31, werden DMSP en glycine betaine gelijktijdig gedemethyleerd. In experimenten uitgevoerd met fructose/DMSP of methanol/DMSP werd DMSP snel verbruikt, maar alleen nadat de fructose of methanol compleet was verbruikt. In steady state van fructose-gelimiteerde chemostaat cultures (met een verdunningssnelheid van 0,03 per uur), met in het voorraadvat DMSP als tweede substraat, werd DMSP omgezet naar MTPA zonder dat dit een hogere biomassa opleverde in vergelijking met cultures zonder DMSP. Cellen van fructose-gelimiteerde chemostaat cultures demethyleerden DMSP met een snelheid van ongeveer met 50 nmol per minuut per mg eiwit, zowel na groei in aanwezigheid als in afwezigheid van DMSP. In extracten van stam PM31, gekweekt op glycine betaine, werden DMSP demethylerende activiteiten gemeten van 20-25 nmol per minuut per mg eiwit. Tetrahydrofolaat diende als methylacceptor in deze reactie; activiteiten met glycine betaine waren een factor 10 lager.

Biochemische aspecten van de DMSP demethylering. Tot een aantal jaren geleden was er niets bekend over de biochemie van de bacteriële demethylering van DMSP. In hoofdstukken 4 en 5 worden de resultaten van de eerste succesvolle studie beschreven naar de DMSP-demethylerende reactie in een sulfaatreducerende bacterie, stam WN. In extracten van stam WN, gekweekt op DMSP, functioneerde tetrahydrofolaat als methylacceptor in de anaërobe demethylering van DMSP naar MTPA. De DMSP-afhankelijke activiteiten waren 0,5-0,6 µmol methyltetrahydrofolaat gevormd per minuut per mg eiwit. Wanneer extracten aan lucht werden blootgesteld, vond een significante afname van de activiteit plaats (35% activiteitsverlies in 3 uur). De reactie vereiste geen ATP of reductieve activering door titanium(III)-nitrilotriazijnzuur. DMSP:tetrahydrofolaat methyltransferase activiteiten van drie andere DMSP-demethylerende sulfaat reduceerders, *Desulfobacterium niacini*, *Db*. *vacuolatum*, en *Db*. stam PM4 waren respectievelijk 0,16, 0,05, en 0,24 µmol per minuut per mg eiwit. Met glycine betaine als substraat kon geen methyltransferase activiteit worden gemeten, zelfs niet in extracten van op glycine betaine gekweekte cellen van bovengenoemde organismen. De demethylering van DMSP in extracten van stam WN werd compleet geremd door 0,5 mM propyl iodide. In het licht was de remming veel minder sterk, wat duidt op de betrokkenheid van een corrinoid-afhankelijke methyltransferase. De verkregen gegevens lieten zien dat het waarschijnlijk mogelijk zou zijn om de DMSP:tetrahydrofolaat methyltransferase te zuiveren.

De zuivering van dit enzym, dat specifiek DMSP demethyleert, is beschreven in hoofdstuk 5. Het enzym werd geïsoleerd uit de mariene sulfaatreducerende bacterie stam WN. DMSP:tetrahydrofolaat methyltransferase van stam WN werd 76 keer opgezuiverd. SDS-polyacrylamide-gelelectroforese resulteerde in twee banden van

ongeveer 10 en 35 kDa. Vooral het polypeptide van 35 kDa werd gedurende de zuivering verrijkt. De methyltransferase activiteit was extreem onstabiel; 99% van de activiteit was verdwenen na twee dagen incubatie bij –20 ^{o}C onder een atmosfeer van stikstof. De activiteit kon gedeeltelijk hersteld worden door toevoeging van 200 µM cyanocobalamine, hydroxocobalamine, of co-enzyme B_{12}. Verder had ATP geen positief effect op de activiteit. Reductie van het analyse-mengsel met titanium(III)-nitrilotriazijnzuur resulteerde in een kleine verhoging van de activiteit. Met behulp van gelfiltratie chromatografie is een schatting gemaakt van de natieve moleculaire massa van de methyltransferase. Deze bleek tussen de 45 en 60 kDa te liggen. De optimale condities voor enzymactiviteit waren 35^{o}C en een pH van 7,8. Glycine betaïne kon niet als methyldonor dienen voor de methyltransferase. Verschillende andere zwavelbevattende DMSP analogen zijn getest, maar alleen methylethylsulfoniopropionaat diende als methyldonor. Geen van deze verbindingen had een remmende werking op de methyl transfer van DMSP naar tetrahydrofolaat. Stam WN groeide op geen van de geteste zwavelbevattende DMSP analogen.

Demethylering van MTPA naar mercaptopropionaat door Archaea. Enkele aërobe mirco-organismen die in staat zijn om MTPA te demethyleren naar mercaptopropionaat waren bekend. Maar niet bekend was of anaërobe organismen ook in staat zouden zijn om deze reactie uit te voeren. Een drietal methanogene Archaea, *Methanosarcina* sp. stam MTP4, *Ms. acetivorans* en *Ms. siciliae*, bleken MTPA te gebruiken als substraat en om te zetten naar mercaptopropionaat (hoofdstuk 6). Ongeveer 0,75 mol methaan werd gevormd per mol afgebroken MTPA; methaanthiol kon niet worden gedetecteerd als tussenproduct. Acht andere methanogenen voerden deze conversie niet uit. In anoxische, mariene sediment suspensies met MTPA (500 µM) werd methaanthiol gevormd. De methaanthiol werd verder omgezet naar methaan (417 µM). In aanwezigheid van ampicilline, vancomycine en kanamycine (20 µg/ml van iedere verbinding) werd ook methaan gevormd, methaanthiol kon echter niet worden aangetoond tijdens deze incubaties. Als een specifieke methanogenese remmer, 2-broomethaansulfonzuur (25 mM) werd toegevoegd aan een sediment suspensie met MTPA, kon methaanthiol wederom worden aangetoond. Deze resultaten laten zien dat in natuurlijke milieus MTPA direct of indirect kan dienen als een substraat voor methanogene Archaea.

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Abbreviations

DMSP	Dimethylsulfoniopropionate
MTPA	Methylthiopropionate
THF	Tetrahydrofolate
SDS	Sodium dodecyl sulfate
ATP	Adenosine 5’-triphosphate
HPLC	High-performance liquid chromatography

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