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Demethylation of Dimethylsulfoniopropionate to 3-*S*-Methylmercaptopropionate by Marine Sulfate-Reducing Bacteria

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The initial step in the anaerobic degradation of the algal osmolyte dimethylsulfoniopropionate (DMSP) in anoxic marine sediments involves either a cleavage to dimethylsulfide and acrylate or a demethylation to 3-*S*-methylmercaptopropionate. Thus far, only one anaerobic bacterial strain has been shown to carry out the demethylation, namely, *Desulfobacterium* sp. strain PM4. The aims of the present work were to study how common this property is among certain groups of anaerobic bacteria and to obtain information on the affinities for DMSP of DMSP-demethylating strains. Screening of several pure cultures of sulfate-reducing and acetogenic bacteria showed that *Desulfobacterium vacuolatum* DSM 3385 and *Desulfobacterium niacini* DSM 2059 are also able to demethylate DMSP; a very slow demethylation of DMSP was observed with a salt-tolerant strain of *Eubacterium limosum*. From a 10^5 dilution of intertidal sediment a new marine DMSP-demethylating sulfate-reducing bacterium (strain WN) was isolated. Strain WN was a short, gram-negative, nonmotile rod that grew on betaine, sarcosine, palmitate, H_2 plus CO_2, and several alcohols, organic acids, and amino acids. Extracts of betaine-grown cells had hydrogenase, formate dehydrogenase, and CO dehydrogenase activities but no α-ketoglutarate oxidoreductase activity, indicating the presence of the acetyl coenzyme A-CO dehydrogenase pathway. Analysis of the 16S rRNA gene sequence of strain WN revealed a close relationship with *Desulfobacter hydrogenophilus*, *Desulfobacter latus*, and *Desulfobacula toluolica*. Strain PM4 was shown to group with *Desulfobacterium niacini*. The K_m of strain WN for DMSP, as derived from substrate progress curves in cell suspensions, was approximately 10 μM. A similar value was found for *D. niacini* PM4.

The algal osmolyte dimethylsulfoniopropionate (DMSP) is the main precursor of dimethylsulfide (DMS), which is the most abundant volatile sulfur compound in the marine ecosystem (5). It has been suggested that DMS plays an important role in the global sulfur cycle (22) and in the formation of cloud condensation nuclei through its atmospheric oxidation products (5). Because of the formation of cloud condensation nuclei, DMS might have an effect on the global climate and even partly counteract global warming. The possible impact of the natural production of DMS on the global climate has resulted in considerable attention for this compound and its precursors over the past 10 years.

DMSP is a structural and functional analog of glycine betaine, a widely used compatible solute (43). The microbial degradation of glycine betaine has been studied extensively, and a variety of aerobic as well as anaerobic glycine betaine-degrading bacteria have been isolated (see, for example, references 13 and 14). DMSP is degraded by bacteria via either an initial cleavage to DMS and acrylate (18) or a demethylation to 3-*S*-methylmercaptopropionate (MMPA) and subsequently to 3-mercaptopropionate (MPA) (17, 32). A number of aerobic marine bacteria have been isolated that degrade DMSP by an initial cleavage to DMS and acrylate (7, 21) or by an initial demethylation to MMPA followed by methanethiol production (32) or a further demethylation to MPA (39). Little is known about the nature of the organisms involved in the demethylation of DMSP. Even less is known about the anaerobic bacteria which are involved in the demethylation pathway of DMSP in anoxic marine sediments. Kiene and Taylor (17) speculated that acetogenic bacteria similar to *Eubacterium limosum* could be responsible for the observed sequential demethylation of DMSP to MPA in sediment slurry experiments. The first steps in understanding the microbial demethylation of DMSP came with the observation of the degradation of DMSP to MMPA by *Desulfobacterium* sp. strain PM4 (36), an organism which had been isolated on glycine betaine (14), and the conversion of MMPA to MPA by certain marine *Methanosarcina* species (35).

The work with strain PM4 combined with the observations of Kiene and Taylor (17) raised the following questions which we address in this paper. (i) Can DMSP-demethylating enrichment cultures of anaerobes be obtained, and how widespread is the ability to demethylate DMSP to MMPA among sulfate-reducing and acetogenic bacteria? (ii) Do DMSP-demethylating anaerobes have K_m values for their substrate that are similar to environmental DMSP concentrations?

MATERIALS AND METHODS

Microorganisms, media, and cultivation. The bicarbonate-buffered medium of Brysch et al. (3) for *Desulfobacterium autotrophicum* was used for dilution or enrichment cultures and growth of strain WN, with the addition of yeast extract (50 to 100 mg/liter). Incubations were at 28°C in 120-ml bottles filled with 50 ml of medium (for growth curves) or 16-ml tubes with 10 ml of medium (for substrate utilization experiments); the headspace consisted of 80% N_2–20% CO_2. Substrates were added from sterile 1 M stock solutions; DMSP was neutralized with 1 M $NaHCO_3$ and filter sterilized.

The following strains were grown in media as described in the 1993 catalog of strains of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and, unless otherwise indicated, precultured at 30°C

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on the substrates shown in parentheses: *Desulfobacterium vacuolatum* IbRM (DSM 3385; 10 mM betaine), *Desulfobacterium autotrophicum* HRM2 (DSM 3382; 10 mM betaine, 28°C), *Desulfobacterium niacini* NAV1 (DSM 2059; 10 mM betaine), *Desulfococcus multivorans* Göttingen 1be1 (DSM 2059; 5 mM benzoate), *Desulfobacter postgatei* Dangast 2ac9 (DSM 2034; 20 mM acetate), *Desulfovibrio salexigens* (DSM 2638; 20 mM lactate), *Desulfovibrio halophilus* SL 8903 (DSM 5663; 20 mM lactate), *Desulfosarcina variabilis* Montpellier 3be13 (DSM 2060; 5 mM benzoate), *Desulfobulbus propionicus* Lindhorst 1pr3 (DSM 2032; 20 mM lactate), *Desulfobulbus* sp. strain 3pr10 (DSM 2058; 20 mM propionate), and *Desulfotomaculum kuznetsovii* (DSM 6115; 20 mM lactate, 60°C). *Desulfobacterium* sp. strain PM4 was cultivated as described previously (14). *Desulfobacula toluolica* Tol2 (DSM 7467) was grown on 5 mM benzoate at 30°C in medium described in reference 27. The following strains were grown as described by Heijthuijsen and Hansen (12): *Acetobacterium woodii* WB1 (DSM 1030; 50 mM methanol), *Sporomusa ovata* H1 (DSM 2662; 50 mM methanol), *Sporomusa sphaeroides* (DSM 2875; 50 mM methanol), *Butyribacterium methylotrophicum* Marburg (DSM 3468; 10 mM glucose, 37°C), and *Eubacterium limosum* PM31 (laboratory collection; 10 mM methanol, 37°C).

Acetogenic bacteria from marine or estuarine sediment were enriched by adding sediment to the same basal medium as was used for strain WN but supplemented with sodium tungstate (0.1 μM) and lacking sodium selenite, sulfate, and yeast extract. For the marine enrichment, betaine was used as the substrate; for the estuarine enrichment, 80% H_2–20% CO_2 was used as the substrate and only 12.5 g of NaCl and 1.5 g of $MgCl_2 \cdot 6H_2O$ were added per liter. Pure cultures were obtained after two transfers of an enrichment culture in liquid medium, followed by anaerobic agar dilutions. Pure-culture studies were done with medium for freshwater acetogenic bacteria (12).

Sediment sampling and dilution series. Sediment was collected from the intertidal zone of the Wadden Sea near Westernieland, The Netherlands, and from the Eems-Dollard estuary near Termuntenzijl, The Netherlands. The sediment consisted of black sulfide-rich mud covered by a thin oxidized layer. Samples were collected as described previously (35). Per gram (wet weight) of sediment, 2 ml of dilution buffer was added. The dilution buffer contained 25 mM potassium phosphate (pH 7.1), 21 g of NaCl per liter, and 3 g of $MgCl_2 \cdot 6H_2O$ per liter and was made anoxic by sparging with oxygen-free N_2 and adding 0.2 mM sodium dithionite. Subsequent dilutions were made from this suspension; 0.5 ml of each dilution was added to 50 ml of the basal medium of Brysch et al. (3) supplemented with 50 mg of yeast extract per liter and approximately 4 mM DMSP unless otherwise indicated. Incubations were at 28°C.

Enzyme measurements. Extracts of cells of strain WN grown on 20 mM betaine were prepared anaerobically as described by Hensgens et al. (15). Enzyme activities were measured anaerobically by bubbling the assay mixture in a cuvette with oxygen-free N_2 for at least 3 min and then closing the cuvette with a grey butyl rubber stopper. Reactions were started by addition of cell extract with an N_2-flushed microsyringe. Carbon monoxide dehydrogenase, hydrogenase, formate dehydrogenase, and α-ketoglutarate oxidoreductase were assayed in accordance with the method of Schauder et al. (30).

Utilization of DMSP by washed-cell suspensions. Cells were grown to the late exponential phase on 20 mM DMSP and harvested anaerobically by centrifugation at 16,000 × *g* for 10 min at 4°C. All of the following steps were performed under anoxic conditions by keeping the cells under a constant flow of oxygen-free N_2. After the pellet was washed twice with anoxic 25 mM potassium phosphate buffer (pH 7.1) containing 21 g of NaCl and 3 g of $MgCl_2 \cdot 6H_2O$ per liter, 4 mM dithiothreitol, and 0.2 mM sodium dithionite, it was resuspended in N_2-sparged buffer containing 21 g of NaCl and 3 g of $MgCl_2 \cdot 6H_2O$ per liter, 20 mM Na_2SO_4, and 0.2 mM sodium dithionite to an optical density at 660 nm of 0.25 to 0.38. This suspension (40 ml) was transferred to a 60-ml bottle, and the experiment was started by the addition of 50 μM anoxic DMSP after flushing the headspace with oxygen-free N_2 for 5 min and sealing the bottle with a butyl rubber stopper. A 1-ml sample was taken through the stopper with an N_2-flushed 1-ml syringe and transferred to a 13-ml vial containing 5 ml of 6 M NaOH, which was sealed with a Teflon-coated rubber stopper. The amount of DMS in the vial was measured after 24 h. The apparent K_m value was estimated from the substrate progress curve by the method described by Dalsgaard and Bak for determining the K_m value for NO_3^- or SO_4^{2-} uptake by *Desulfovibrio desulfuricans* (6).

DNA analysis. The moles percent G+C of the DNA was determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen by high-performance liquid chromatography (HPLC) based on the method of Mesbah et al. (24). DNA for 16S rRNA gene sequence analysis was extracted by incubating cells, which had been resuspended in 2.5 ml of a solution containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, with lysozyme (2 mg/ml) for 30 min at 37°C, followed by lysis of the protoplasts with *N*-lauroylsarcosine (1% [wt/vol]), sodium dodecyl sulfate (0.5% [wt/vol]), and DNase-free RNase (50 μg/ml). After incubation with proteinase K (100 μg/ml) for 30 min at 55°C, the DNA was extracted as described by Sambrook et al. (29). The 16S rRNA gene was amplified via PCR with a set of oligonucleotide primers corresponding to positions 8 to 27 (5′-AG AGTTTGATC[C/A]TGGCTCAG-3′) and 1492 to 1513 (5′-TACGG[C/T]TAC CTTGTTACGACTT-3′) of *Escherichia coli* 16S rRNA. The PCR reaction mixture (100 μl) contained 33 ng of DNA, 10 pmol of each primer, 20 pmol of each nucleoside triphosphate, 1 U of *Pwo* DNA polymerase (Boehringer, Mannheim, Germany), 10 mM Tris-HCl (pH 8.85), 25 mM KCl, 5 mM $(NH_4)_2SO_4$, and 2 mM $MgSO_4$. The reaction mixture was preincubated at 94°C for 180 s and subsequently subjected to 27 cycles of 94°C for 60 s, 52°C for 120 s, and 72°C for 120 s; in the final cycle, the incubation at 72°C was extended to 300 s. The DNA sequence of the amplified product was determined by using the Sequenase PCR product sequencing kit (U.S. Biochemical Corporation, Cleveland, Ohio) in accordance with the manufacturer's manual and with custom primers based on the conserved regions. The sequence phylogeny was analyzed by using PHYLIP 3.5c and PAUP. A phylogenetic tree based on the neighbor-joining method of Saitou and Nei (28) as implemented in the NEIGHBOR program was constructed from a distance matrix in accordance with the two-parameter model of Kimura (19). Insertions and deletions were not taken into account.

Analytical procedures. Sulfide was measured colorimetrically (33). DMSP concentrations were determined after conversion to DMS and acrylate by overnight treatment with 1 to 5 M NaOH. Acrylate concentrations were determined by gas chromatography in accordance with the method of Laanbroek et al. (20) for measuring lactate. DMS, methanethiol, and methane were measured by headspace gas chromatography, and MMPA was quantified by gas chromatography or HPLC (35, 36). Glycine betaine and *N*,*N*-dimethylglycine were determined by HPLC (13). Protein was measured colorimetrically by the method of Lowry et al. (23) for whole cells after treatment with 1 M NaOH at 100°C for 10 min and by the method of Bradford (2) for cell extracts. The presence of desulfoviridin was determined by the method of Postgate (26) and by recording the absorption spectrum of a cell extract. Cell extract of *Desulfovibrio gigas* was used as a positive control. The cellular fatty acid composition was determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen in accordance with the procedure of Vainshtein et al. (34).

Chemicals. DMSP was synthesized from acrylic acid and DMS by the method of Chambers et al. (4). MMPA was obtained by alkaline hydrolysis of its methyl ester (Aldrich, Steinheim, Germany) as described by Wackett et al. (40). The identity and purity of the product were checked by hydrogen nuclear magnetic resonance; the DMSP or MMPA content was estimated by total carbon analysis (13) and gas chromatography or HPLC analysis (35, 36).

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA genes of strains WN and PM4 have been assigned GenBank accession numbers U51844 and U51845, respectively.

RESULTS

Sediment dilution and isolation of strain WN. Addition of 2.5 ml of suspended anoxic intertidal sediment to mineral medium supplemented with sulfate, DMSP, and yeast extract always resulted in the formation of DMS; MMPA was not detected in these incubations. To avoid a possible rapid DMSP cleavage by free DMSP lyase from the sediment or by DMSP-cleaving bacteria, triplicate 10^3, 10^4, and 10^5 dilutions of the sediment were made to study the conversion of DMSP (Table 1). In the absence of sulfate, up to 50% of the DMSP was degraded and converted to DMS after 26 days of incubation. No MMPA, methane, or methanethiol was detected in these incubations. However, when sulfate was present, all the DMSP was degraded. In the highest dilution, DMSP was converted almost stoichiometrically to MMPA; in the first two dilutions, the concentration of MMPA was lower than the amount of DMSP that was added. Only traces of methane and/or methanethiol were detected (Table 1).

The 10^4 and 10^5 dilutions contained small rod-shaped cells, some oval- to rod-shaped cells that were slightly refractile, and vibrios. A pure culture of a rod-shaped DMSP-demethylating bacterium (strain WN) was obtained by three successive transfers of colonies on agar plates with DMSP-containing medium which were incubated under anoxic conditions.

Characteristics of strain WN. Cells of strain WN were short, nonmotile rods with a length of 1.0 to 2.5 μm and a width of 1.0 to 1.5 μm (Fig. 1); colonies were beige and smooth. Strain WN was able to use the following growth substrates: formate, acetate, propionate, isobutyrate (5 mM), butyrate (5 mM), valerate (5 mM), lactate, fumarate, pyruvate, succinate, propanol, butanol, ethanol, glycerol, alanine, glutamine, glycine, glutamate, palmitate (0.5 mM), betaine, sarcosine, and H_2-CO_2 (80:20 [vol/vol]). No growth or sulfide production was observed on glucose, fructose, lysine, arginine, choline, methanol, DMS (1 mM), vanillate, syringate, benzoate (5 mM), or 3,4,5-trimethoxybenzoate. All substrates were tested at 10 mM in the

TABLE 1. Anaerobic conversion of DMSP and product formation in medium inoculated with diluted anoxic intertidal sediment[a]

Sediment dilution	DMSP concn (mM) at:		DMS concn at t = 26 days (μmol/ml)	MMPA concn at t = 26 days (mM)	Methanethiol concn at t = 26 days (μmol/ml)	Methane concn at t = 26 days (μmol/ml)
	t = 0	t = 26 days				
2×10^3	4.0	2.4	1.5	0	0	0
2×10^4	4.6	2.2	2.5	0	0	0
2×10^5	4.3	4.0	0.4	0	0	0
2×10^3 (sulfate)[b]	3.7	0	0.04	2.7	0.1	0.05
2×10^4 (sulfate)[b]	4.3	0	0.05	3.6	0	0.04
2×10^5 (sulfate)[b]	4.6	0	0.08	4.5	0	0

[a] Conversions were studied in sulfide-reduced (1 mM) bicarbonate-buffered mineral medium containing 50 mg of yeast extract per liter, with and without 20 mM sulfate, at 28°C.
[b] Medium with 20 mM sulfate.

presence of 20 mM sulfate unless otherwise indicated. Fermentative growth occurred only on pyruvate; no growth occurred on betaine and sulfate under oxic conditions. Toluene, which was supplied via an inert hexadecane phase (1 ml per 50 ml of medium, containing 100 mM toluene), could not be utilized by strain WN. This concentration of toluene was not inhibitory for a culture growing with betaine. Thiosulfate and sulfite were used as alternative electron acceptors; nitrate, nitrite, and elemental sulfur were not. Growth on 10 mM betaine was observed in medium with biotin, *p*-aminobenzoate, and nicotinate as the only vitamin supplements. The fastest growth was observed between pH 6.6 and 7.0 (6.4 to 8.5 tested), at a temperature of 28°C (4 to 45°C tested; no growth at 37°C) and a salinity of 3.0% (0.1% to 4.0% tested; no growth at 1.2% and 4.0%). Desulfoviridin could not be detected in cell extracts of strain WN. The moles percent G+C was 49.8%.

During growth on 7.5 mM DMSP, strain WN converted DMSP stoichiometrically to MMPA and produced 5.6 mM sulfide (Fig. 2). Growth was exponential only in the early growth phase, with a maximum specific growth rate of 0.04 h^{-1} (doubling time, 17 h). Betaine was converted to dimethylglycine.

Cellular fatty acid composition. The fatty acid profile of cells of strain WN grown on 20 mM ethanol and 20 mM sulfate was similar to those described for *Desulfobacterium autotrophicum* and *Desulfobacter hydrogenophilus* (Table 2). The 17:0 *cis*-11 fatty acid, which is typical for *Desulfobacterium* species, was clearly present in strain WN (4.6%) and *Desulfobacula toluolica* (4.1%), while the cyclopropane 17:0 fatty acid, which is a biomarker fatty acid of *Desulfobacter curvatus* (33), was absent in strain WN as well as *Desulfobacula toluolica*. Strain WN did not contain the 10-methyl 16:0 fatty acid, which was present in *Desulfobacter curvatus*, *Desulfobacula toluolica*, and *Desulfobacterium autotrophicum.*

Enzyme activities. In extracts of strain WN grown on 20 mM betaine and 20 mM sulfate, the following enzyme activities were detected: carbon monoxide dehydrogenase (5.3 μmol/min/mg of protein), formate dehydrogenase (0.6 μmol/min/mg of protein), and hydrogenase (1.5 μmol/min/mg of protein). No α-ketoglutarate oxidoreductase activity was found; this activity was detected in *Desulfobacter hydrogenophilus* (0.2 μmol/min/mg of protein) grown on 20 mM acetate and 20 mM sulfate.

Phylogenetic analysis. Initially, strain WN was identified as a strain of the genus *Desulfobacterium* on the basis of a positive hybridization (data not shown) of its RNA with the *Desulfobacterium*-specific 16S rRNA probe of Devereux et al. (8). However, a study of the relationship of strain WN to other sulfate-reducing bacteria based on 16S rRNA gene sequence analysis (Fig. 3) showed that strain WN is related to *Desulfobacter hydrogenophilus* (94.7% similarity), *Desulfobacter latus* (93.1% similarity), and *Desulfobacula toluolica* (92.2% similarity). We also determined the 16S rRNA gene sequence of strain PM4 and the relationship of this strain to other *Desulfobacterium* species. Strain PM4 is closely related to *Desulfobacterium nia-*

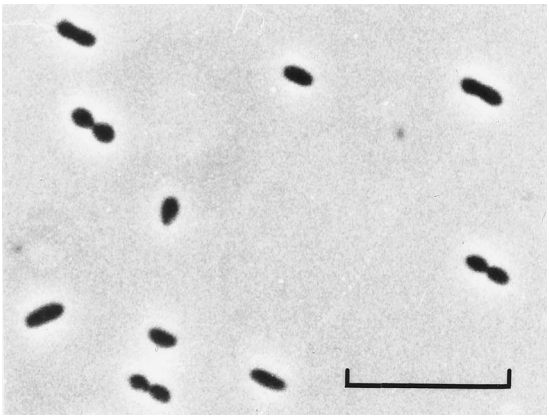

FIG. 1. Phase-contrast micrograph of DMSP-grown cells of strain WN. Bar, 10 μm.

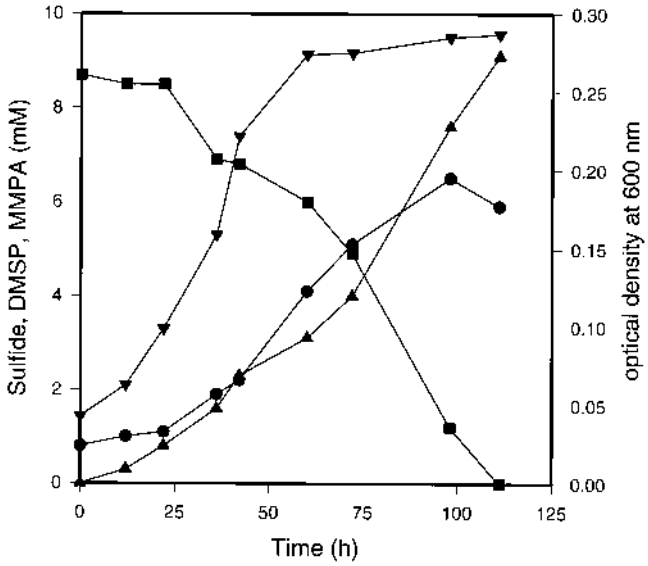

FIG. 2. Growth of strain WN on DMSP and sulfate. Symbols: ■, DMSP; ▲, MMPA; ●, sulfide; ▼, optical density at 600 nm.

TABLE 2. Cellular fatty acid compositions of strain WN, *Desulfobacula toluolica*, *Desulfobacter curvatus*, and *Desulfobacterium autotrophicum*

ECL[a]	Fatty acid	Cellular fatty acid composition (%) of:			
		Strain WN	*Desulfobacula toluolica*	*Desulfobacter curvatus*[b]	*Desulfobacterium autotrophicum*[b]
14.00	14:0	13.5	15.8	9.4	3.2
14.86	15:1 c9	1.7	0.8	0.4	6.8
15.00	15:0	5.0	5.8	2.7	4.8
15.77	16:1 c7	1.8	1.5	1.1	
15.82	16:1 c9	33.1	16.3	10.3	32.1
16.00	16:0	26.2	27.4	34.2	13.3
16.43	10-Methyl 16:0		10.15	7.0	7.6
16.86	17:1 c11	4.6	4.1		8.6
16.89	17:0 cyc			7.6	
17.52	3-OH 16:0	2.4	3.0	1.1	0.5
17.79	18:1 c9	1.9	0.7	0.7	1.0
18.00	18:0	2.1	1.4	0.7	1.0

[a] ECL, equivalent chain length.
[b] Data were taken from reference 34.

cini (97.6% identity). Comparison of the 16S rRNA gene sequence of strain WN with the *Desulfobacterium*-specific probe sequence (8) shows that there is a T instead of a C at position 226 (5′-TTTGAAGATGAGTC*T*GCGCA-3′). However, there is also one mismatch with the *Desulfobacter*-specific probe (8), i.e., an A instead of a G at position 141 (5′-AATCT*A*CTTTC AAGCCTG-3′). The nucleotide sequences of strains WN and PM4 were deposited with GenBank under accession numbers U51844 and U51845, respectively.

Kinetics of DMSP conversion. Initial experiments with washed-cell suspensions of strain WN grown on DMSP indicated that after the addition of 0.2 mM DMSP the concentration of DMSP decreased linearly in time to about 10 μM. Experiments to determine the K_m values of strains WN and PM4 for DMSP were therefore done with a DMSP concentration of approximately 50 μM and cell suspensions with optical densities (at 660 nm) of 0.2 to 0.4. Figure 4 shows progress curves for DMSP conversion by strain PM4 (Fig. 4A) and strain WN (Fig. 4B). The lines represent the theoretical progress curve for the plot that gives the best approximation of the measured values. By using this method, maximum average K_m values of 10 and 9 μM DMSP were obtained for strains WN and PM4, respectively. Strain WN degraded DMSP to a level below the detection limit (0.2 μM), but in suspensions of strain PM4 about 2 μM DMSP was left unconverted. We do not have an explanation for this difference.

Conversion of DMSP by pure cultures of sulfate-reducing and acetogenic bacteria. A wide variety of sulfate-reducing bacteria from marine as well as nonmarine habitats were tested for their ability to demethylate DMSP (for a complete list, see Materials and Methods). Two *Desulfobacterium* strains were found to be able to demethylate DMSP: *Desulfobacterium vacuolatum* DSM 3385 and *Desulfobacterium niacini* DSM 2650. Interestingly, *Desulfobacterium autotrophicum* DSM 3382, which is able to demethylate glycine betaine to *N*,*N*-dimethylglycine (14), was not able to grow on DMSP.

Two strains of acetogenic bacteria were isolated from marine sediment to test whether they could utilize DMSP. Strain ACES was isolated from anoxic intertidal estuarine sediment of the Eems-Dollard estuary near Termuntenzijl, The Netherlands, with H_2-CO_2 as the substrate; strain ACM was isolated from anoxic marine intertidal sediment of the Wadden Sea

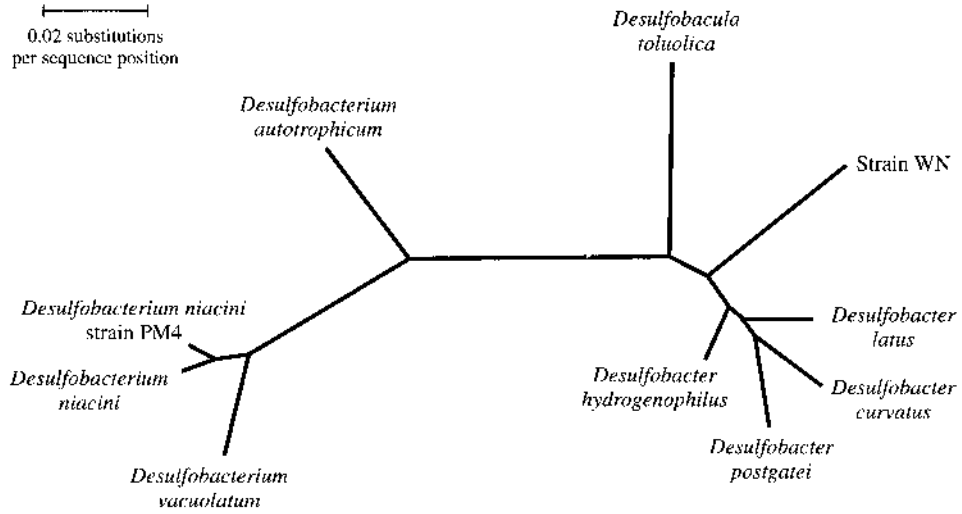

FIG. 3. Phylogenetic relationship of strain WN with other sulfate-reducing bacteria. The unrooted tree is based on a distance matrix of the 16S rRNA gene sequence (base pairs 8 to 1513) and was constructed via the neighbor-joining method as implemented in the Phylip 3.5c software package.

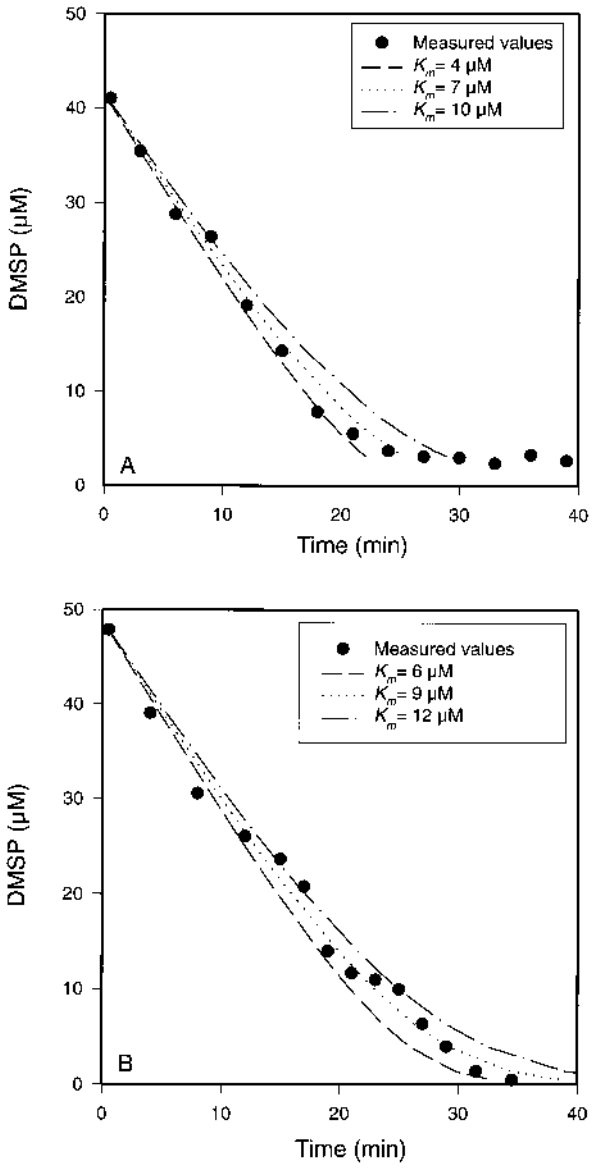

FIG. 4. DMSP degradation in washed-cell suspensions of strain PM4 (A) and strain WN (B). The closed circles represent the measured DMSP concentrations, and the various lines represent calculated substrate progression curves for different K_m values (indicated in the insets).

near Westernieland, The Netherlands, with glycine betaine as the substrate. Both strains could grow in freshwater medium for acetogenic bacteria (12) and can therefore be regarded as salt-tolerant strains. Strain ACES converted H_2-CO_2 exclusively to acetate and was also able to demethylate glycine betaine to *N*,*N*-dimethylglycine; strain ACM converted glycine betaine to acetate and an equimolar amount of *N*,*N*-dimethylglycine. Both strains had an American football shape, which is characteristic for *Acetobacterium* species (31). On the basis of morphology, product formation, and substrate utilization (data not shown), both strains were tentatively identified as *Acetobacterium* species. Strains ACM and ACES were not able to grow on DMSP.

Pure cultures of the following nonmarine acetogenic bacteria were found to be unable to grow on DMSP: *A. woodii* DSM 1030, *S. ovata* DSM 2662, *S. sphaeroides* DSM 2875, and *B. methylotrophicum* Marburg DSM 3468. A salt-tolerant strain of *Eubacterium limosum* (strain PM31), which was isolated by Heijthuijsen (11) from anoxic intertidal sediment of the Wadden Sea with vanillic acid as the substrate, grew very poorly and converted 7.1 mM DMSP to 7.0 mM MMPA, 0.4 mM acetate, and 2.0 mM butyrate in 31 days of incubation.

DISCUSSION

These results show that the ability to demethylate DMSP to MMPA under anoxic conditions is not a property exclusive to strain PM4 but is found in several members of the *Desulfobacter-Desulfobacterium* cluster of the delta-*Proteobacteria*. None of the DMSP-degrading anaerobes demethylated DMSP to MPA; thus, pure-culture studies suggest that the observed demethylation of DMSP to MPA in anoxic sediments is the result of the combined activities of more than one type of organism. The isolation of strain WN demonstrates that it is possible to obtain anaerobic DMSP-demethylating bacteria via enrichment cultures with DMSP as a substrate, provided sufficient precautions are taken to prevent the rapid cleavage of DMSP. Strain WN differs from the DMSP-demethylating strains of the genus *Desulfobacterium* and is difficult to affiliate with any of the known members of the *Desulfobacter-Desulfobacterium* cluster. With respect to lipid composition, substrate utilization, and moles percent G+C, strain WN is similar to members of the genus *Desulfobacterium* (34, 41). Because of its autotrophic growth and hybridization with a *Desulfobacterium*-specific 16S rRNA probe (8), strain WN was initially identified as a strain of *Desulfobacterium autotrophicum* (3), although the type strain of this *Desulfobacterium* species does not demethylate DMSP. However, phylogenetic positioning based on the 16S rRNA gene sequence showed that strain WN is closely related to *Desulfobacter hydrogenophilus* and, to a lesser extent, to *Desulfobacula toluolica*. Neither of these strains is able to demethylate DMSP to MMPA. We are reluctant to assign strain WN to the genus *Desulfobacter* because it has the acetyl coenzyme A-CO dehydrogenase pathway, which is characteristic for *Desulfobacterium* species and is absent in *Desulfobacter* species (42). A similar taxonomic problem was described for a toluene-degrading sulfate-reducing bacterium (27). The new genus *Desulfobacula* was created to solve that taxonomic problem. Assignment of strain WN to the genus *Desulfobacula* would be improper because of the large difference in moles percent G+C and the different substrate utilization patterns.

Bacteria such as strain WN and *Desulfobacterium niacini* PM4 probably play a significant role in the demethylation of DMSP in anoxic intertidal sediments, since they are in relatively high abundance and have low K_m values for DMSP, which are in the range of the concentrations of DMSP that have been found in marine sediments (16, 35, 38). Our K_m value results for strain WN, strain PM4, and a DMSP-cleaving *Desulfovibrio* strain (37) which had a far higher K_m value for DMSP (0.4 mM) are in line with the observations made by Kiene and Taylor (17) that at higher DMSP concentrations in sediments the cleavage pathway becomes more important than the demethylation pathway.

Interestingly, the demethylation of DMSP was not restricted to sulfate-reducing bacteria but was also found to occur in a strain of *Eubacterium limosum*. The demethylation of DMSP by *Eubacterium limosum* was already suggested by Kiene and Taylor (17) on the basis of its ability to demethylate the DMSP analog glycine betaine (25). It is not clear whether organisms such as *Eubacterium limosum* PM31 play an important role in the conversion of DMSP in marine intertidal sediments, be-

cause this bacterium might have been a temporary guest in the intertidal sediment, originating from the rumen of sheep (see reference 10), which graze on the dikes adjacent to the intertidal flat. However, the better growth of strain PM31 at higher NaCl concentrations compared with *E. limosum* RF (10, 11) indicates that strain PM31 is better adapted to the marine environment and therefore might be active in such an environment. However, the results shown in Table 1 indicate that sulfate was required for the demethylation of DMSP, which suggests a dominant role of sulfate-reducing bacteria in this process.

The demethylation of glycine betaine by a certain strain does not imply that such a strain is able to demethylate DMSP, as was shown for two salt-tolerant strains of acetogenic bacteria of the genus *Acetobacterium* and for *Desulfobacterium autotrophicum*. The three DMSP-demethylating species of the genus *Desulfobacterium* and strain WN utilize the acetyl coenzyme A-CO dehydrogenase pathway for the oxidation of their substrate (42); the methyl group of betaine or DMSP is probably transferred by a methyltransferase system to a tetrahydropterin, yielding methyltetrahydropterin, which is an intermediate in this pathway. Because not all glycine betaine-demethylating strains demethylate DMSP, most probably the methyltransferase system in these strains is specific for only one methyl donor, as is also the case for methanol and trimethylamine conversion by methanogens (1).

A remarkable difference between anaerobes and aerobes in the demethylation of DMSP and betaine is that the anaerobes carry out a single demethylation of the substrate to yield MMPA and dimethylglycine. For aerobic betaine-demethylating bacteria, the dimethylglycine is not an end product but is metabolized further (see, e.g., reference 9). Similarly, MMPA is usually not the end product of DMSP demethylation by aerobes. It is further demethylated to MPA (39) or metabolized in a pathway leading to the formation of methanethiol (9, 32).

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