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Isolation and characterization of *Methylophaga sulfidovorans* sp. nov.: an obligately methylotrophic, aerobic, dimethylsulfide oxidizing bacterium from a microbial mat

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Abstract

Sediment from a microbial mat from the South-West coast of the Netherlands consumed dimethylsulfide (DMS) under oxic and anoxic conditions. From this sediment, a Gram-negative, oval DMS oxidizing bacterium, strain RB-1, was isolated. Its substrate range is typical of an obligately methylotrophic organism. Enzyme analysis revealed the presence of the ribulose monophosphate pathway for carbon assimilation, and the ability to use the linear dissimilatory pathway via formate to carbon dioxide, as well as the cyclic pathway via the ribulose monophosphate route for carbon dissimilation. 16S rRNA sequence analysis showed high similarity with species belonging to the genus *Methylophaga*. Because of the specific dimethylsulfide and hydrogen sulfide oxidizing capacity, the new isolate was named *Methylophaga sulfidovorans*.

Keywords: Microbial mat; Dimethylsulfide oxidation; *Methylophaga sulfidovorans*

1. Introduction

Dimethylsulfide (DMS) is an important volatile organic sulfur compound in the global sulfur cycle. Its significance, as the main carrier of sulfur from sea to land, was first noted by Lovelock and Maggs [1], who detected DMS in trace quantities in soils and seawater. DMS might also have an important role in climate regulation since it contributes to the formation of cloud condensation nuclei in the atmosphere. Cloud condensation nuclei affect the radiation balance of the earth [2]. The main natural source of DMS in marine environments is dimethylsulfoniopropionate (DMSP), which serves as an osmolyte in some, but not all algae [3]. Due to its volatile nature, DMS evaporates into the atmosphere in large quantities (40×10^{12} g sulfur per year). It has been estimated that about 90% of the DMS produced in marine systems is microbiologically removed by conversion to sulfate and carbon dioxide, and that just 10% is ventilated into the atmosphere [4].

In spite of the important role of marine bacteria as a major sink for DMS, the number of marine bacteria known to be capable of using DMS as an energy source is still small. *Thiobacillus thioparus* T5 and *Thiobacillus* ASN-1 are able to oxidize DMS aerobically, and were both isolated from marine samples [5,6]. Recently, an aerobic, facultatively methylotrophic bacterium, strain BIS-6 was isolated from marine sediment [7]. This bacterium grew on the

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osmolytes DMSP and glycine betaine as well as their degradation products. Further details of its growth on DMS were not given. DMS metabolizing *Hyphomicrobium* species, apparently common in fresh water environments [8], have not been isolated from marine sources. Most research on marine DMS oxidation has focused on ecosystems as a whole. Salt marsh sediments, especially, have been used as model systems in which the production and consumption processes of organic sulfides under anoxic conditions have been investigated in some detail [9–12]. The isolation of new species capable of degrading DMS, and the investigation of their DMS degrading capabilities, should thus enhance our understanding of the role of microorganisms in DMS consumption in the natural environment.

This paper reports on the isolation and characterization of an obligately methylotrophic bacterium, strain RB-1, from a microbial mat sediment. This isolate was designated *Methylophaga sulfidovorans* sp. nov. Its physiology and taxonomy are described.

2. Materials and methods

2.1. Bacterial culture and natural samples

Microbial mat sediment samples were obtained in 1993 and 1994 from an estuarine intertidal region on the South-West coast of the Netherlands (51° 26.3′ NL, 4° 7.5′ EL). Strain RB-1 was isolated from mat samples collected in 1993. *Methylophaga marina* and *Methylophaga thalassica* were acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 5689 and DSM 5690, respectively).

2.2. Culture media

Medium contained (per l): 15 or 25 g NaCl, 0.5 g $(NH_4)_2SO_4$, 0.33 g $CaCl_2 \cdot 6H_2O$, 0.2 g KCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.02 g KH_2PO_4, 2 g Na_2CO_3, 1 mg $FeSO_4 \cdot 7H_2O$, 1 ml trace element solution [13], 1 ml vitamin solution. pH was set at 7.5 ($\pm$0.3) with 1 N HCl. The trace element solution contained additional 0.6 mM Na_2SeO_4. The vitamin solution contained (per l): 20 mg biotin, 200 mg nicotinic acid, 100 mg thiamine, 100 mg *p*-aminobenzoic acid, 50 mg pantothenate, 500 mg pyridoxine · HCl, 10 mg riboflavine and 10 mg vitamin B_{12}. Medium used for purification of strains was supplemented with HEPES (1 mM). Artificial sea water, used for suspension of sediment samples, contained (per l) 22.5 g NaCl, 24.6 g $MgSO_4 \cdot 7H_2O$, 1.5 g KCl and 2.2 g $CaCl_2$. pH was set at 7.5 with 1 N NaOH. Liquid medium containing DMS was kept in glass bottles, which were sealed with butyl rubber or teflon stoppers to avoid DMS loss.

The purity of the cultures was routinely checked by streaking on Brain Heart Infusion plates supplemented with 1.5% NaCl.

2.3. Analytical techniques

DMS from the headspace of the cultures was measured with a gas chromatograph with a sulfur-specific, flame photometric detector, equipped with a Hayesep R column [8]. Measurements of DMS were accurate (less than 5% variance) down to 0.5 ppm in the gas phase. The equilibrium constant of DMS at room temperature (13.5) was used for calculating the dissolved DMS concentration [14].

Oxygen consumption rates of bacterial suspensions were measured with a polarographic Clark-type electrode in a Biological Oxygen Monitor at 28°C. Oxygen concentrations in the gas phase were determined by gas chromatography.

Thiosulfate and tetrathionate were determined according to Sörbo [15]. Sulfide was determined by iodometric titration for sulfide concentrations higher than 1 mM [16], and by the methylene blue method for sulfide concentrations lower than 1 mM [17].

Biomass concentration was determined as organic carbon using Total Organic Carbon analysis with a Tocamaster type 815-B. The biomass obtained from continuous cultures was analyzed for its carbon, hydrogen and nitrogen content (molar ratio) with a Carlo Erba elemental analyzer, type EA 1108 and was found to be $CH_{1.75}O_{0.46}N_{0.19}$ for strain RB-1. This shows that about 50% of the biomass was carbon. Biomass was also measured by dry weight determination.

Chemicals used were of analytical grade. 3-methiol propionate (MMPA) and dimethylsulfoniopropionate (DMSP) were a generous gift from M. van der Maarel of the University of Groningen.

2.4. DMS consumption in microbial mat sediment samples and Most Probable Number (MPN) counts of aerobic DMS oxidizing bacteria

DMS oxidation rates in natural samples were determined in 100 ml serum bottles filled with 20% (v) sediment, leaving the larger part of the bottle as headspace. The sediment was diluted 5-fold (weight basis) to ensure a concentration of biomass that was high enough to result in a detectable DMS removal rate and low enough to keep the headspace aerobic. A large variety of microorganisms with readily available substrates are present in the sediment, leading to a depletion of oxygen. Aerobic and anaerobic headspaces were created by flushing with air or nitrogen gas, respectively. The bottles were sealed with butyl rubber septa and teflon stoppers, in order to prevent inward oxygen diffusion and outward DMS diffusion. DMS was added to the samples (< 1 mM DMS) with a syringe from a stock solution (27 mM DMS). As the bottles were not shaken during incubation, it must be assumed that even with an air-flushed headspace, part of the sediment in the flask rapidly became anoxic. The total DMS removal was therefore the sum of the oxic and anoxic disappearance of DMS. The oxygen concentration in the headspace was followed during one 'oxic' experiment, and it showed that oxygen was present during the whole experiment. When the headspace was flushed with nitrogen, only anoxic DMS degradation could occur. To check the biological activity of the sediment, oxic and anoxic serum bottles with sediment and DMS were heated 5 min to 90°C. The DMS disappearance in these bottles was measured during a period of 10 days.

MPN counts were carried out by making 5 ml dilution series in 10 ml tubes in mineral, carbonate buffered, medium in a desiccator with DMS in the headspace. The desiccators were kept at 20–25°C. DMS concentrations in the liquid did not exceed 1 mM. Positive tubes were scored on the basis of turbidity after a maximum of 6 weeks of incubation.

2.5. Isolation procedure

The tubes containing the highest positive dilution in the MPN counts were used for enrichment and strain purification. One enrichment culture was selected for further study on the basis of its high DMS-oxidizing capacity. As this culture (strain RB-1) did not grow on agar or agarose plates with DMS in the headspace, the aerobic DMS-oxidizing bacterium was isolated using several dilutions to extinction with mineral medium supplemented with 1 mM HEPES. Purity of strain RB1 was checked with phase contrast microscopy and BHI agar plates. Culture purity was consistent with the observation that DNA of the culture gave only one visible 16S rDNA band after PCR amplification.

2.6. Cultivation of cells

Strain RB-1 was cultivated in batch and chemostat cultures. Batch cultures were grown at 25°C at a pH of 7.5 ± 0.5 in a rotary shaker at 100 rpm. Growth in stainless steel fermentors with DMS as a substrate resulted in severe corrosion which gave toxic levels of nickel and chromate in the fermentor. For this reason, fermentors for continuous cultivation were made from glass and polycarbonate. Continuous cultures with growth limiting methanol (10/20 mM), methanol/sulfide (10 mM/0–10 mM) or DMS (2.4 mM) as substrate were grown at 27°C, a pO_2 of 50% and pH 7.6, and dilution rates of 0.05, 0.05 and 0.01 h^{-1} respectively. A steady state on DMS was obtained by switching from a medium supply containing methanol (10 mM) at a dilution rate of 0.05 h^{-1} (in steady state) to a medium supply containing DMS (2.4 mM) and reducing the dilution rate.

Methylophaga thalassica and *Methylophaga marina* were grown in batch cultures (25°C, pH 7.5 ± 0.5 in a rotary shaker at 100 rpm). DMS oxidation for these bacteria was tested in serum bottles (20/80 v% medium/headspace) with 1 mM DMS.

2.7. Physiology of strain RB-1 for methanol, DMS and H_2S

Aerobic growth was tested in batch cultures (unless otherwise stated) for the following substrates in mineral medium at a concentration of 10 mM, unless otherwise indicated: DMS (1 mM), methane (50/50 methane/air), methanol, formate, thiosulfate, hydrogen sulfide (chemostat), methylamine (5 mM), dimethylamine (5 mM), trimethylamine (5 mM), dimethylsulfoxide (5 mM), acrylate (5 mM), acetate,

ethanol, propanol, lactate, galactose, glucose, fructose, dimethylsulfoniopropionate, 3-methiolpropionate, 3-mercaptopropionate, methanesulfonic acid. Denitrification capacity with methanol as electron donor and nitrate as electron acceptor was tested in an anaerobic jar.

Kinetic parameters and biomass yields of strain RB-1 on DMS, methanol and hydrogen sulfide were determined. The maximum specific growth rate on methanol was measured in batch culture. The affinity constant and dry weight were determined with cell suspensions from methanol-limited continuous cultures. The maximum specific consumption rate for DMS (nmol DMS min^{-1} mg $biomass^{-1}$) was determined in cell suspensions in an air-tight, pH, temperature and oxygen controlled fermentor. Cells, pregrown on methanol, were adapted to DMS. The maximum DMS consumption rate was measured by following DMS disappearance in this suspension by gas chromatography. The kinetic parameters, q_{MAX} (nmol O_2 min^{-1} mg $biomass^{-1}$) and K_s (μM), and the biomass yield were determined with cells obtained from a continuous culture with DMS as the sole substrate ($D = 0.01$ h^{-1}). Biomass yield on DMS was also determined in batch cultures, incubated at 25°C in a rotary shaker at 100 rpm. Biomass formation was measured by Total Organic Carbon (TOC) analysis. Biomass yield and the affinity constant for H_2S were determined with cells from a continuous culture supplied with methanol and sulfide.

DMS oxidation stoichiometry was measured with cell suspensions obtained from a DMS-limited continuous culture by measuring the oxygen uptake in 5 ml tubes in a Biological Oxygen Monitor. Thiosulfate production was measured after DMS had been oxidized completely.

2.8. Enzyme activities in cell free extracts

The carbon assimilation and dissimilation pathway for methanol and DMS grown cells of strain RB-1 were determined with enzyme assays. Cell free extracts were made in 50 mM phosphate buffer by sonification of whole cells from continuous cultures. The sonicated cells were centrifuged at $50\,000 \times g$ at 4°C and the supernatant was used for enzyme determinations. The protein concentration was measured with the method of Bradford [18]. D-Ribulose-1,5-bisphosphate carboxylase was determined according to Beudeker et al. [19]. RUBP reacts with $^{14}CO_2$ to 3-P-glycerate; the radioactivity of biomass was counted in a Beckman liquid scintillation system type LS 3801. Hydroxypyruvate reductase was determined according to Large and Quayle [20]. 3-Hexulose-6-phosphate synthase was tested with the indirect method described by van Dijken et al. [21], which is based on the ribulose-5-P and formaldehyde-dependent production of NADPH, and also with the direct method of Kato [22]. The latter method is based on ribulose-5-P and formaldehyde uptake by cell free extract. Formaldehyde was measured by the method of Nash [23]. Methanol dehydrogenase activity was measured by the method described by Frank and Duine [24] and formaldehyde dehydrogenase according to Attwood [25]. Formate dehydrogenase was assayed with the method of Jollie and Lipscomb [26], except that the cell free extract was prepared in phosphate buffer (50 mM, pH = 7) instead of MOPS (50 mM, pH = 6.5). Glucose 6-P dehydrogenase was assayed according to Kletsova et al. [27]. Gluconate 6-P dehydrogenase activity was determined with the method of Beardsmore et al. [28], but the cell free extract was prepared in phosphate buffer (50 mM, pH = 7) instead of Tris · HCl (pH = 8.2) and 2-mercaptoethanol (5 mM). Extinctions were measured in a Hitachi UV-VIS spectrophotometer, type 100-60.

2.9. Identification tests and taxonomy of strain RB-1

Standard identification tests API 20 NE and API 32 GN (commercial substrate range and substrate assimilation tests for C_{3-6} sugars (acids), lipids and amino acids, BiO MERIEUX SA, France), Gram-stain, oxidase and catalase tests were carried out. For the determination of the guanine + cytosine content (GC%) of the DNA, cells were obtained from methanol-limited continuous cultures. The DNA was obtained by a double phenol/chloroform extraction and following precipitation in ethanol. Prior to final purification of the DNA with Qiagen genomic tips (Qiagen GmbH, Germany), the RNA was digested with RNA-ase. The GC% was determined from the melting curve of DNA, followed at 260 nm in a Perkin Elmer UV-VIS spectro photometer, type

lambda 15. Temperature, pH optima and salt tolerance were determined in batch cultures grown on 10 mM methanol.

For further identification the full (1500 base pairs) 16S rDNA sequence was determined for strain RB-1, *Methylophaga marina* and *Methylophaga thalassica* at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM). These sequences were aligned manually against representatives of the Proteobacteria at DSM. The 16S rRNA sequence of *Methylophaga thalassica* (EMBL/Genbank/DDBJ data base, X87339), which was already available was compared with the sequence of *M. thalassica* made at the DSM using a BESTFIT program (Genetics Software, University of Leiden, the Netherlands). The sequences made at DSM were deposited at the EMBL Nucleotide Database under accession numbers X95459, X95460 and X95461 for *Methylogphaga marina*, *Methylophaga thalassica* and strain RB-1 respectively.

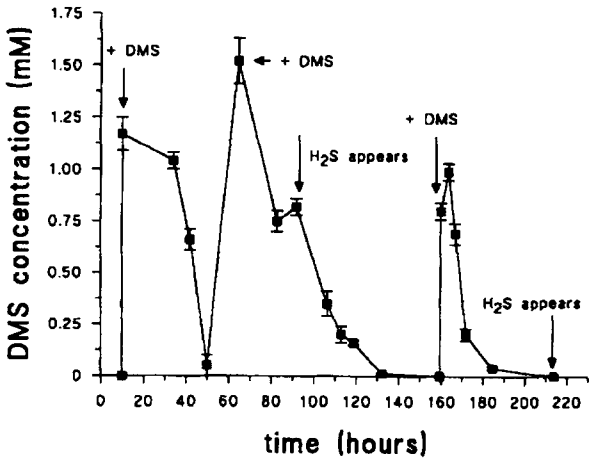

Fig. 1. Oxic (0–55 h, 160–240 h) and anoxic (55–160 h) DMS consumption in microbial mat sediment. In the 'oxic' experiment, the gas phase above the sediment samples was oxic, but parts of the sediment in the bottle were anoxic, due to low oxygen diffusion from the headspace to the sediment. This experiment therefore represents a sum of oxic and anoxic DMS conversion. In the anoxic experiment, DMS was added to the sediment, which was flushed with nitrogen in order to make the sediment and the headspace fully anoxic.

3. Results

3.1. DMS consumption in microbial mat sediments: MPN experiments and isolation

Different samples from an intertidal coastal area were tested for their DMS-degrading capabilities under oxic and anoxic conditions. The heated samples showed no DMS disappearance under oxic or anoxic conditions, indicating that the removal of DMS by the sediment was a biological process. Most of the fresh samples (which had been stored in the dark) produced DMS. After 1 to 3 days, the production of DMS ceased, and DMS disappearance rates under oxic and anoxic conditions could be determined, as shown for one sample in Fig. 1. As long as oxygen could be detected in the gas phase, the experiment was termed 'oxic'. Hydrogen sulfide was detectable in the headspace of anoxic serum bottles with sediment and no DMS added within hours of incubation, indicating that sulfate reduction was an active process in the sediment. When the oxygen and DMS were depleted (at the end of the oxic experiment, or during the anoxic experiment), hydrogen sulfide was detected. Most probably, the hydrogen sulfide produced in the anoxic part of the sediment by sulfate reduction had been previously oxidized with oxygen by chemolitho (auto)trophic bacteria present in these sediments. The average DMS-consumption rate with an oxic headspace was 2.5 ($\pm$ 1.0) μmol ml sediment^{-1} day^{-1} (7 samples). Under anoxic conditions, a DMS consumption rate of 1.5 ± 0.2 μmol ml sediment^{-1} day^{-1} was found. The aerobic DMS oxidation rate was therefore estimated to be 1.0 μmol ml sediment^{-1} day^{-1} by subtracting the DMS oxidation rate found under anoxic conditions from the rate found under 'oxic' conditions.

Similar samples were used for MPN experiments using bicarbonate-buffered medium with DMS supplemented in the headspace. The counts ranged from 10^4 to 10^5 cells g sediment^{-1}. The addition of 1 mM HEPES to the mineral medium increased the counts by a factor 10 to 100 (i.e. to give 10^5–10^7).

A DMS-metabolizing bacterium, strain RB-1, was isolated from one of the highest dilutions, by repeated serial dilution followed by thorough checks for purity. This organism was selected for further identification and study because of its relatively rapid rate of DMS metabolism. Strain RB-1 was deposited at the Delft Culture Collection (LMD 95.210).

Table 1
Growth kinetics and biomass yield of the methylotrophic bacterium, strain RB-1

Kinetic parameter	Growth on methanol	Growth on DMS
μ_{MAX} (h^{-1})	0.30 ± 0.02 *	0.04–0.05
K_s (μM)	2.6 ± 0.5	1.5 ± 0.3
Yield (g biomass mol^{-1})	10.0 ± 0.5	9.2 ± 0.4

Unless otherwise indicated, the data were obtained with continuous culture experiments. The dilution rates for methanol and DMS were 0.05 h^{-1} and 0.01 h^{-1}, respectively.
* Data obtained in batch cultures.

3.2. Physiology of strain RB-1

Strain RB-1 grew on methanol, methylamine, dimethylamine and DMS. No growth was detected on trimethyl amine, methane, methane sulfonic acids and for a wide range of organic substrates tested in batch culture and in the standard identification tests. H_2S could be used as supplementary energy source (see below). The organism could not denitrify under the conditions tested. It therefore appears from its limited substrate range that strain RB-1 is an obligately methylotrophic organism.

Strain RB-1 could easily be grown at relatively high growth rates on methanol, both in batch and in continuous culture. Its kinetic growth parameters and yield are presented in Table 1. Growth on DMS was considerably slower, and batch cultures suffered from DMS toxicity which became apparent at concentrations above 2.5 mM DMS. Growth under DMS limitation (2.4 mM in the in-flowing medium) in continuous cultures resulted in a stable culture with 22 mg biomass l^{-1}, corresponding to an optical density of 0.20 (Fig. 2). DMS in the culture was below the detection limit. The yield on DMS in this case was 9.2 g biomass mol DMS^{-1}. This was within the range (10.4 ± 1.4, 15 experiments) obtained from batch yield measurements, made in very diluted cultures. The maximum specific growth rate on DMS was estimated from direct substrate uptake measurements in the chemostat, and from the maximum specific oxygen uptake by cells from a DMS-limited continuous culture ($D = 0.01\ h^{-1}$). The maximum specific substrate uptake rate was 74 ± 7 nmol DMS mg $biomass^{-1}$ min^{-1}. The maximum specific oxygen uptake rate was 383 ± 16 nmol oxygen min^{-1} mg $biomass^{-1}$. With a yield of ± 9.8 g biomass mol^{-1} DMS and an oxygen/DMS ratio of 4 (see below), the maximum specific growth rates were $0.044 \pm 0.04\ h^{-1}$ and $0.055 \pm 0.02\ h^{-1}$, respectively.

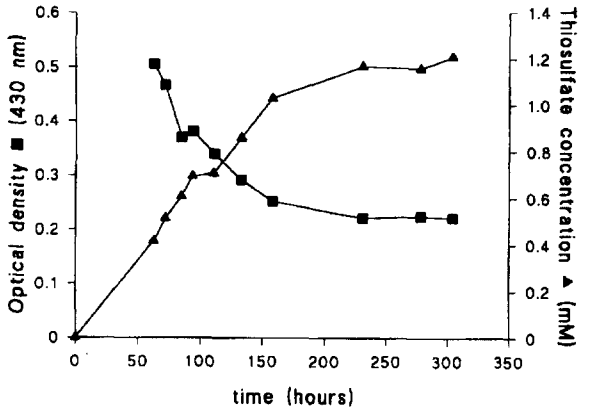

Fig. 2. Optical density and thiosulfate concentration in a continuous culture. At $t = 0$ the medium supply was switched from methanol (10 mM) to DMS (2.4 mM). Thiosulfate, product of the DMS oxidation, which is stoichiometrically formed, is shown to indicate that steady state conditions with DMS as a sole substrate occurs after 250 h. Optical density of 0.2 at 430 nm corresponds with a biomass concentration of 22 mg l^{-1}.

Unexpectedly, thiosulfate, rather than sulfate, was found to be the main product of DMS metabolism (Fig. 2).

Hydrogen sulfide could be biologically oxidized, and it served as supplementary energy source. This was evident from the dry weight increase in chemostat cultures with increasing H_2S supplies. Thiosulfate was stoichiometrically formed from sulfide in batch cultures (Table 2). Growth on H_2S was tested by switching the methanol/sulfide (10/8.15 mM)

Table 2
Dry weight and thiosulfate measurements for the methylotroph, strain RB-1, in steady state continuous cultures supplied with mixtures of methanol and sulfide at a dilution rate of 0.05 h^{-1}

Substrate (mM) methanol/sulfide	Dry weight (mg)	$S_2O_3^{2-}$ (mM)
10/0	100 ± 10	0
10/0.75	102 ± 10	0.33 ± 0.03
10/3.5	115 ± 12	1.01 ± 0.17
10/8.15	146 ± 15	3.88 ± 1.14
0/10	no growth	no growth

Tetrathionate was not detectable.

culture to sulfide alone. The culture washed out, the optical density decreased at a slightly higher rate than the wash out rate would predict, indicating that autotrophic growth was not possible (detection limit $< 0.001\ h^{-1}$).

Cells cultivated on DMS in continuous culture and incubated in 5 ml air saturated mineral medium converted 79 μM DMS to 34 ± 1 μM thiosulfate. The oxygen uptake for DMS oxidation was 4.0 ± 0.2 mol O_2 mol^{-1} DMS (6 measurements). From these results, it was concluded that the reaction stoichiometry for DMS oxidation was: $2\ (CH_3)_2S + 8\ O_2 \rightarrow 4\ CO_2 + S_2O_3^{2-} + 2\ H^+ + 5\ H_2O$. These cells also oxidized methanethiol (0.2 mM), formaldehyde (0.1 mM), hydrogen sulfide (0.05 mM) and methanol (1 mM), and did not oxidize thiosulfate (0.1 mM), elemental sulfur (0.1 mM) and formate (0.1 mM).

3.3. Key enzymes of C_1-metabolism

Strain RB-1 was tested for the presence or absence of key enzymes for carbon assimilation and dissimilation (Table 3). RuBP carboxylase and hydroxypyruvate reductase were not detectable in cell free extracts from cells grown with methanol or DMS, while controls with organisms known to possess these enzymes were positive. High activity was found for hexulose 6P synthase in extracts from cells grown on methanol or DMS. The minimum carbon fixation rates theoretically required for growth in continuous culture on methanol (10 mM, dilution rate of $0.05\ h^{-1}$) and DMS (2.4 mM, dilution rate of $0.01\ h^{-1}$) are 69 and 14 nmol C min^{-1} mg $protein^{-1}$ respectively, assuming that half of the biomass consists of protein. This is strong evidence that the ribulose monophosphate route is used for carbon assimilation.

There was significant activity of the carbon dissimilation enzymes for the direct route via formate to carbon dioxide, as well as for the cyclic route via 6-P gluconate in cell free extracts of strain RB-1. As about 60% of the methanol and DMS is dissimilated, the minimum required activity would be 95 and 20 nmol min^{-1} mg $protein^{-1}$, respectively. The observed activity would thus account for about 50% of the theoretical activity for methanol dissimilation and is sufficient for the observed rate of DMS dissimilation.

NH_4Cl (50 mM) was required for methanol dehydrogenase activity, indicating that this enzyme is PQQ dependent [24]. Methanol dehydrogenase activity in cells grown on DMS can be attributed to the production of formaldehyde from DMS. Formaldehyde is known to induce the methanol dehydrogenase activity.

3.4. Taxonomy of strain RB-1

The API 20 NE and API 32 GN tests gave negative results for the isolate, confirming its limited

Table 3
Enzyme activities for the carbon assimilation and dissimilation pathways of strain RB-1

Enzyme	Coenzyme	Strain RB-1 on methanol	Strain RB-1 on DMS
Hydroxy pyruvate reductase	NAD(P)H	N.D.	N.D.
Ribulose bisP carboxylase	–	N.D.	N.D.
Hexulose 6-P synthase	–	511 ± 58 (6) *	81 ± 8 (2)
Hexulose 6-P synthase	–	1895 ± 114 (3) **	N.M.
Glucose 6-P dehydrogenase	NAD	240 ± 14 (3)	262 ± 5 (3)
Glucose 6-P dehydrogenase	NADP	465 ± 83 (3)	448 ± 185 (3)
Methanol dehydrogenase	PMS/DCPIP	1086 ± 110 (17)	462 ± 68 (14)
Formaldehyde dehydrogenase	NAD	17 ± 7 (7)	21 ± 2 (2)
Formate dehydrogenase	NAD	78 ± 18 (10)	59 ± 12 (3)
Gluconate 6-P dehydrogenase	NADP	52 ± 29 (3)	17 ± 11 (2)

The activities are expressed as nmol min^{-1} mg $protein^{-1}$. Positive controls for the activity of hydroxypyruvate reductase and RUBP carboxylase were *Hyphomicrobium* EG (LMD 84.101) and *Thiobacillus neopolitanus* (LMD 94.73).
The activities found indicated with * and ** represent the indirect method described by van Dijken et al. [21] and the direct method described by Kato [22], respectively. The numbers in brackets indicate the number of experiments.
N.D., not detectable; N.M., not measured.

range of metabolic capabilities. The GC% content was 42.4 ± 0.2. Standard tests revealed that the organism was Gram-negative and catalase and oxidase positive. The optimum temperature for growth was 22 ± 1°C. The optimum pH was 7.4–7.8. The organisms was salt tolerant up to 1.5 M NaCl.

The physiological data show that strain RB-1 belongs to the groups of obligate methylotrophs. The largest phenotypical resemblance can be found with *Methylophaga* spp. [29]. The data on 16S rRNA sequence for strain RB-1, *Methylophaga marina* and *Methylophaga thalassica*, acquired from the DSM revealed similarities for strain RB-1 of 98.8% with *Methylophaga thalassica* and 98.3% with *Methylophaga marina*. The third best similarity was found with *Methylomonas methanica* (89.9%). The 16S rRNA sequence of *Methylophaga thalassica* (X873339) was 99.4% similar with the sequence of *Methylophaga thalassica*, determined at DSM. *M. thalassica* and *M. marina* could not oxidize DMS in batch cultures, whereas strain RB-1 could oxidize DMS successfully.

4. Discussion

4.1. DMS consumption in microbial mat sediments and MPN counts

Microbial mat samples from the South-West coast of the Netherlands showed a significant potential for aerobic and anaerobic DMS metabolism. The rate of metabolism of slurry under anoxic conditions was in the range of rates found by Kiene and Capone [12], who reported removal of about 100 μmol DMS in 12 days by 25 ml sediment slurry. Recalculated for these experiments, this is about 0.33 μmol DMS ml slurry^{-1} day^{-1}.

As expected in these actively DMS producing and consuming sediments, aerobic DMS oxidizing bacteria were present. Most probable number counts of aerobic DMS oxidizing bacteria in the sediment samples gave results that were similar to results obtained by Visscher et al. [5] for a marine microbial mat on Texel (the Netherlands). These counts underestimate the aerobic DMS oxidizing bacteria in the sediment samples [30]. It has been found recently, that the addition of trace amounts of HEPES increased the viability counts at least by a factor 10–100, indicating that sub-optimal media might be (partly) responsible for the lower counts. Further work will be necessary to show whether strain RB-1, which was isolated from one of the highest dilutions, belongs to the dominant population of DMS oxidizers in the sediment.

4.2. Taxonomy of strain RB-1

Obligately methylotrophic bacteria, of which strain RB-1 is a representative, have been classified under three different genera; *Methylopilus*, *Methylobacillus* and *Methylophaga* [31]. The greatest similarity of the taxonomic data of strain RB-1 (carbon assimilation route, GC content, substrate range) is with the two members of the (marine) genus *Methylophaga*. 16S rRNA sequence analysis revealed a great similarity between members of the genus *Methylophaga* and strain RB-1. It can therefore be concluded that strain RB-1 belongs to the genus *Methylophaga*. On the basis of the sulfide oxidizing properties of strain RB-1 a new species is defined: *Methylophaga sulfidovorans*. In an earlier publication *Methylophaga sulfidovorans* was provisionally named *Methylopila sulfovorans* [30].

4.3. Description of Methylophaga sulfidovorans sp. nov.

Methylophaga sulfidovorans sp. nov. (sul.fi.do.vo'.rans. L. adj: *vorans* devouring: M.L. sulfidovorans sulfide consuming) cells are irregular, oval shaped rods 0.2 by 0.9 μm, polar flagelum. Catalase and oxidase positive. The GC% content of the DNA is 42%. Optimum temperature 22°C. Grows at 17 to 35°C. Growth substrates are methanol, methylamine, dimethylamine and dimethylsulfide. Hydrogen sulfide is used as energy source. The type strain is strain LMD 95.210.

4.4. Physiology of Methylophaga sulfidovorans

M. sulfidovorans used the RuMP route for carbon assimilation. This is common for obligately C_1-utilizing bacteria [32]. Carbon dissimilation can take place through the cyclic RuMP route, since gluconate 6-P dehydrogenase activity was detected. The

enzymes for direct oxidation of formaldehyde, through formate, were also detected. In oxygen uptake experiments, formate was not oxidized. This, however, may be related to transport problems of formate through the bacterial membrane. It thus remains unclear whether one or both of the routes are used in vivo. Oxygen uptake experiments further showed that methanethiol, formaldehyde and hydrogen sulfide may be intermediates in the degradation of DMS to thiosulfate and carbon dioxide.

The kinetics of *M. sulfidovorans* were similar to those of other known DMS-utilizing bacteria ($\mu_{MAX} \approx 0.05\ h^{-1}$, $K_s \approx 1\ \mu M$). The biomass yield was ± 10 g biomass mol^{-1} DMS and ± 10 g mol $methanol^{-1}$. This is unexpected, because the RuMP route, which has a relatively large energy efficiency for carbon assimilation, is used [33]. Yields on methanol for obligately methylotrophic organisms using the RUMP route for carbon assimilation, in general, are ± 18 g mol $methanol^{-1}$ [32]. An explanation for this remains to be found. For *Hyphomicrobium* spp. and *Thiobacillus* spp. significantly higher biomass yields (± 18 g biomass mol^{-1} DMS) have been found [5,8]. The relatively low yield of *M. sulfidovorans* on DMS is clearly related to the low yield on methanol, since the pathways for both compounds are very similar.

The stoichiometric formation of thiosulfate, rather than sulfate, as end product of the aerobic oxidation of sulfide and DMS, was detected in (chemostat) cultures of *M. sulfidovorans*. Intermediate thiosulfate production during the oxidation of sulfide has been reported for oxygen limited *Thiobacillus thioparus* cultures, but sulfate was the end product [34]. Thiosulfate was found as the end product of sulfide oxidation in some anoxygenic phototrophs (Chromatiaceae and Chlorobiaceae). These bacteria possess a flavocytochrome which converts sulfide directly into thiosulfate [35]. Whether or not a similar system operates in *M. sulfidovorans* remains to be seen.

Thus far, *Hyphomicrobium* spp. [6,36] and *Thiobacillus* spp. [37] have generally been found to be responsible for aerobic DMS degradation in (fresh water) waste systems and from marine systems, *Thiobacillus* spp. capable of aerobic DMS degradation have been isolated [5,6]. Recently, a versatile aerobic methylotrophic bacterium, which could degrade a wide range of substrates as well as DMS was isolated from marine samples [7]. Our new isolate, *Methylophaga sulfidovorans*, is an obligately methylotrophic bacterium that can degrade DMS aerobically. It had already been concluded from inhibition experiments that methylotrophic organisms are mainly responsible for DMS degradation in sea water [4]. The presence of significant numbers of *Methylophaga sulfidovorans* in marine samples provides the first direct evidence that obligate methylotrophs may play a significant role in the metabolism of DMS in marine environment.

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